Role of the Cyclic AMP-dependent Protein Kinase in Homologous Resensitization of the β1-Adrenergic Receptor*

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A fundamental question in biology is how the various motifs in G protein-coupled receptors participate in the divergent functions orchestrated by these molecules. Here we describe a fundamental role for a serine residue at position 312 in the third intracellular loop of the human β1-adrenergic receptor (β1-AR) in endocytic recycling of the agonist-internalized receptor. In receptor recycling experiments that were monitored by confocal microscopy, the agonist-internalized wild-type (WT) β1-AR recycled with a t½ of 14 ± 3 min. Mutagenesis of Ser312 to alanine (Ser312 → Ala β1-AR) or to the phosphoserine mimic aspartic acid (Ser312 → Asp β1-AR) resulted in β1-AR constructs that were pharmacologically indistinguishable from the WT β1-AR. The internalized Ser312 → Asp β1-AR recycled efficiently with a t½ of 11 ± 3 min, whereas the internalized Ser312 → Ala β1-AR was not recycled or functionally resensitized through the endosomal pathway. Because this serine is a putative residue for phosphorylation by the cyclic AMP-dependent protein kinase (PKA), we examined the role of this kinase in recycling of the internalized β1-AR. Inhibition of PKA biochemically or genetically using a dominant negative PKA construct blocked the recycling of the internalized WT β1-AR. Phosphorylation studies revealed that the β1-AR is partially phosphorylated by PKA and that phosphorylation of the β1-AR by the catalytic subunit of PKA occurs exclusively at Ser312. Our results identify a new signaling paradigm in which homologous activation of a kinase provides a reversible modification that shifts the itinerary of the internalized receptor toward recycling and resensitization. Therefore, PKA-mediated phosphorylation of G protein-coupled receptors might result in motif-dependent desensitization or resensitization.

The β1-AR mediates many of the cardiovascular actions of catecholamines such as the regulation of heart rate and the force of myocardial contraction (1, 2). Activation of the β1-AR and other GPCR in turn causes marked changes in the receptor protein and its associated signaling components (reviewed in Ref. 3). In the case of GPCR, the activated receptor becomes a substrate for modification by specific kinases that serve to uncouple the receptor from the G protein (4). One such example is homologous desensitization of the β2-AR by β-agonists, whereby ligand-dependent phosphorylation of the β2-AR by G protein-coupled receptor kinases (GRKs) is rapidly followed by receptor interaction with cytoplasmic β-arrestins that disrupt the interaction between the receptor and the G protein (5). This interaction presumably occurs in the cell membrane and causes rapid desensitization of the receptor and is intimately associated with endocytosis of the ligand-activated receptor via the clathrin-coated pit pathway (5–8).

Initially, internalization of the GPCR was viewed as a means to uncouple the receptor from its signaling components, thereby dampening the overall response (9–12). The results of many studies indicate that the itinerary of the internalized GPCR is receptor- and cell-specific (13). For example, in human embryonic kidney (HEK-293) cells, the μ-opioid receptor is internalized and then recycled, whereas the internalized δ-opioid receptor is significantly degraded (14–16). Cell-specific outcomes have been encountered with the β2-AR, which is recycled in HEK-293 cells but degraded in A-431 cells (17, 18). Intracellular trafficking for some GPCR, therefore, appears to promote their resensitization and recovery from desensitization (19–23). However, little is known about the molecular mechanisms or the motifs within the GPCR that are involved in its various outcomes. The carboxyl-terminal tails of the β2-AR and the β2-AR have distinct PDZ-like domains. The PDZ-like domain of the β2-AR interacts with PSD-95 (postsynaptic associated protein 90) and membrane-associated guanylate-inverted-2 (24, 25), whereas the PDZ domain of the β2-AR interacts with the Na+/H+ exchanger regulator factor (NHERF, also known as EBP50 (ezrin-binding protein 50)) (26). Mutagenesis of the PDZ of the β2-AR interferes with its efficient recycling and its functional properties (27). The motifs involved in regulating the recycling of the β2-AR are more obscure. Our results demonstrate that a critical serine in the third intracellular loop of the human β1-AR that is a substrate for reversible phosphorylation by PKA is involved in recycling and functional resensitization of the human β1-AR.

