Role of the G Protein-coupled Receptor Kinase Site Serine Cluster in β2-Adrenergic Receptor Internalization, Desensitization, and β-Arrestin Translocation*

Received for publication, January 10, 2005, and in revised form, December 29, 2005 Published, JBC Papers in Press, January 3, 2006 DOI 10.1074/jbc.M500328200

David J. Vaughan, Ellen E. Millman, Veronica Godines, Jacqueline Friedman, Tuan M. Tran, Wenping Dai, Brian J. Knoll, Richard B. Clark, and Robert H. Moore

From the Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030; Department of Integrative Biology and Pharmacology, University of Texas Health Sciences Center, Houston, Texas 77030; and Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, Texas 77204

There is considerable evidence for the role of carboxyl-terminal serines 355, 356, and 364 in G protein-coupled receptor kinase (GRK)-mediated phosphorylation and desensitization of β2-adrenergic receptors (β2ARs). In this study we used receptors in which these serines were changed to alanines (SA3) or to aspartic acids (SD3) to determine the role of these sites in β-arrestin-dependent β2AR internalization and desensitization. Coupling efficiencies for epinephrine activation of adenylyl cyclase were similar in wild-type and mutant receptors, demonstrating that the SD3 mutant did not drive constitutive GRK desensitization. Treatment of wild-type and mutant receptors with 0.3 nM isoproterenol for 5 min induced ~2-fold increases in the EC50 for agonist activation of adenylyl cyclase, consistent with protein kinase A (PKA) site-mediated desensitization. When exposed to 1 μM isoproterenol to trigger GRK site-mediated desensitization, only wild-type receptors showed significant further desensitization. Using a phospho site-specific antibody, we determined that there is no requirement for these GRK sites in PKA-mediated phosphorylation at high agonist concentration. The rates of agonist-induced internalization of the SD3 and SA3 mutants were 44 and 13%, respectively, relative to that of wild-type receptors, but the SD3 mutant recruited enhanced nonvisual arrestins (1) at the plasma membrane, whereas the SA3 mutant did not. EGFP-β-Arrestin2 overexpression triggered a significant increase in the extent of SD3 mutant desensitization but had no effect on the desensitization of wild-type receptors or the SA3 mutant. Expression of a phosphorylation-independent β-arrestin 1 mutant (R169E) significantly rescued the internalization defect of the SA3 mutant but inhibited the phosphorylation of serines 355 and 356 in wild-type receptors. Our data demonstrate that (i) the lack of GRK sites does not impair PKA site phosphorylation, (ii) the SD3 mutation inhibits GRK-mediated desensitization although it supports some agonist-induced β-arrestin binding and receptor internalization, and (iii) serines 355, 356, and 364 play a pivotal role in the GRK-mediated desensitization, β-arrestin binding, and internalization of β2ARs.

The β2-adrenergic receptor (β2AR) is a member of the extended G protein-coupled receptor (GPCR) family. As with other cell membrane signaling receptors, the signaling capacity of β2ARs is rapidly attenuated after activation by agonist. This process, termed desensitization, is initiated by receptor phosphorylation by a variety of protein kinases including protein kinase A (PKA) and by members of the G protein-coupled receptor kinase family (GRKs). At low agonist concentrations, β2ARs undergo phosphorylation at serine 262 within the third intracellular loop (1), whereas under conditions of high occupancy by agonist, receptor phosphorylation is mediated by one or more GRKs in addition to PKA. GRK-mediated phosphorylation of residues within the receptor cytoplasmic tail triggers the translocation of nonvisual arrestins (β-arrestin 1 and β-arrestin 2) to the cell surface where they interact with phosphorylated receptors, causing receptor uncoupling from stimulatory G protein (2–4). β-Arrestins also interact directly with two structural components of clathrin-coated pits, clathrin and AP-2, which promote the endocytosis of β2ARs into early endosomes via clathrin-coated vesicles (5–7).

