Protein Kinase A and G Protein-coupled Receptor Kinase Phosphorylation Mediates β-1 Adrenergic Receptor Endocytosis through Different Pathways*§

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Agonist-induced phosphorylation of β-adrenergic receptors (βARs) by G protein-coupled receptor kinases (GRKs) results in their desensitization followed by internalization. Whether protein kinase A (PKA)-mediated phosphorylation of βARs, particularly the β1AR subtype, can also trigger internalization is currently not known. To test this, we cloned the mouse wild type PKA receptor (H9252) and created 3 mutants lacking, respectively: the putative PKA phosphorylation sites (PKA β1AR, the putative GRK phosphorylation sites (GRK β1AR), and both sets of phosphorylation sites (PKA/GRK β1AR). Following agonist stimulation, both PKA β1AR and GRK β1AR mutants showed comparable increases in phosphorylation and desensitization. Saturation concentrations of agonist induced only 50% internalization of either mutant compared with wild type, suggesting that both PKA and GRK phosphorylation of the receptor contributed to receptor sequestration in an additive manner. Moreover, in contrast to the WT β1AR and PKA β1AR, sequestration of the GRK β1AR and PKA/GRK β1AR was independent of β-arrestin recruitment. Importantly, clathrin inhibitors abolished agonist-dependent internalization for both the WT β1AR and PKA β1AR, whereas caveolae inhibitors prevented internalization only of the GRK β1AR mutant. Taken together, these data demonstrate that: 1) PKA-mediated phosphorylation can trigger agonist-induced internalization of the β1AR and 2) the pathway selected for β1AR internalization is primarily determined by the kinase that phosphorylates the receptor, i.e. PKA-mediated phosphorylation directs internalization via a caveolae pathway, whereas GRK-mediated phosphorylation directs it through clathrin-coated pits.

β-Adrenergic receptors (βARs)† belong to the large family of G protein-coupled receptors (GPCRs) characterized by a typical structure of seven transmembrane domains (1, 2). Three types of βARs, designated β1, β2, and β3ARs, have been cloned from mammalian tissues (1, 3). Both β1 and β3ARs contain phosphorylation sites located in the third intracellular loop and the C-terminal tail of the receptor, which serve as targets for cAMP-dependent protein kinase A (PKA), protein kinase C (PKC), and G protein-coupled receptor kinases (GRKs) (2). Furthermore, site-specific mutagenesis studies of the human β2AR suggest that low concentrations of agonist preferentially induce phosphorylation at PKA sites, whereas higher concentrations of agonist induce phosphorylation at both PKA and GRK sites (4).

Continuous exposure of cells to a stimulus causes βARs to undergo rapid phosphorylation in a process that dampens receptor signaling known as desensitization (4–8). βARs demonstrate two different mechanisms of desensitization. Agonist-specific or homologous desensitization of βARs consists of a two-step process in which phosphorylation at the C terminus of the βAR is mediated by GRKs followed by binding to an arrestin protein, which sterically interrupts signaling to the G protein (5, 8). Heterologous or non-agonist-specific desensitization is mediated by the second messenger-stimulated protein kinase A and C, which phosphorylate the receptor and effect a change in receptor conformation such that interaction with the G protein is impaired (5). An important consequence of agonist-mediated receptor phosphorylation and desensitization by GRKs is the subsequent internalization of phosphorylated receptors into the cell (9). This process is mediated by β-arrestin, which binds to components of the clathrin-mediated endocytic machinery and targets the ligand-bound receptor to clathrin-coated pits for endocytosis (10, 11). Interestingly, PKA phosphorylation, although an important mechanism for desensitization (4–8), appears to play only a small role in β1AR

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† The abbreviations used are: βAR, β-adrenergic receptor; GPCR, guanine nucleotide-binding regulatory protein-coupled receptor; PKA, protein kinase A; PKC, protein kinase C; GRK, G protein-coupled receptor kinase; HER, human embryonal kidney; IBMX, 3-isobutyl-1-methylxanthine; ISO, (-)-isoproterenol bitartrate; β-CID, 2-hydroxypropyl-β-cyclodextrin; MDC, monodansylcadaverine; WTβ1AR, wild type β1AR; PKA β1AR, β1AR lacking putative PKA phosphorylation sites; GRK β1AR, β1AR lacking putative GRK phosphorylation sites; PKA/GRK β1AR, β1AR lacking both sets of phosphorylation sites; GFP, green fluorescent protein; MEM, minimum essential medium; WT, wild type; Gβγ, beta-gamma subunits of G protein; Ga13, stimulatory G protein alpha subunit; Gαs, stimulatory G protein alpha subunit; GFX, bisindolylmaleimide I; PM, phorbol 12-myristate 13-acetate; VASP, vasodilator- and A kinase-stimulated protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Erk, extracellular signal-regulated kinase; ANOVA, analysis of variance.