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**PKA-mediated Phosphorylation Triggers β1-AR Resensitization**

**EXPERIMENTAL PROCEDURES**

**Measurement of β1-AR Cell Surface Expression by Radioligand Binding Assays**—Membranous β1-AR expression in HEK-293 cells stably transfected with FLAG-tagged wild-type or Ser 312 point mutants was measured as described previously (18). The density of the β1-AR in these cell lines were 0.95 ± 0.12 pmol of WT β1-AR/mg, 1.1 ± 0.2 pmol of Ser312→Ala β1-AR/mg; and 0.85 ± 0.1 pmol of Ser312→Asp β1-AR/mg of protein. The supernatant was subjected to electrophoresis on SDS-containing 12% gels, transferred to nitrocellulose, and probed with the anti-FLAG antibody. The density of the membrane fraction of membranes as described below. The third set was used as the control for resensitization, and the fourth set was used for resensitization assays. Cells for resensitization were exposed to 1 mM ascorbic acid (control) or to 10 μM isoproterenol for 3 h at 37 °C and then washed with serum-free DMEM supplemented with 10 mM HEPES. The cells were then incubated with 100 μM alprenolol for 1 h at 37 °C and then washed with serum-free DMEM supplemented with 10 mM HEPES. Membranes were prepared from all cells by hypotonic lysis of the cells with 20 mM HEPES, pH 7.4, 2 mM MgCl2, 1 mM EDTA, and 1 mM 2-mercaptoethanol supplemented with 10 μM leupeptin and 10 μg/ml aprotinin for 10 min on ice. The cells were then transferred into a glass-plate homogenizer and lysed by 30 up-and-down strokes. Cell lysates were centrifuged at 2,500 × g for 5 min to pellet the nuclei, and the supernatant was centrifuged at 15,000 × g for 20 min to pellet the membranes. Then 50 μg of membrane proteins were incubated at 30 °C in a final volume of 0.1 ml containing 50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 10 mM phosphocreatine, 1 mM cyclic AMP, 2 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.4 mM EGTA, 2 mg/ml creatinine kinase, 0.2 mM ATP containing 1 μCi of [α-33P]ATP, 1 mM GTP, and the various concentrations of isoproterenol. The assay was initiated by the addition of membranes and terminated after 10 min (31). The cyclic AMP that formed was isolated by column chromatography and quantitated by liquid scintillation counting (31). Assays were performed in triplicate and replicated n = 4 times. The Kact, S.E. for each β1-AR was calculated using the Graphpad Prism 4 program and statistical comparisons were analyzed using Graphpad Instat program.

**Confocal Microscopy**—HEK-293 cells stably transfected with FLAG-tagged WT β1-AR or its Ser312 mutants were grown on poly-l-lysine-coated coverslips and serum-starved for 16 h prior to transfection with 25 μM HEPES, pH 7.4. The receptors were labeled with the M2 anti-FLAG fluorescein isothiocyanate-conjugated antibody (0.5 μg/ml) for 1 h. The cells were treated with 10 μM isoproterenol for 30 min, followed by an acid wash (0.2 M NaCl, 0.5 M acetic acid) to strip off the excess of antibodies. Then 100 μM alprenolol was added, and the coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature at different time points to establish the recycle time. In those experiments where the slides were fixed after 1 h from the removal of isoproterenol, the slides were pretreated with 100 μg/ml of the lysosomal inhibitor leupeptin for 1 h in serum-free medium.

In another series of experiments, a 1 μM concentration of the specific PKA inhibitor H-89 (32) and 1 μM concentration of the myristoylated PKA peptide inhibitor (PKI), TTYADFIASGRTGRRNAIHD (33), that inhibits phosphorylation of target proteins by binding to the protein-substrate site of the catalytic subunit of PKA were added cells prior to isoproterenol.