A small region between positions 355 and 364 in the cytoplasmic tail of the β2AR has been shown to be essential for agonist-mediated β2AR internalization, phosphorylation, and desensitization (8, 9). Using a phosphoserine-specific antibody (β2AR Ser(P)-355,356), it recently has been demonstrated that serines 355 and 364 are phosphorylated in an agonist concentration-dependent manner and that these residues are substrates for phosphorylation by both GRK2 and GRK5 in vivo, consistent with recently published mass spectrometry data (1, 10, 11). Mutation of serines 355, 356, and 364 to alanines almost completely eliminates receptor level desensitization and reduces GRK-mediated receptor phosphorylation by at least 90% (9). Furthermore, receptor internalization is significantly impaired, suggesting that phosphorylation of these sites is essential for high affinity β-arrestin interaction. However, in these studies we utilized β2ARs in which the PKA consensus sites were mutated and a 6-histidine tag was added to the carboxyl terminus (9), modifications that may independently alter receptor desensitization or cellular localization (12). Furthermore, although we have shown that modification of the PKA consensus sites does not affect GRK site phosphorylation (1), it is not known if the GRK sites have a hierarchal effect on PKA site phosphorylation.

The goal of the current study was to determine the specific effects of

---

8 This work was supported by National Institutes of Health Grants HL64934 to R. H. M. and GM031208 to R. B. C. and by American Heart Association, Texas affiliate Grant 0455072Y to B. J. K. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Pediatrics, Baylor College of Medicine, 6621 Fannin, CCC1040, Houston, TX 77030. Tel.: 832-824-3312; Fax: 832-825-4255; E-mail: rmoore@bcm.tmc.edu.

2 The abbreviations used are: β2AR, β2-adrenergic receptor; PKA, cyclic AMP-dependent protein kinase; GRK, G protein-coupled receptor kinase; AC, adenylyl cyclase; HXK, human embryonic kidney; EPR, epinephrine/ISO; SD, isoproterenol/AT, ascorbate/thiourea; CGP12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazole-2-one; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-3′-O-(thio)triphosphate.
GRK site mutations on β2AR desensitization and internalization and to explore the role of arrestin in these processes without other potentially confounding receptor modifications. We used receptors in which only the putative GRK sites (Ser-355, -356, and -364) have been mutated to either alanine (β2AR-SA3) to eliminate phosphorylation or to aspartic acid (β2AR-SD3), a substitution that may mimic the phosphorylated state. To accomplish these goals we studied the effects of GRK site mutations on receptor desensitization, internalization, cellular trafficking, and EGFP-β-arrestin 2 translocation. Furthermore, we determined if a phosphorylation-independent mutant of β-arrestin 1 (R169E) altered the internalization of mutant receptors or the GRK-mediated phosphorylation of wild-type receptors. Our data provide insights into the β2AR domains governing β-arrestin interaction, receptor internalization, and desensitization.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human embryonic kidney (HEK) 293 cells (purchased from American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Mutations were introduced into serines 355, 356, and 364 of human β2ARs expressing an amino-terminal FLAG epitope using the QuikChange kit (Stratagene, La Jolla, CA) per the manufacturer’s instructions. The mutants were sequenced throughout the entire coding region and epitope tag to ensure accuracy of the mutagenesis. Plasmids were stably transfected into HEK293 cells using FuGENE 6 transfection reagent (Roche Applied Science), and multiple stable clones were selected using 400 μg/ml G418 (Invitrogen) and analyzed with similar results.

EGFP-β-arrestin 2 cDNA was a gift from Dr. M. Caron, and the untagged wild-type β-arrestin 1 and β-arrestin 1-(R169E) cDNAs were gifts from Dr. V. Gurvich. HA-dynamin-1 K44A cDNA was a gift from Dr. S. Schmid. Monoclonal antibodies against the FLAG epitope (M2), the HA epitope, and β-arrestin 1 were obtained from Sigma, Berkeley Antibody Co. (Berkeley, CA), and BD Transduction Laboratories, respectively. The polyclonal antibodies against the β2AR cytoplasmic tail and β2AR phosphoserines 355 and 356 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Species-appropriate fluorescently tagged goat secondary antibodies and transferrin-Alexa594 were obtained from Molecular Probes (Eugene, OR).