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PKA-mediated Phosphorylation Triggers β2AR Internalization

PKA-mediated phosphorylation plays a critical role in the desensitization of β2ARs, particularly the β2AR. Although mechanisms of phosphorylation, desensitization, and internalization by GRKs have been well studied for the β2AR (4–8), little is known of the role that PKA-mediated phosphorylation plays in the internalization of β2ARs. GPCRs can internalize via at least two distinct pathways, namely clathrin-coated pits and caveolae. Although very different structurally, clathrin-coated pits and caveolae both serve as microdomains, which, in addition to functioning as transport machinery, also serve as platforms for integrating the cell's signaling pathways. These microdomains serve to facilitate cross-talk between different proteins from a particular signaling pathway contained within these distinct regions (15). Proteins to be transported are now thought to have “molecular addresses” located in the amino acid sequences of their cytoplasmic tail regions or to contain a binding site for a particular adaptor protein that directs the molecule to a particular cellular domain (16, 17).

In this regard, although a number of studies have demonstrated a critical role for GRK phosphorylation and β-arrestin binding in the process of clathrin-mediated internalization of the β2AR (5), the molecular mechanisms that are involved in the internalization of the β2AR are less known. The aim of the present study was to determine the specific role of PKA- and GRK-catalyzed phosphorylation of the β2AR in determining the cellular pathway for agonist-promoted receptor internalization.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were procured from Invitrogen. Human embryonal kidney (HEK) 293 cells were obtained from American Type Culture Collection. H-89 was obtained from BIOMOL Research Laboratories. Nonidet P-40 and bisindolylmaleimide I (GFX) was from Calbiochem. 3-Isobutyl-1-methylxanthine (IBMX), the anti-flag affinity gel, (-)-isoproterenol bitartrate (ISO), filipin III, 2-hydroxypropyl-β-cyclodextrin (β-CD), water-soluble cholesterol, monodansylcadaverine (MDC), and phosphor 12-mytastate 13-acetate (PMA) were obtained from Sigma. The β2AR antagonist ICI 118,551- HCl was procured from Research Biochemicals International. [32P]Orthophosphate, and [125I]iodocyanopindolol came from PerkinElmer Life Sciences. Restriction enzymes were obtained from Invitrogen. A cyclic AMP (cAMP) assay kit and ECL Western blotting detection reagents were obtained from Amersham Biosciences. An colchicine phosphate substrate and protein assay kit were obtained from Pharmacia Bio-Rad.

Plasmid Constructs—We generated mouse wild type β2AR (WTβ2AR) as previously described (18). Three different mutants lacking, respectively: the putative PKA phosphorylation consensus sites (PKA β2AR), the putative GRK phosphorylation sites (GRK β2AR), and both sets of sites (PKA/GRK β2AR) (Fig. 1A) were generated using a combination of primers (for details see Supplemental Material). All recombinant DNA-containing plasmids were verified for sequence authenticity and subcloned into mammalian expression vectors.

Mammalian Cell Culture and Transfection—HEK 293 cells were maintained as previously described (18). The evening before transfection, 4 × 10^6 cells were plated per 75-cm² flask. These cells were transfected on day 1 by FuGENE6™ method (Roche Applied Science). Human embryonal kidney (HEK) 293 cells were transfected with cDNA containing WT or one of the mutants and GFP-arrestin2 plasmid. Cells were split on day 2 into assay dishes as previously described (18). Bound ligand was separated on glass fiber filters (Whatman, GFC) by vacuum filtration. The filters were washed three times with cold wash buffer (10 mM Tris, 5 mM EDTA (pH 7.4)) and counted in a scintillation counter. Protein concentration was measured with an Amersham Biosciences PhosphoImager.