Confocal fluorescence microscopy was performed using Zeiss Axiovert LSM 510 (100 × 1.4 DIC oil immersion objective). Fluorescein isothiocyanate was excited with the 488-nm argon-krypton laser and imaged through the 520-nm long-pass emission filter. Z-stacks of images were acquired with a Zeiss AxioVision 3.1 program with a threshold setting. The data are presented as the mean ± S.E. for each experiment.

**Generation of HEK-293 Cells Expressing Dominant Negative PKA**—Two dominant negative PKA vectors, MT-REVAC and MT-REVAC-neo, were used in our studies (34). In both vectors, the metallothionine gene promoter drives the expression of a PKA RIIα subunit mutant in which the two cyclic AMP-binding sites were mutagenized. MT-REVAC was used for transient expression, whereas MT-REVAC-neo was used to generate a stable construct in HEK-293 cells. The metallothionine promoter is induced by Zn2+ ions and inhibited in the absence of Zn2+. The activity of PKA in these cells was determined by an in vitro kinase assay in which cyclic AMP-mediated phosphorylation of biotinylated Membranous-α1A-Ser-Lys-Arg (Kemptide) was used as an activity (SignaTECT for PKA from Promega Corp.). An MT-REVAC-neo expressing cell line was selected in which basal PKA activity was undetectable and cyclic AMP-mediated induction of the activity of PKA was 1.8 ± 0.3-fold compared with about 20 ± 4-fold in the cell line expressing the empty MT-neo vector.

The HEK-293 cells that are described earlier using the MT-REVAC-neo were grown on coverslips and transiently transfected with the WT β1-AR for 2 days. The cells were then cultured with MEM supplemented with 10% fetal bovine serum and 1 μM ZnSO4 overnight to induce MT-REVAC-neo expression or in the absence of zinc to repress MT-REVAC-neo expression. Slides were processed for the recycling assay by confocal microscopy as described earlier.
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RESULTS AND DISCUSSION

Human WT β₁-AR expressed in HEK 293 cells that were exposed to isoproterenol for 30 min followed by agonist washout, exhibited rapid internalization and recycling (Fig. 1A). These data are consistent with the effect of isoproterenol on β₁-adrenergic receptors in this cell line (6). However, mutagenesis of the serine residue at position 312 in the third intracellular loop to alanine (Ser₃₁₂→Ala β₁-AR) prevented the recycling of the agonist-internalized receptor as determined by radioligand binding (Fig. 1A). Surface biotinylation of cells after 3 h from the removal of isoproterenol showed that the incorporation of biotin into the Ser₃₁₂→Ala β₁-AR was less than the WT β₁-AR (Fig. 1B). Functional assays were used to determine whether this mutation affected the coupling of the WT β₁-AR or the Ser₃₁₂→Ala β₁-AR to adenyl cyclase in response to isoproterenol (Fig. 1C). Expression of the β₁-AR did not markedly increase basal activity of adenyl cyclase when compared with its activity in cells expressing the empty expression pCDNA vector. Increasing concentrations of isoproterenol increased the activities of adenyl cyclase by ~7-fold in each β₁-AR-expressing cell line with comparable coupling affinities (Fig. 1C) (36).