Measurement of Equilibrium Binding Constants for Epinephrine and [125I]Labeled Cyanopindolol — The Kd values for 125I-labeled cyanopindolol and epinephrine (EPI) binding were determined for wild-type and mutant β2ARs as previously described (9, 13). All reactions were performed in triplicate using 0.2 or 1 μg of membrane protein for determinations of Kd and EPI displacement, respectively, and a range of 125I-labeled cyanopindolol concentrations from 1 to 200 nm. To determine the Kd for EPI binding, assays included 50 pm 125I-labeled cyanopindolol and 10 μM GTPγS, and the concentrations of EPI ranged from 0.1 to 100 μM. Nonspecific binding was measured by adding 1 μM alprenolol. The data were analyzed using GraphPad Prism software (San Diego, CA) and fitted to a one-component sigmoid curve using a Hill coefficient of 1.

Adenylyl Cyclase Assays—Adenylyl cyclase (AC) activity was determined as previously described (9, 14). In brief, 5 μg of membrane protein was incubated at 30 °C for 10 min in 100 μl of buffer containing 40 mM HEPES, pH 7.7, 6 mM MgCl2, 1 mM EDTA, 100 mM ATP, 1 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, 8 mM creatine phosphate, 16 units/ml creatine kinase, and 2 μCi of [α-32P]ATP (30 Ci/mmol; PerkinElmer Life Sciences). AC activities for six to eight concentrations of EPI were assayed in triplicate, and the EC50 and Vmax values were determined using GraphPad Prism software. Coupling efficiencies for EPI activation and β2AR desensitization were determined from the measurements of Kd and EC50 as previously described in detail (9, 15). The fractional activity remaining is the extent of receptor desensitization determined from the ratio of receptor coupling capacity in the desensitized relative to the naïve state and is calculated using equation 3 from Seibold et al. (9).

To determine whether EGFP-β-arrestin 2 overexpression enhances the desensitization of wild-type or mutant β2ARs, cells stably expressing wild-type β2ARs or the SA3 or SD3 mutants were grown for 24 h on 100-mm dishes and then transfected with 15 μg of empty EGFP vector or with plasmid DNA encoding EGFP-β-arrestin 2 using 22.5 μl of FuGENE 6 transfection reagent. Forty-eight hours later the efficiencies of transfections were determined by direct visualization using epifluorescence microscopy, and plates with a transfection efficiency of at least 70% were used for experimentation. The extent of arrestin overexpression was determined by Western blot analysis using a polyclonal antibody (3.0 μg/ml) that recognizes both β-arrestin 1 and 2 (Affinity BioReagents, Golden, CO) and quantified by densitometry. Cells were treated with AT (0.1 mM ascorbate/1 mM thiourea) with or without 1 μM isoprenaline (ISO) for 5 or 30 min before measurement of AC activity.

Measurement of β2AR Internalization—HEK293 cells stably expressing wild-type or mutant β2ARs were grown on poly-l-lysine-coated 24-well clusters. Cells were exposed to 5 μM ISO at 37 °C for varying times up to 30 min to promote β2AR internalization. In some experiments cells were pretreated for 60 min with either filipin (1 μg/ml) or Me6SO (1:1000). After agonist treatment, the cells were chilled and extensively with Dulbecco’s modified Eagle’s medium (DMEM) containing 20 mM HEPES, pH 7.4 (DMEM-H), and surface receptors were measured by incubation in DMEM-H containing 6 nM [125I]CGP12177 at 0 °C for 2 h followed by washing with cold DMEM-H. The monolayers were then harvested with 0.1% SDS and 0.1% Nonidet P-40, and the lysates were counted by scintillation spectroscopy. For every experiment, binding at each time point was measured in triplicate. The fraction of receptors left on the surface was plotted versus time of exposure, and the curves were fitted by nonlinear regression as previously described (16). First-order rate constants for receptor endocytosis were determined as previously described (16), and the level of statistical significance was determined using a paired Student’s t test. Endocytic rate constants are presented as the means ± S.E. of three independent experiments.