Receptor Binding by ELISA—To determine agonist concentration dependence for internalization of the wild type and three mutants, the receptor number was measured by the ELISA method, as described previously. Cell lines expressing either the PKA or GRK mutants were plated onto 24-well tissue culture dishes. To improve adhesion of cells to plastic, the wells were treated with 20 μg/ml poly-D-lysine (Whatman, GFC) by vacuum filtration. The filters were washed three times with PBS followed by blocking with 1% BSA in PBS for 45 min. Mouse monoclonal M2 anti-FLAG IgG was added at a dilution of 1:1000 for 1 h, followed by three subsequent washes with PBS. The samples were then briefly aspirated and incubated with goat anti-mouse-conjugated alkaline phosphatase at a concentration of 1:1000 in PBS/BSA for 1 h, and washed three times with PBS before the addition of a colorimetric alkaline phosphatase substrate. When adequate color change was reached, 100-μl samples were taken for colorimetric readings at 405 nm using a scanning multiwell spectrophotometer. Non-transfected cells were studied concurrently to determine background signal and all experiments were done in triplicate.

GFP-β-arrestin2 Translocation in Live Cells—GFP-β-arrestin2 translocation was visualized in real-time at a 37 °C heated stage Zeiss laser scanning microscope (LSM-510) as previously described (22). Cells expressing either the WTβ2AR or one of the mutants and GFP-β-arrestin2 plasmid were stimulated with 10 μM ISO in serum-free medium buffered with 10 mM HEPES. Images were collected sequentially every minute for a period of 10 min using a single line excitation filter 488 nm and emission filters at 505–550 nm. For each single experiment, quantitation was performed in the image in which maximal agonist-dependent β-arrestin2 translocation occurred. β-Arrestin2 translocation was calculated and expressed as agonist-promoted percent loss of the green fluorescent color from the cytosol due to GFP-β-arrestin2 translocation.

Intact Cell β2AR Desensitization: cAMP Assay—HEK 293 cells were incubated for 1 h in serum-free MEM (10 mM HEPES, pH 7.4) prior to the assay. Two identical sets of 100-mm plates were set up; one set was used as controls and the other for desensitization assays. Cells used for desensitization were exposed for 30 min at 37 °C and were then washed with serum-free MEM (10 mM HEPES, pH 7.4). Control cells were washed in an identical manner. Both sets of cells were then replaced with assay buffer (serum-free MEM, 10 mM HEPES, 1 mM IBMX, 100 mM NaCl) and were then incubated with 200 nM ISO for 10 min at 37 °C. Reactions were stopped by addition of cold wash buffer (4°C) and the plate was washed with cold wash buffer (10 mM Tris, 5 mM EDTA (pH 7.4)). The resultant amount of receptor-bound cAMP was determined using a cAMP-ELISA kit to each plate. Plates were frozen at −80 °C for subsequent processing of cells. Cells were scraped and collected, boiled for 10 min and then placed on ice. Samples were then centrifuged at 15,000 × g for 15 min, and the supernatant was transferred to a tube for cAMP measurement. cAMP was measured using the procedure outlined in the assay kit. The amount of protein per fraction was determined using a dye-binding protein assay kit.

Confocal Microscopy—Confocal microscopy was carried out as previously described (18). HEK 293 cells were transfected with the plasmids...
containing cDNAs encoding either the FLAG-WT β1AR, FLAG β1, or one of the mutant FLAG-β1ARs as well as pRK5. Live cells were incubated in the absence or presence of filipin, β-CD, cholesterol, MDC along with sucrose, H-89, and GFX for the indicated times and stimulated with ISO (10 μM) for 30 min. All incubations were carried out at 37 °C. Staining of FLAG-tagged receptor was carried out as previously described (23). Transferrin uptake was carried out as described previously (24). All samples were visualized under the Olympus IX70 laser scanning confocal microscope, using single sequential line excitation filters of 568 nm and emission filters of 585 nm for Texas Red detection. Images were viewed using Fluoview software and processed using Adobe Illustrator 9.0.1 and Adobe Photoshop 6.0.1.