To investigate the role of Ser₃₁₂ in the trafficking of the β₁-AR, we visualized membrane trafficking of antibody-labeled receptors using confocal microscopy. Both the WT β₁-AR and Ser₃₁₂→Ala β₁-AR underwent rapid endocytosis following their activation by isoproterenol, as indexed by the translocation of antibody labeled β₁-AR from the plasma membrane to endocytic vesicles (Fig. 2, B and D). Acid treatment of these cells removed the surface-exposed antibody and revealed that intracellular β₁-AR staining was in discrete punctate vesicular μCi of [³²P]ATP (Amersham Biosciences) for 1 h with either 0.03% Me₂SO or 1 μM H-89. Stimulation with 10 μM isoproterenol or with 20 μM forskolin were for 10 min, followed by the addition of RIPA extraction buffer composed of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 1 mM phenethylsulfonyl fluoride, and 1 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin. Equivalent amounts of dissolved proteins were incubated with M2-FLAG-agarose beads (Sigma) at 4°C overnight. For in vitro cell phosphorylation, 50 μg of membrane protein from HEK-293 cells stably expressing the WT β₁-AR or the Ser₃₁₂→Ala β₁-AR were incubated in 200 μl of PKA kinase buffer composed of 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 2 mg/ml sodium fluoride, 1 mM phenethylsulfonyl fluoride, and 1 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin. At the beginning of the experiment, the buffer was supplemented with 1 mM ATP and 0.3 mCi of [γ⁻³²P]ATP. To some samples, ascorbic acid or 10 μM isoproterenol without or with 20 μM of the catalytic subunit of PKA (Promega) were added and incubated for 10 min at 30°C. At the end of the incubation, the tubes were centrifuged, and the supernatant was collected. Equal amounts of proteins were mixed with 50 μl of anti-M2-FLAG antibody coupled to agarose beads (Sigma) at 4°C overnight. The next day, the resins were washed in RIPA buffer, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis in 10% or 12% gels, electroblotted to nitrocellulose, and probed with anti-FLAG M2 antibody (Sigma) at 4°C overnight. For immunoprecipitation, the cell signaling technology. The blots were stripped and reprobed with anti-P42/44 MAPK antibody (Upstate Biotechnology Inc., Lake Placid, NY) to ascertain that equal amounts of protein were loaded onto the gels.

A, cells expressing either the WT β₁-AR or a mutant in which serine at position 312 in the third intracellular loop of the β₁-AR was converted to alanine were exposed to isoproterenol for 30 min to induce internalization. Then the cells were washed, and the distribution of recycled receptors over the next 8 h was assessed by radioligand binding using [³²H]CGP-12177. B, cells expressing the WT β₁-AR or the Ser₃₁₂→Ala β₁-AR were exposed to buffer or isoproterenol for 15 min to induce internalization and then washed extensively and exposed to alprenolol for 3 h. Cells were surface-biotinylated using sulfo-NHS-SS-biotin for 20 min at 4°C followed by immunoprecipitation and immunoblotting to quantify cell surface distribution of each β₁-AR. In C, isoproterenol-mediated activation of adenyl cyclase in membranes prepared from cells expressing the empty pCDNA 3.1 vector, WT β₁-AR, Ser₃₁₂→Ala β₁-AR, or Ser₂₁₁→Asp β₁-AR were compared. The Kᵣₐₐₐ was 0.4 ± 0.06 μM for the WT β₁-AR, 0.12 ± 0.05 μM for the Ser₃₁₂→Ala β₁-AR and 0.8 ± 0.06 for the Ser₃₁₂→Asp β₁-AR (p > 0.05, n = 4).
structures (Fig. 2, c and k). In a confocal recycling assay, involving initial exposure of the cells to isoproterenol, followed by an acid wash and then the addition of the adrenergic antagonist alprenolol to prevent further internalization, it was revealed that the WT $\beta_1$-AR recycled rapidly and completely (Fig. 2A). Isoproterenol-treated cells were visualized after they were exposed to a 100 µM concentration of the $\beta_1$-adrenergic antagonist alprenolol for up to 24 h. Receptor recycling was observed in cells expressing either the WT $\beta_1$-AR (d-g) or the Ser$^{312}$ $\rightarrow$ Asp$\beta_1$-AR (t-w) but not the Ser$^{312}$ $\rightarrow$ Ala$\beta_1$-AR even after 24 h (l-p). Each scale bar represents 5 µm.

B, quantification of $\beta_1$-AR recycling kinetics by confocal recycling assays. The LSM-510 software was used to determine the distribution of pixels between the membranous and intracellular compartments of acid washed cells. The ratios of membranous to intracellular pixels were determined for each time point after the washout of isoproterenol. The $t_{0.5}$ for recycling was calculated by fitting the relevant data to a single exponential function of time from $y = y_0 + A(1 - e^{-t/t_0})$, where $y_0$ and $A$ are constants.