To determine the effects of β-arrestin 1 overexpression on receptor internalization, HEK293 cells growing on 35-mm dishes were transiently transfected using 0.6 μg of cDNA encoding wild-type β2ARs, β2AR-SA3, or β2AR-SDS and 2.4 μg of either empty vector, wild-type β-arrestin 1, or the β-arrestin 1-(R169E) mutant using FuGENE 6 transfection reagent with a DNA to FuGENE 6 ratio of 3 μg to 4.5 μl. After 24 h cells were split into 24-well clusters and allowed to grow an additional 24 h. Cells were then treated with AT alone or 10 μM ISO for 30 min, and receptor internalization was measured using [125I]CGP12177, as described above. The data shown are the means ± S.E. from three separate experiments performed in triplicate.

Fluorescence Microscopy—HEK293 cells growing on poly-l-lysine-coated coverslips in 6-well clusters were treated as indicated in the figure legends. In some experiments cells were fed transferrin-Alexa594 (50 μg/ml) as indicated. After treatment the cells were washed with phosphate-buffered saline (PBS) containing 1.2% sucrose (PBSS) and fixed with 3.6% paraformaldehyde in PBSS at 4 °C for 10 min. For studies of EGFP-β-arrestin 2 translocation, coverslips were immediately mounted after fixation. For other studies, fixed cells were permeabilized.
β2-Adrenergic Receptor Sites Mediating Desensitization

with 0.2% Triton X-100 and labeled with antibodies as previously described (17). We used the following concentrations of antibodies: monoclonal anti-FLAG (M2), 5 μg/ml; monoclonal anti-HA, 5 μg/ml; polyclonal anti-β2AR carboxyl terminus, 2 μg/ml; Alexa488-anti-mouse IgG, 1:200 dilution; Alexa594 anti-rabbit IgG, 1:500 dilution. The coverslips were mounted in Mowiol and viewed using a DeltaVision deconvolution microscopy system (Applied Precision Inc., Issaquah, WA) equipped with a Zeiss Axiovert microscope. Imaging was performed using a Zeiss 100× (1.4 numerical aperture) oil immersion lens, and sections were collected at an optical depth of 150 nm in the z-plane. Images were optimized using DeltaVision deconvolution software and transferred to Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) for the production of final figures.

Measurement of β2AR Phosphorylation—To measure PKA-mediated phosphorylation, HEK293 cells stably expressing wild-type β2ARs, β2AR-SD3, or β2AR-SA3 were grown to confluence in 12-well plates and stimulated with either AT alone or AT with 1 μM ISO for varying times up to 30 min. Cells were solubilized, and aliquots were resolved on SDS-PAGE and blotted (without receptor purification) as described in Tran et al. (1). Blots were probed first with monoclonal antibody against Ser-262 at 0.5 μg/ml and then stripped and probed with an antibody to the receptor carboxyl tail at a 1:5000 dilution to determine receptor levels. The blots were imaged using a CCD camera system (Syngene GeneGnome, Frederick, MD) and quantified using Syngene software. To compare data from different gels, an internal standard of PKA-phosphorylated β2AR was run with each gel. The data from one experiment were normalized to the internal standard of gel and corrected for receptor levels by normalizing to the carboxyl-tail antibody.