**Immunoblotting**—Pretreatment of cells with inhibitors and stimulation with agonist were carried out at 37 °C in serum starvation medium as described in the figure legends. After stimulation, cells were lysed directly with 100 μlWell Laemmli sample buffer and proteins (30 μg/ lane) were resolved by SDS-PAGE. Phosphorylation of Erk1/2 was detected by protein immunoblotting using a 1:1000 dilution of rabbit secondary antibody. Blots were developed in ECL reagents for 1 min.

**RESULTS**

**Agnost-induced Phosphorylation of the β1AR in Intact 293 Cells**—To study the role of PKA- and GRK-mediated phosphorylation of the β1AR in an agonist-dependent manner, we used HEK 293 cells transiently transfected with plasmids containing the WTβ1AR, PKA β1AR, GRK β1AR, or PKA- GRK β1AR cDNAs. In unstimulated cells, the WTβ1AR exists as a phosphoprotein migrating with a molecular mass of ~70 kDa (Fig. 1B). Upon stimulation, phosphorylation of the WTβ1AR as well as PKA β1AR and GRK β1AR mutants increased ~2-fold above basal levels (Fig. 1B). However, when both the PKA and GRK sites were mutated (PKA- GRK β1AR), no agonist-dependent phosphorylation of the receptor was observed.

**Agnost-induced Desensitization of the β1AR in Intact 293 Cells**—To determine the role of PKA and GRK phosphorylation in the desensitization of the β1AR we measured cAMP accumulation in HEK 293 cells transiently transfected with the various β1AR mutants. The PKA and GRK phosphorylation mutants caused a 6- to 7-fold increase in cAMP, similar to the wild type receptor, indicating that they were fully coupled to Gs (Fig. 2). In contrast, stimulation of the PKA- GRK β1AR mutant produced only a 3-fold increase in cAMP, suggesting that removal of all of the sites resulted in a general impairment of receptor function (Fig. 2). Furthermore, cells overexpressing the WTβ1AR, PKA β1AR, or GRK β1AR showed ~70% desensitization measured as a reduction in catecholamine-induced cAMP generation on repeated exposure to the β1-selective agonist dobutamine. In contrast to the wild type receptor, which fully desensitized even at agonist concentrations that produced a less than maximal increase in intracellular cAMP, cells transfected with the PKA- GRK β1AR were not significantly desensitized by pretreatment with agonist. Consistent with the β1AR model of PKA-mediated heterologous desensitization and GRK-mediated homologous desensitization, these data suggest that β1ARs could become fully desensitized by either phosphorylation of PKA and/or GRK sites and only when all the phosphorylation sites were removed did a dramatic reduction in agonist-promoted desensitization occur.

**Both PKA and GRKs Mediate Agonist-induced β1AR Internalization**—As opposed to desensitization, agonist-induced internalization of β1ARs is thought to be mediated predominately through GRK phosphorylation and β-arrestin binding. To determine the contribution of PKA and GRK phosphorylation to agonist promoted β1AR internalization, we measured the loss of β1ARs from the cell surface in response to isoproterenol by radioligand binding. As shown in Fig. 3A, agonist stimulation resulted in a marked loss of WTβ1ARs from the cell surface. In contrast, at saturating agonist concentrations the extent of agonist-induced sequestration of both the PKA β1AR and GRK β1AR was about half that of the WTβ1AR. The double mutant exhibited minimal agonist-induced internalization. These results contrast with the desensitization data (Fig. 2), which indicate that either PKA or GRK sites alone were sufficient for full receptor desensitization, and suggest that both PKA and GRK phosphorylation are required for full internalization of the β1AR. To further examine the role of PKA and GRK phosphorylation in β1AR receptor endocytosis, we determined the dose dependence of isoproterenol-stimulated sequestration of the wild type and mutant receptors (Fig. 3B). Whereas WTβ1AR and PKA β1AR exhibited half-maximal internalization at similar agonist concentrations, the EC50 for GRK β1AR internalization was ~10-fold higher (EC50 values for WTβ1AR = 56 nM, PKA β1AR = 46 nM, GRK β1AR = 632 nM, and PKA- GRK β1AR = 534 nM). Thus, two distinct mechanisms appear to contribute additionally to β1AR sequestration, one a GRK site-dependent mechanism that predominates at lower agonist concentrations, and the other a PKA site-dependent mechanism that accounts for approximately half of the
agonist-dependent β1AR sequestration at higher agonist concentrations. Importantly, the reduced efficiency of sequestration for the β1AR mutants was observed over a broad range of agonist concentrations (Fig. 3B).