In a biochemical recycling assay was performed (Fig. 3A). In this assay, cells expressing the WT $\beta_1$-AR or the Ser$^{312}$ $\rightarrow$ Ala$\beta_1$-AR were surface-biotinylated with cleavable biotin and then exposed to isoproterenol or buffer for 30 min, followed by cleavage of the remaining cell surface biotin. The first cleavage step permits the analysis of the recycling kinetics of internalized biotinylated receptors without interference from de novo synthesized nonbiotinylated receptors. The data reveal complete cleavage of cell surface biotin from cells exposed to buffer only (compare lanes 1 and 2 in Fig. 3A). Exposure of the biotinylated cells to isoproterenol protected a significant amount of internal biotin from cleavage (compare lanes 3 and 4 in Fig. 3A). The biotinylated fraction of WT $\beta_1$-AR that was resistant to cleavage in lane 4 was less than the comparable fraction of Ser$^{312}$ $\rightarrow$ Ala$\beta_1$-AR.
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Fig. 3. Role of Ser\(^{312}\) in recycling and functional resensitization of the β1-AR. A, several culture dishes of cells expressing the WT β1-AR or Ser\(^{312}\rightarrow Asp β1-AR\) were surface-biotinylated for 20 min. Two sets of culture dishes from each cell type were either cleaved or uncleaved to determine total biotinylation and cleavage efficiency for each cell line (lanes 1 and 2). The remaining cultures were exposed to isoproterenol for 30 min to induce internalization, and the remaining surface biotin was cleaved (first cleavage) with glutathione (lanes 3 and 4). The remaining cultures were returned to 37 °C to allow each β1-AR to recycle for 1, 12, and 24 h and then recleaved (second cleavage) to ensure cleavage of any newly appearing surface biotin. The loss of biotinylated WT β1-AR after the second biotin cleavage in lanes 5–7 provides confirmation that the WT β1-AR recycled rapidly, whereas retention of biotin in lanes 5–7 of the Ser\(^{312}→Ala β1-AR\) samples indicates intracellular retention of this receptor. B, functional resensitization of the β1-AR assessed by a membrane adenylyl cyclase assay. Cells expressing WT β1-AR, Ser\(^{312}→Ala β1-AR\), and Ser\(^{312}→Asp β1-AR\) were exposed to buffer or to 10 μM isoproterenol for either 10 min or 3 h at 37 °C. For the 3-h condition, the buffer or isoproterenol was replaced with 100 μM of alpenrol for 1 h. Membranes were prepared from these cells by hypotonic lysis followed by differential centrifugation. Fifty μg of membranes were exposed to 10 μM isoproterenol or to 20 μM forskolin in cyclase assay buffer for 10 min at 30 °C. The ratio for the specific activity of adenylyl cyclase in response to isoproterenol to that for forskolin in each sample was determined to calculate the percent of maximal adenylyl cyclase activity in each type of membrane. These experiments were replicated (n = 6) each in triplicate to determine the S.E.

Ala β1-AR because some WT β1-AR apparently recycled during the period when isoproterenol was present. After the first cleavage, the cells were returned to 37 °C to allow the WT β1-AR and the Ser\(^{312}→Ala β1-AR\) to recycle for 1, 12, or 24 h. After each time period, the cells were cooled to 4 °C and cleaved for the second time to ensure cleavage of any newly appearing surface biotin. The data reveal that by 1 h, no biotin was detected in the WT β1-AR blots, reflecting membrane recycling of the WT β1-AR and subsequent biotin cleavage (Fig. 3A, lanes 5–7). In contrast, the internalized (biotinylated) Ser\(^{312}→Ala β1-AR\) was not changed after 1, 12, or 24 h from the addition of isoproterenol, reflecting their internal distribution (compare lanes 5–7 in Fig. 3A with lane 4). Importantly, the internalized (biotinylated) Ser\(^{312}→Ala β1-AR\) experienced no detectable nonspecific loss of biotin or protein degradation under the experimental conditions used (i.e. with 100 μg/ml leupeptin), indicating that the loss of biotin by the WT β1-AR in lanes 5–7 of Fig. 3A was specifically due to membrane reinsertion.