For studies of Ser-355 and -356, HEK293 cells growing in 6-well clusters were transfected with 0.6 μg of β2AR cDNA and 2.4 μg of either empty vector, wild-type β-arrestin 1, or the β-arrestin 1 (R169E) mutant using FuGENE 6 transfection as described above. The transfection mixture remained on cells for 48 h, at which time the cells were treated with 10 μM ISO or vehicle for 5 min. Cells were harvested, collected by centrifugation, and solubilized in lysis buffer as previously described (1). The solubilized cells were centrifuged for 30 min at 45,000 rpm in a Beckman 50Ti rotor, and the lysates were digested with 1500 units of N-glycosidase F (New England Biolabs, Beverly, MA) for 2 h at 37 °C before heating at 60 °C for 15 min in SDS sample buffer. Samples were resolved on identically loaded SDS-polyacrylamide gel electrophoresis (12%) gel and transferred to nitrocellulose for immunoblotting. The membranes were probed with a rabbit β2AR phosphoserine 355- and 356-specific antibody at a dilution of 1:500 or with the anti-carboxyl-tail antibody at a dilution of 1:1000 to determine total receptors and quantified by densitometry using SigmaGel software (SPSS Science, Chicago, IL). Separate blots with identical protein loads were probed with a monoclonal antibody against β-arrestin 1 at a dilution of 1:1000. The resulting figure depicts the extent of receptor phosphorylation at serines 355 and 356 normalized to total receptor load for each transfection as determined using the anti-receptor carboxyl-tail signal and compared with ISO-treated cells expressing receptors alone.

RESULTS

Determination of Coupling Efficiencies for Wild-type β2ARs and GRK Site Mutants—Because the introduction of mutations within the β2AR may alter its coupling to Gs-adenylyl cyclase, we measured the coupling efficiencies for wild-type and mutant receptors as previously described (9). We thought it possible that aspartic acid substitution of the GRK sites (β2AR-SD3) would increase agonist-independent β-arrestin binding to receptors and increase the EC50 for AC activation. Alternatively, we expected that β2AR-SA3 mutants would be normally coupled to AC. In testing multiple stable clones with varying expression levels, we found that both mutant receptors were as well coupled to Gs-AC as wild-type receptors and that there were no significant differences in their Kmax values for EPI binding. The coupling efficiencies, Kmax values for EPI binding, and EC50 values for AC activation are summarized in Table 1.

Desensitization of Wild-type and Mutant β2ARs—Next we tested the capacity of GRK site mutants to undergo receptor-level desensitization in response to agonist, which is defined experimentally by a rightward shift in the EC50 for AC activation after agonist treatment (9, 15). We have shown that treatment with low concentrations (0.1–0.3 nM) of agonist for 5 min induces the PKA-mediated phosphorylation of Ser-262, whereas higher concentrations (1 μM) are needed to stimulate GRK-mediated phosphorylation of Ser-355 and -356 (1). In this study we exposed cells to low and high concentrations of ISO to determine whether mutant β2ARs are impaired in PKA- or GRK-mediated desensitization. Cells expressing wild-type or mutant receptors were treated with 0.3 nM or 1 μM ISO for 5 min and washed, and membranes were purified over sucrose step gradients. These membrane preparations were then exposed to increasing concentrations of EPI, and dose response curves for AC activation were constructed (Fig. 1A). Treatment with 0.3 nM ISO triggered ∼2-fold increases in the EC50 values for AC activation for wild-type receptors and SA3 and SD3 mutants, consistent with PKA-mediated desensitization.

Wild-type receptors were significantly further shifted in their EC50 values (10-fold increase compared with base line) when treated for 5 min with a high concentration of ISO (1 μM). Alternatively, the additional increase in EC50 for both the SA3 and SD3 mutants was only 0.6-fold, indicating a marked impairment in receptor desensitization after a high concentration of agonist. The Vmax was reduced after a 5-min treatment with 1 μM ISO for wild-type and mutant receptors. However, we previously have shown that this decrease is downstream of receptor activation and does not reflect receptor-mediated events under conditions of receptor overexpression (9, 15). Desensitization data are summarized in Table 2.

To directly assess the effects of the GRK mutants on PKA-mediated desensitization, we measured the phosphorylation of Ser-262 using a specific monoclonal antibody (1). The phosphorylation of this site was not impaired in SA3 or SD3 mutant receptors after exposure to both low (0.1 nM, data not shown) and high (1 μM, Fig. 1B) concentrations of ISO. From 0 to 5 min, the level of phosphorylation of wild-type and mutant receptors was similar. However the phosphorylation of Ser-262 for both mutants remained fairly constant over time, whereas that of wild-type receptors decreased with prolonged agonist exposures such that by 30 min the fold stimulation in the wild-type, SA3, and SD3 receptors was 2-, 3-, and 4-fold over basal, respectively.