**PKA-mediated β1AR Internalization Does Not Involve β-Arrestin Recruitment to the Membrane**—Previous studies have shown that GRK-mediated desensitization involves recruitment of β-arrestin to the phosphorylated β1AR (5). We therefore studied the ability of the wild type and mutant receptors to recruit GFP-β-arrestin2 to the membrane. As shown in Fig. 4A, agonist stimulation of the WTβ1AR resulted in marked translocation of β-arrestin from the cytosol to the plasma membrane. In the absence of PKA sites (PKA−β1AR), β-arrestin recruitment was significantly increased. In contrast, cells expressing the GRK β1AR mutant showed marked impairment of β-arrestin recruitment to the membrane (Fig. 4, A and B). Removal of all phosphorylation sites in the double mutant resulted in very low β-arrestin recruitment comparable to that of the GRK β1AR (Fig. 4, A and B). Importantly, the ability of the GRK β1AR (PKA sites intact) to internalize (Fig. 3), despite the marked reduction of β-arrestin recruitment (Fig. 4), suggests that internalization of the β1AR can proceed via multiple mechanisms.
PKA-mediated Phosphorylation Triggers β1AR Internalization

Because the GRK−/β1AR mutant, we sought to determine the pathway(s) of internalization following PKA- and GRK-mediated phosphorylation of the β1AR. In these experiments cells expressing FLAG epitope-tagged WTβ1AR, PKA−β1AR, GRK−β1AR, or β1AR (used as a control) were used for the internalization studies assessed by laser scanning confocal microscopy. Because caveolae have been reported to play a significant role in both the signaling and internalization of several GPCRs, cells were pretreated with different caveolae pathway inhibitors: filipin, a sterol-binding agent that disrupts caveolae, prevented internalization of the GRK−β1AR (PKA sites intact) (panel l). Similar treatment of the β1AR, WTβ1AR, and PKA−β1AR had no effect indicating internalization by a caveolae-independent pathway (panels c, f, and i). Treatment by filipin alone had no effect on unstimulated, transfected cells (data not shown).

Because caveolae contain many of the proteins involved in GPCR signal transduction, they may function as signaling microdomains as well as locations for receptor internalization. Therefore to determine whether the caveolae inhibitors filipin and β-CD indirectly block internalization of the GRK−β1AR mutant by preventing PKA activation, we used a highly sensi-

Phosphorylation of the β1AR Directs Internalization via Two Separate Pathways—β-Arrestin recruitment has been shown to be important for targeting the β1AR to clathrin-coated pits for internalization (25). Because the GRK−β1AR mutant showed marked impairment of β-arrestin recruitment compared with the WTβ1AR, we used a sterol-binding agent that disrupts caveolae, prevented internalization of the GRK−β1AR (PKA sites intact) (Fig. 5, panel l). Pretreatment with filipin, a sterol-binding agent, and β-CD, which causes disruption of the caveolae by cholesterol depletion (26), showed distribution of GRK−β1AR, PKA−β1AR, and PKA−GRK−β1AR translocates to WTβ1AR, WTβ1AR, and PKA−β1AR had no effect indicating internalization by a caveolae-independent pathway (panels c, f, and i). Treatment by filipin alone had no effect on unstimulated, transfected cells (data not shown).

Because caveolae contain many of the proteins involved in GPCR signal transduction, they may function as signaling microdomains as well as locations for receptor internalization. Therefore to determine whether the caveolae inhibitors filipin and β-CD indirectly block internalization of the GRK−β1AR mutant by preventing PKA activation, we used a highly sensi-

Fig. 5. Inhibition of caveolae pathway in cells by filipin prevents internalization of the β1AR. Each panel shows representative images from 3–4 experiments. Confocal microscopy was used to visualize HEK 293 cells transfected with the plasmids containing cDNAs that were FLAG-tagged encoding for β1AR, WTβ1AR, PKA−β1AR, and GRK−β1AR. Cells were serum-starved for 3 h and incubated in the absence or presence of 1 μg/ml filipin, for 1 h. Following stimulation with ISO (10 μM) for 30 min, cells were fixed and stained with Texas Red. Unstimulated cells in panels a, d, g, and j showed distribution of βAR at the plasma membrane. Following agonist stimulation, all βARs internalized into cytoplasmic puncta with complete loss of membrane fluorescence (panels b, e, h, and k). Pretreatment with filipin, a sterol-binding agent that disrupts caveolae, prevented internalization of the GRK−β1AR (PKA sites intact) (panel l). Similar treatment of the β1AR, WTβ1AR, and PKA−β1AR did not have any effect indicating internalization by a caveolae-independent pathway ( panels c, f, and i). Treatment by filipin alone had no effect on unstimulated, transfected cells (data not shown).
PKA-mediated Phosphorylation Triggers βAR Internalization