Next we examined whether the differences in postendocytic trafficking between the WT β1-AR or the Ser\(^{312}→Asp β1-AR\) and the Ser\(^{312}→Ala β1-AR\) were associated with significant effects on the ability of the Ser\(^{312}→Ala β1-AR\) to be functionally resensitized after agonist stimulation of cells (Fig. 3B). In this assay, rapid desensitization of adenylyl cyclase in membranes expressing all three β1-AR was observed after a 10-min exposure of cells to isoproterenol, indicating that mutagenesis of Ser at position 312 did not affect short term desensitization (37). In the next series of experiments, the cells were exposed to isoproterenol for 3 h to desensitize and internalize the β1-AR, followed by agonist washout and recycling in the presence of alpenrol for 1 h. In this assay, we observed significant differences between the cell lines in their recovery from desensitization. WT β1-AR and Ser\(^{312}→Asp β1-AR\) cells recovered from desensitization and their activation of adenylyl cyclase in response to isoproterenol were comparable with control cells, whereas Ser\(^{312}→Ala β1-AR\)-expressing cells were significantly desensitized under the same conditions (Fig. 3B). Thus, the modification of Ser at position 312 to alanine apparently disrupted the recycling itinerary involved in functional resensitization of this receptor.

The sequence around the serine 312 residue in the human β1-AR is RRPS\(^{312}\), which corresponds to a putative site for phosphorylation by PKA (38). To investigate whether phosphorylation of the β1-AR by PKA is involved in recycling of the internalized β1-AR, we measured the recycling kinetics of the agonist-internalized WT β1-AR using the radioactive antagonist [\(^{3}H\)]CGP-12177 binding to cells that were exposed to an initial 30-min pulse of PKA in HEK-293 cells. This construct (MT-REVAB) contains mutations in each of the two cyclic AMP binding sites in the PKA receptor, allowing the MT-REVAB to be functionally resensitized after agonist stimulation of cells under conditions in which PKA was inhibited. Inhibition of PKA chemically with its specific inhibitors H-89 (32) or with the cell-permeable myristoylated PKI (33) had no appreciable effect on agonist-induced endocytosis but prevented the recycling of the internalized β1-AR (compare q and y to h in Fig. 4A). Because these agents might inadvertently inhibit other kinases (39), we used a dominant negative construct of the regulatory type 1 subunit (RⅠα) of cyclic AMP-dependent protein kinase (34) to inhibit the activity of PKA in HEK-293 cells. This construct (MT-REVAB) contains mutations in each of the two cyclic AMP binding sites in the RⅠα subunit to inactivate endogenous PKA or β1-AR-stimulated PKA activity. Co-expression of the WT β1-AR with MT-REVAB in the amounts described in Fig. 4C, completely inhibited basal PKA activity and reduced cyclic AMP-stimulated PKA activation in cell extracts by 96 ± 3% (data not shown).

Cell surface β1-AR expression was assessed by \(^{[3]H}\)CGP-12177 binding to cells that were exposed to an initial 30-min pulse with isoproterenol followed by a 3-h washout (Fig. 4C). Co-expression of β1-AR cDNA with the empty control vector resulted in 90 ± 3% restoration of the original cell surface β1-AR complement, whereas co-expression of β1-AR cDNA with the MT-REVAB vector reduced cell surface expression by 62 ± 5% (p < 0.01).

Recycling assays were carried out in a HEK-293 cell line stably expressing the MT-REVABneo construct in which the expression of the double mutant RⅠα subunit of PKA was under the control of the metallothionine promoter. Repression of MT-REVABneo expression in the absence of zinc ions, allowed the recycling of the internalized β1-AR (d→h' in Fig. 4A). Activation of MT-REVABneo expression by prior treatment of the cells with Zn\(^{2+}\) ions prevented the efficient recycling of the agonist-internalized β1-AR (k→p' in Fig. 4A). Zn\(^{2+}\) (1 μM) had no effect on the recycling of the WT β1-AR (data not shown).
Therefore, biochemical and genetic approaches indicate that PKA is involved in recycling of the internalized β1-AR.