**TABLE 1**

Characterization of β2ARs stably expressed in HEK293 cells

| Cell line | EC50 for epinephrine (μM) | Epinephrine Kmax (nM) | Coupling efficiency ± S.E. |
|-----------|---------------------------|-----------------------|---------------------------|
| Wild type | 2.19 ± 0.290              | 693.0 ± 152.0         | 0.031 ± 0.006             |
| SD3       | 2.40 ± 0.459              | 364.8 ± 54.1          | 0.034 ± 0.006             |
| SA3       | 1.80 ± 0.208              | 639.3 ± 53.0          | 0.029 ± 0.003             |

Membranes from unstimulated HEK293 cells were used to measure the Kmax for epinephrine binding and the EC50 for epinephrine-stimulated adenyl cyclase activity, as described under “Experimental Procedures.” The coupling efficiencies of wild-type and mutant receptors were not significantly different (n = 4–9 experiments).
Effect of GRK Site Mutations on β₂AR Internalization—The finding that the β₂AR-SD3 mutant is severely defective in its capacity to undergo receptor-level desensitization while the coupling efficiency remained unchanged suggests that acidic substitutions at positions 355, 356, and 364 do not mimic the phosphorylated state. To further address this question, we measured agonist-induced internalization, which also is dependent on phosphorylation of these sites by GRK (9). By measuring the loss of binding of the hydrophilic radioligand [3H]CGP12177 over time, we were able to determine rate constants (kₜ) and extents of internalization for wild-type and mutant β₂ARs (Fig. 2). The extent of internalization of the β₂AR-SA3 mutant was markedly reduced by ≈75% compared with wild-type receptors. The rate and extent of internalization of the β₂AR-SD3 mutant was also reduced compared with wild-type receptors, but the extent of internalization of SD3 was more than 2-fold greater than that of the SA3 mutant. The kₜ values for wild-type, SD3, and SA3 receptors were 0.242, 0.106, and 0.031 min⁻¹, respectively, indicating that the rates of SD3 and SA3 mutants were 44 and 13% that of wild-type receptors, respectively.
Because β2AR-SD3 internalized more efficiently than β2AR-SA3, we next determined if mutant receptors supported the agonist-triggered translocation of β-arrestin to the cell surface, which is requisite for efficient receptor internalization (6, 18, 19). Cells stably expressing wild-type or mutant receptors and transiently expressing EGFP-β-arrestin 2 were exposed to agonist for 2 min or left untreated. Cells were immediately fixed, and the localization of EGFP-β-arrestin 2 was determined by fluorescence microscopy (Fig. 3). In the basal state, EGFP-β-arrestin 2 is localized diffusely throughout the cytosol regardless of which receptor type was expressed. In both wild-type and β2AR-SD3-expressing cells, agonist-mediated EGFP-β-arrestin 2 translocation to the cell surface was readily observed as clusters along the cell surface in images from the middle of cells or a punctate pattern in images from the bottoms of cells, consistent with β-arrestin 2 localization to clathrin-coated pits (20, 21). However, in β2AR-SA3-expressing cells, little or no EGFP-β-arrestin 2 translocation occurred after 2 min or even 15 min of agonist treatment (data not shown). These findings suggest that Ser-355, -356, and -364 are important for the interaction of receptor with β-arrestin and indicate that aspartic acid substitutions can support the translocation of β-arrestin to the cell surface.

The extent of receptor internalization can be affected by changes both in endocytosis and in recycling. We and others have shown that after agonist treatment β2ARs undergo endocytosis into early endosomes where they colocalize with internalized transferrin receptors (22, 23). Furthermore, GRK-mediated phosphorylation of a serine cluster within the carboxyl tail of the V2 vasopressin receptor regulates its endosome sorting and recycling (24). Thus, we considered it important to determine whether the increased internalization of β2AR-SD3 compared with β2AR-SA3 was due to endocytosis into an aberrant intracellular compartment or to a failure of receptors to recycle. For these studies we utilized deconvolution microscopy to monitor the subcellular distribution of β2ARs and transferrin during endocytosis and recycling (Fig. 4).