PKA-mediated phosphorylation triggers βAR internalization. VASP (vasodilator- and A kinase-stimulated phosphoprotein) is a major substrate of cyclic nucleotide-dependent protein kinases in response to agonist stimulation. VASP is phosphorylated by PKA and is visualized as a phosphorylation-induced electrophoretic mobility shift from 46 to 50 kDa by SDS-PAGE (27, 28). PKA phosphorylates VASP and is visualized as a phosphorylation-induced electrophoretic mobility shift (27, 28). Addition of cholesterol to the cells treated with β-CD reverses the inhibitory effect on the GRK β AR (PKA sites intact) (panel l). Addition of cholesterol to the cells treated with β-CD reversed the inhibitory effect on the GRK β AR (PKA sites intact) (panel l). Similar treatment by β-CD on β AR, WT β AR, and PKA β AR shows no effect on internalization (panels c, f, and i). Treatment by β-CD alone had no effect on unstimulated transfected cells (data not shown). B, filipin and β-CD do not affect endogenous PKA activity. HEK 293 cells stably expressing WT β ARs, GRK β ARs, and PKA β ARs were transiently transfected with FLAG-VASP cDNA (2 μg/10-cm culture dish). Cells were serum-starved for 24 h in MEM and 0.1% BSA and incubated in the absence or presence of the caveolae inhibitors filipin and β-CD for indicated times and stimulated with ISO (10 μM) for 5 min. After agonist stimulation, cell lysis was followed by immunoblotting of cell with FLAG antibody (27, 28). PKA phosphorylates VASP and is visualized as a phosphorylation-induced electrophoretic mobility shift from 46 to 50 kDa by SDS-PAGE (27, 28). PKA-mediated phosphorylation of VASP was confirmed by preincubation with the PKA specific inhibitor H-89 (10 μM) for 15 min. To further demonstrate a role for PKA phosphorylation in internalization of the β AR, we pretreated cells with the selective PKA inhibitor H-89 followed by agonist stimulation. Importantly,
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![Fig. 7. Inhibition of clathrin-coated pit pathway in cells by MDC and sucrose prevents internalization of the β1AR. A, each panel shows representative images from 3–4 experiments. Confocal microscopy was used to visualize HEK 293 cells transfected with the plasmids containing cDNAs that were FLAG-tagged encoding for WTβ1AR, PKA-β1AR, and GRK-β1AR. Cells were serum-starved for 3 h and incubated in the absence or presence of treatment of a combination of clathrin inhibitors 300 μM MDC and 0.45 μM sucrose for 30 min. Following stimulation with isoproterenol (10 μM) for 30 min, cells were fixed and stained with Texas Red. Unstimulated cells in panels a, d, and g show distribution of β1AR at the plasma membrane. Following agonist stimulation, all β1ARs are internalized into cytoplasmic puncta with complete loss of membrane fluorescence (panels b, e, and h). WTβ1AR and PKA-β1AR (GRK sites intact) show 80–90% inhibition of internalization (panels c and f). Similar treatment of cells expressing GRK-β1AR mutant does not block internalization (panel i). B, pretreatment with MDC and sucrose abolishes transferrin internalization confirming that clathrin-mediated processes are effectively inhibited. Treatment by MDC combined with sucrose alone had no effect on unstimulated transfected cells (data not shown). Treatment by either MDC or sucrose alone inhibited internalization of the WTβ1AR and PKA-β1AR by ~40–50% without an effect on GRK-β1AR internalization (data not shown).]