PKA-mediated phosphorylation of GPCR has been implicated in functional effects ranging from desensitization (40, 41) or switching in G protein-coupling specificity from Gs to Gi (42). Therefore, we tested the effect of ablating or mimicking the phosphorylation state of Ser312 on these parameters. Agonist-mediated phosphorylation of ERK1/2 in the three β1-AR-expressing cell lines were comparable and were insensitive to pertussis toxin, suggesting that coupling of β1-AR to Gs was maintained (Fig. 5A). These data, when combined with those in Figs. 1–4 and (37) concerning the role of Ser312 in desensitization and resensitization of the β1-AR, indicate that the putative PKA phosphorylation site in the human β1-AR is exclusively

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involved in trafficking of the internalized β₁-AR in this cell line.

If PKA is involved in recycling, its effect is achieved through phosphorylation of the β₁-AR in response to agonist activation. Robust phosphorylation of the β₁-AR was observed within 10 min of exposing the cells to isoproterenol (Fig. 5B). We have already determined that phosphorylation of the β₁-AR occurs exclusively on serine residues (35). Phosphorylation of the β₁-AR in response to forskolin, a general activator of all isoforms of adenyl cyclase that activates PKA in a receptor-independent manner, increased the magnitude of receptor phosphorylation to about 15% of that attained by isoproterenol (Fig. 5C). H-89 pretreatment of cells abolished forskolin-induced β₁-AR phosphorylation, indicating that it was mostly due to PKA. H-89 reduced isoproterenol-mediated phosphorylation by about 13 ± 4%, which was significant (p < 0.05). In the next series of experiments, we sought to determine whether the catalytic subunit of PKA (cPKA) phosphorylates the WT β₁-AR in membranes and whether mutagenesis of the putative PKA-phosphorylated Ser^{312} to Ala affects this parameter (Fig. 5D). The data in Fig. 5D reveal that cPKA markedly increased the phosphorylation of the WT β₁-AR and that the majority of the incorporated ^32P was in Ser^{312}. Pretreatment of these membranes with 5 μM of PKI peptide 5 min prior to the addition of cPKA. After phosphorylation, the β₁-AR was immunoprecipitated and subjected to SDS-PAGE, followed by autoradiography and electronic counting.
carboxyl-terminal tail of the human β1-AR, there are six serine residues with an acidic amino acid in their proximity that are potential sites for phosphorylation by GRK. Therefore, by analogy to the β2-AR, the expected stoichiometry for GRK versus PKA in phosphorylating the β1-AR would be about 6:1 (or 85:15%), which is in close agreement with the magnitude by which H-89 inhibited the phosphorylation of the β2-AR (Fig. 5C). The data in Fig. 5D also indicate that pretreatment of the isolated membranes with isoproterenol, which promotes conformational changes in the receptor, had little effect on cPKA-mediated phosphorylation of the β1-AR. Therefore, it does not appear that steric hindrance of another phosphorylation site is caused by the Ser112 → Ala substitution; rather, cPKA is efficient in phosphorylating the agonist-unoccupied β2-AR. These data indicate that PKA can phosphorylate the β1-AR in cells in response to agonist activation and in isolated membranes, thereby providing a reversible signal that favors receptor recycling and recovery.

Many GPCRs undergo agonist-induced endocytosis (8, 14, 22, 23). Endocytosis seems to mediate opposite effects, namely resensitization (13–15, 19–22) and down-regulation of the GPCR (12, 14, 22, 23). The identification of PKA as the kinase involved in endocytic recycling of the β2-AR identifies a novel role for PKA in receptor resensitization. Because the activation of PKA is but a consequence of catecholamine-mediated activation of the β2-AR, this suggests that PKA is involved in homologous resensitization of the β2-AR. Another facet concerning the relationship between the chronology and localization of PKA-mediated phosphorylation of the β1-AR on the recycling itinerary of the agonist-occupied β1-AR is revealed in Fig. 5D. The data reveal that cPKA phosphorylation of the β1-AR is independent of agonist occupancy and occurs rapidly in the membranes. Therefore, it is conceivable that PKA-mediated phosphorylation of Ser112 preaddresses the trafficking itinerary of the agonist-internalized β2-AR toward recycling and resensitization.