In all experiments endosomes were labeled by feeding cells with Alexa594-transferrin (50 μg/ml) for 15 min simultaneously with agonist treatment. In the basal state, wild-type and mutant β2ARs were completely localized to the cell surface. After a 15-min exposure to ISO, wild-type receptors and the SD3 and SA3 mutants internalized into punctate endosomes, where they largely overlapped with pulsed transferrin. As expected, agonist triggered the redistribution of only a small amount of β2AR-SA3 into vesicles, and most of the receptors remained localized to the plasma membrane. After removal of agonist, wild-type and mutant β2ARs were largely recycled to the plasma membrane within 30 min. Direct measurement of β2AR-SD3 recycling using radioligand binding confirmed that its recycling is similar in rate to that of wild-type receptors (data not shown). For the SA3 mutant, the immuno-localization data could not be corroborated using radioligand binding techniques to measure recycling due to its limited internalization. Together these data indicate that the enhanced internalization of the β2AR-SD3 mutant compared with β2AR-SA3 is not due to a defect of receptor recycling or to trafficking through an aberrant intracellular pathway.

β2ARs are known to internalize in a dynamin-dependent manner via clathrin-coated vesicles (23, 25–27). However, a recent report shows that GRK-phosphorylated β2ARs internalize via clathrin-coated vesicles, whereas PKA phosphorylation induces β2AR internalization through caveolae (28). To determine whether this is the case for β2ARs, we used two treatments to disrupt dynamin- or caveolae-dependent endocytic pathways in cells expressing wild-type receptors or receptors mutated at their GRK sites. First, we transiently expressed a dominant negative mutant of dynamin (dynK44A), which inhibits clathrin-mediated endocytosis and also caveolae internalization (28, 29). In other experiments we pretreated cells with the caveolae inhibitor filipin in a manner that inhibits the internalization of GRK site defective β2ARs in this cell line (data not shown). As seen in Fig. 5A, expression of dynamin-K44A almost completely inhibited the internalization of wild-type and mutant β2ARs after a 15-min exposure to agonist, whereas filipin had no effect on the internalization of wild-type or mutant receptors (Fig. 5, B and C). These data indicate that PKA-mediated phosphorylation in the absence of GRK site phosphorylation does not induce β2AR endocytosis via caveolae.

Overexpressing EGFP-β-Arrestin 2 Enhances the Desensitization of β2AR-SD3—Our observations that β2AR-SD3 internalize to an extent that is approximately twice that of β2AR-SA3 and are able to support the agonist-stimulated translocation of β-arrestin 2 to the cell surface suggest that the SD3 mutant is able to interact with β-arrestin 2, albeit with an affinity that is below that necessary for GRK site receptor desensitization. If true, we predicted that under conditions of β-arrestin overexpression the extent of desensitization of the SD3 mutant after a high concentration of agonist would increase. To test this hypothesis, we transiently expressed EGFP-β-arrestin 2 in cells stably expressing wild-type or mutant β2ARs using a protocol that resulted in a transfection efficiency of at least 70% (data not shown) and extents of overexpression that ranged from 8- to 15-fold (Fig. 6). As shown in Table 3, after a 5-min treatment with agonist, we observed a 39% greater right
underestimate the true effect of β-arrestin 2 overexpression on receptor desensitization by as much as 30% given the fraction of untransfected cells. However, it is unlikely that higher transfection efficiencies would alter the results in the case of wild-type or SA3 receptors given the small differences in EC50 shifts we observed. Nor would we expect that the level of desensitization of βAR-S3D would approach that seen with wild-type receptors even with higher levels of β-arrestin overexpression.