![Fig. 8. Phosphorylation plays a role in internalization of the β1AR. Each panel shows representative images from 3–4 experiments. Confocal microscopy was used to visualize HEK 293 cells transfected with the plasmids containing cDNAs that were FLAG-tagged encoding for WTβ1AR, PKA-β1AR, or GRK-β1AR. Cells were serum-starved for 3 h and incubated in the absence or presence of PKA selective inhibitor H-89 (10 μM) for 15 min. Following stimulation with isoproterenol (10 μM) for 30 min, cells were fixed and stained with Texas Red. Unstimulated cells in panels a, d, and g show distribution of β1AR at the plasma membrane. Following agonist stimulation, all β1ARs internalize into cytoplasmic puncta with complete loss of membrane fluorescence (panels b, e, and h). WTβ1AR form puncta on the membrane following agonist stimulation, indicating partial inhibition likely due to the presence of both PKA and GRK phosphorylation sites (panel c). PKA-β1AR (GRK sites intact) shows robust agonist-mediated internalization in the presence of H-89 (panel f), while GRK-β1AR (PKA sites intact) internalization was completely prevented (panel i).]

the GRK-β1AR mutant (PKA sites intact) showed no internalization upon agonist stimulation (Fig. 8, panel i), whereas robust agonist-mediated internalization of the PKA-β1AR occurred (Fig. 8, panel f). In the case of the WTβ1AR, puncta were visible on the membrane, indicating partial inhibition likely due to the presence of both phosphorylation sites (Fig. 8, panel c). The lack of an H-89 effect on the internalization of PKA-β1AR indicates that other potential PKA phosphorylation sites were not present on the PKA-β1AR mutant. Taken together these data demonstrate that PKA-mediated phosphorylation of the β1AR directs internalization via a caveola pathway, whereas GRK-mediated phosphorylation of the β1AR directs internalization through the clathrin-coated pit machinery.

PKC-mediated Phosphorylation Does Not Play a Role in β1AR Internalization—To determine whether PKC-mediated phosphorylation directs β1AR internalization through a mechanism of heterologous desensitization, cells stably expressing either the FLAG epitope-tagged WTβ1AR, PKA-β1AR, or GRK-β1AR were used in the internalization studies. Pretreatment of WTβ1AR-, PKA-β1AR-, or GRK-β1AR-expressing cells with the PKC inhibitor GFX, followed by agonist stimulation resulted in the formation of intracellular aggregates with complete loss of membrane fluorescence (Fig. 9, panels d, h, and l), similar to cells treated with agonist alone (Fig. 9, panels c, g, and k). The lack of a GFX effect on cells expressing the wild type or the phosphorylation mutant receptors indicates that PKC-mediated phosphorylation does not play a role in β1AR internalization under these conditions. To confirm the specificity of GFX to inhibit PKC-mediated phosphorylation (Fig. 9B), we assessed its effect on cells expressing the WTβ1AR by immunoblotting for phospho-ERK alongside confocal microscopy studies. Whereas pretreatment with PMA alone caused a large increase in the activation of ERK, the pretreatment of GFX with PMA completely blocked the ERK activation. No differences in ERK activation were observed between treatment of ISO alone or ISO with GFX. Taken together the results demonstrated that, first, GFX was able to inhibit endogenous PKC in the cells, and, second that PKC-mediated phosphorylation does not play a role in β1AR signaling.

**DISCUSSION**

In this study we demonstrate that, in addition to the established role of GRKs in the process of receptor internalization, PKA-mediated phosphorylation plays a critical role in agonist-induced internalization of the β1AR. Furthermore, although GRK-mediated phosphorylation directs internalization through a clathrin-coated pit pathway, PKA-mediated phosphorylation directs internalization via a caveola pathway. These data contrast dramatically with previous findings obtained using PKA and GRK mutants of the β1AR. Although both PKA and GRK phosphorylation contribute to desensitization of the β1AR (4, 12), PKA phosphorylation does not play a significant role in endocytosis of this receptor (12). Our data suggest that at maximally efficacious concentration of agonist, β1AR endocytosis occurs via both clathrin-coated pits and caveolae. Each pathway contributes approximately to half of the observed response, and the two pathways are additive. Thus, endocytosis through clathrin-coated pits cannot compensate for loss of the caveolar pathway, and vice versa.
Whether this indicates that β2ARs naturally exist in two distinct pools, inside and outside of caveolae, and that endocytosis of each pool is independently regulated, is unclear. Because the EC_{50} for isoproterenol-stimulated endocytosis of the wild type and PKA β1AR is ~10-fold lower than that for the GRK β1AR, our data suggest that clathrin-mediated endocytosis is the preferred mechanism for β1AR internalization at low agonist concentrations, whereas at higher agonist concentrations, both pathways contribute equally to endocytosis. Importantly, however, the GRK β1AR still undergoes significant internalization at low agonist concentrations, suggesting that the clathrin-independent mechanism of β1AR internalization does contribute to receptor endocytosis over a wide range of agonist concentrations. Data from this study also highlights other differences in the internalization mechanism for the two βAR subtypes in that PKC-mediated phosphorylation does not play a role in the internalization of the β1AR. This is in contrast to previous findings that showed contribution of PKC-mediated phosphorylation to internalization of the β2AR.