Persistent activation of PKA in cells that endogenously express the β1-AR by chronic treatment with high levels of isoproterenol causes the destabilization of β1-AR mRNA that ultimately results in down-regulation of the β1-AR protein (44–46). This effect is countered by PKA-mediated desensitization of the β1-AR. These two apparently opposing effects of PKA, namely resensitization and mRNA destabilization of the β1-AR, highlight an unexpected but a potentially important function for PKA as a regulator (rheostat) that strives to stabilize the flow of the signal (current) generated by the β2-AR to a preset intensity (voltage). These findings also underscore an important physiological interplay between GRK- and PKA-mediated phosphorylation of this GPCR. Since the phosphorylation by GRK and PKA are reversible, homologous desensitization through GRK and homologous resensitization through PKA are capable of generating several cycles of receptor activation, desensitization, and recovery, which potentially can maintain the signaling output from cells with low density of β1-AR.

PKA is a plethoric regulator that is also involved in regulating the trafficking itinerary of many proteins such as the translocation of aquaporin-2 in response vasopressin in kidney cells and recycling of the N-methyl-D-aspartate receptor in cortical neurons (30, 47). The effect of PKA on recycling of the N-methyl-D-aspartate receptor highlights its potential role in heterologous resensitization of this receptor (30). Furthermore, PKA is involved in heterologous desensitization of the β2-AR and others (40, 41). Maturation of the four serines that are putative sites for phosphorylation by PKA in the β2-AR uncouples the receptor from Gq and prevents β2-AR-mediated activation of ERK1/2 (42). However, maturation of the corresponding serine in the human β1-AR caused no adverse effects on its coupling to Gq (37) (Fig. 1) or on β2-AR-mediated activation of ERK1/2 (Fig. 5A). The motifs around the PKA-phosphorylation sites in each of these receptors are different but appear to play a major role in dictating the outcome for each of these modifications. A better understanding of the extended motifs surrounding PKA phosphorylation sites is fundamental in understanding their role in biochemical regulation of signal transduction.
PKA-mediated Phosphorylation Triggers β1-AR Resensitization

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Role of the Cyclic AMP-dependent Protein Kinase in Homologous Resensitization of the β1-Adrenergic Receptor
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MDM2 binding induces a conformational change in p53 that is opposed by heat-shock protein 90 and precedes p53 proteasomal degradation.

Mark Sasaki, Linghu Nie, and Carl G. Maki

In Fig. 4 we reported that the p53-ubiquitin fusion protein has a mutant conformation (pAb1620+/pAb240−). This p53-ubiquitin fusion protein has been used to mimic p53 mono-ubiquitinated in its C terminus. Subsequent to our manuscript being accepted, we discovered that the p53-ubiquitin fusion protein we used has a deletion of valine 218 near the pAb240 epitope. We have subsequently generated a new p53-ubiquitin fusion protein with valine 218 intact and compared its conformation to that of wild-type p53. This involved immunoprecipitation with the wild-type (pAb1620) and mutant (pAb240) conformation-specific antibodies followed by immunoblotting for p53. We did not detect an appreciable difference in conformation between wild-type p53 and the p53-ubiquitin fusion in these subsequent experiments. Therefore, the observation that the p53-ubiquitin fusion protein has an altered conformation compared with wild-type p53 was made in error. It remains possible that MDM2-mediated ubiquitination of p53, particularly at lysines within the p53 DNA-binding domain, could alter p53 conformation. However, the suggestion that C-terminal mono-ubiquitination might hold p53 in a mutant conformation is not supported.

Role of the cyclic AMP-dependent protein kinase in homologous resensitization of the β1-adrenergic receptor.

Lidia A. Gardner, Noel M. Delos Santos, Shannon G. Matta, Michael A. Whitt, and Suleiman W. Bahouth

The images shown in Figs. 2A (p. 21138) and 4A (p. 21140) were obtained from slides prepared simultaneously from the same colony of cells. Panel c in Fig. 2A and panel K′ in Fig. 4A, due to an inadvertent error, were derived from slides that were not part of the set shown. The correct Fig. 2A, panel c, and Fig. 4A, panel K′, are shown below. The distribution of pixels in the revised panel c (Fig. 2A) and panel K′ (Fig. 4A) is similar to that in the corresponding published images. The legends for Figs. 2 and 4 and the text remain unchanged.

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