A number of previous studies have found that other GPCRs, including bradykinin B1, cholecystokinin, endothelin subtype A, and muscarinic acetylcholine receptors localize in caveolae (31–35). Indeed, both β2ARs and β1ARs have also previously been shown to localize in caveolar microdomains (36–41), including endogenous β2ARs in neonatal rat cardiomyocytes (37, 38). Our study adds to this literature by showing that β1ARs lacking phosphorylation sites for GRK internalize through caveolae. Our results are also in accordance with findings obtained for the endothelin receptor, where agonist-induced internalization was found to proceed either via clathrin-coated pits or caveolae, depending on the oxidative state of cell surface cholesterol (42). A similar scheme has also been shown for the cholecystokinin receptor, which can utilize either pathway for internalization but internalizes predominantly via the clathrin-coated pit pathway (32).

Both caveolae and clathrin-coated vesicles serve as scaffolds that integrate signal-transduction complexes, providing microdomains for cross-talk between specific signaling molecules (15, 16, 49). Several studies indicate that proteins involved in adrenergic signaling (e.g. Gβγ, Gαi, adenylate cyclase, β1ARs, and β2ARs) co-localize within the same microdomains (37, 39), possibly because they contain an “address” for that specific domain (16). The cytoplasmic tail of the receptor is a potential region that likely contains an address site (16). Indeed, scaffold proteins containing PDZ domains have been shown to associate with the C termini of βARs (17), and these interactions can be disrupted through phosphorylation by GRK5 (44, 45). Thus cellular signaling and trafficking is precisely controlled by phosphorylation-dependent regulation of the receptor and its association with a variety of scaffold proteins (17). For example, a recent study in cardiac neonatal myocytes has shown that the PDZ motif at the C terminus of the β1AR is responsible for its limited internalization and that the mutation of this domain increases internalization to levels similar to those observed with the β2AR (31). Indeed, we show here that differential phosphorylation of the β1AR plays a critical role in determining its internalization pathway, indicating that the site of phosphorylation may serve as a molecular address that directs receptor internalization.

Determination of the internalization pathway utilized by each receptor in this study was based on specific inhibitors for either caveolae or clathrin-coated pits. Specifically, cholesterol depletion by β-CD was one of the reagents used to demonstrate caveolae-mediated internalization. In contrast, several studies have suggested that disruption of cholesterol by this agent can interfere with clathrin-coated pit formation (46–48). However, our data supporting internalization of the GRK β1AR mutant through caveolae are based on: 1) the use of two different caveolae inhibitors, filipin that binds sterols, and β-CD that depletes cholesterol (26); 2) reintroduction of cholesterol to cells depleted of cholesterol by β-CD rescued internalization of the GRK β1AR mutant (49); and 3) transferrin receptor internalization insensitivity to filipin (46). Finally, we showed that the use of the caveolae inhibitors filipin and β-CD did not affect the ability of the various mutant receptors to increase endogenous PKA activity in response to agonist.

In conclusion, we demonstrate that PKA-mediated phosphorylation plays an important role in agonist-induced internalization of the β1AR in addition to the recognized role of GRKs in receptor internalization. Furthermore, we have shown that, although either set of phosphorylation sites is sufficient to induce desensitization of the β1AR, both PKA and GRK sites are required to accomplish complete internalization. These sites may serve as “molecular addresses” that target the receptor into microdomains thus allowing different signal transduction pathways to be triggered.

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REFERENCES

PKA-mediated Phosphorylation Triggers βAR Internalization