**TABLE 3**

Effect of EGFP-β-arrestin 2 overexpression on β2AR desensitization

| Cell line          | -Fold shift EC50 (ISO 5 min) | -Fold shift EC50 (ISO 30 min) |
|--------------------|------------------------------|------------------------------|
| Wild-type          | 10.07 ± 1.21                 | 11.73 ± 1.70                 |
| β-Arrestin 2       | 10.23 ± 1.34                 | 13.60 ± 1.62                 |
| SD3                | 3.38 ± 0.34                  | 3.37 ± 0.43                  |
| β-Arrestin 2       | 4.69 ± 0.13*                 | 4.78 ± 0.82                  |

*a* Significantly different from vector control as determined by paired t test, *p* < 0.05, *n* = 3–4 for each condition.

FIGURE 5. Internalization of mutant β2ARs is dynamin-dependent. A, to determine whether GRK site mutations alter dynamin-dependent internalization of β2ARs, HEK293 cells expressing the indicated β2ARs were transfected to transiently express HA-dynamin-K44A. Forty-eight hours later cells were treated with 5 μM ISO for 15 min, fixed, and labeled with antibodies against the HA epitope (to identify dynamin-expressing cells) and against the β2AR cytoplasmic tail. Cells were imaged using deconvolution microscopy. The expression of mutant dynamin almost completely inhibited the internalization of wild-type and GRK site mutant β2ARs (for comparison, see Fig. 5B). Scale bar = 10 μm. B, HEK293 cells stably expressing wild-type or mutant β2ARs were pretreated for 60 min with 1 μg/ml filipin or vehicle (Me2SO, 1:1000), then exposed to 5 μM ISO for 15 min. After fixation, cells were labeled with the anti-carboxyl-tail antibody and imaged as described. Scale bar = 10 μm. C, to assess if filipin alters the rate of wild-type mutant β2AR internalization, cells were pretreated with filipin (dashed lines) or Me2SO (solid lines) as above and then exposed to 5 μM ISO for varying times up to 30 min. Internalization of wild-type β2ARs (●), β2AR-S3D (○), and β2AR-SA3 (▲) were measured as previously described. Each point represents the means ± S.E. of three independent experiments performed in triplicate.

FIGURE 6. EGFP-β-arrestin 2 overexpression in HEK293 cells stably expressing wild-type (WT) or mutant β2ARs. Cells were transfected to transiently express either EGFP alone (○) or EGFP-β-arrestin 2 (▲). Cells were immediately lysed, and proteins were resolved by SDS-PAGE or were treated as indicated in Table 3. Cellular extracts were probed with a pan-arrestin polyclonal antibody to determine the extent of overexpression of EGFP-β-arrestin 2 and the expression of endogenous β-arrestin 1 and 2 and quantified by densitometry. Shown is a representative Western blot.

shift in the EC50 for AC activation for β2AR-S3D in cells overexpressing EGFP-β-arrestin 2 as compared with vector control transfectants (*p* < 0.05, *n* = 4). After a 30-min treatment, there was a 42% greater right shift in the EC50 in cells overexpressing EGFP-β-arrestin 2, although in this case the difference was not statistically significant (*p* = 0.14, *n* = 4). Overexpressing EGFP-β-arrestin 2 had little or no effect on the desensitization of either wild-type receptors or the SA3 mutants after either treatment period (*n* = 3). It should be noted that these results likely
**DISCUSSION**

Based on crystallographic studies of rhodopsin and visual arrestin as well as mutagenesis studies of nonvisual arrestins, current data support a multisite model of GPCR-arrestin interaction, with an activation recognition site and a phosphorylation recognition site that synergistically mediate high affinity binding of arrestin to receptor (34–39). It has been recently shown for the leukotriene B4 receptor (40), our data showed normal receptor coupling to AC and localization to the plasma membrane in the naïve state. Furthermore, the SD3 mutant also was impaired in GRK-mediated desensitization, similar to the SA3 mutant, but both mutants showed full PKA-mediated desensitization and phosphorylation (Fig. 1). Second, internalization of both mutants was significantly reduced compared with wild-type receptors, although the SD3 mutations allowed much better internalization than the SA3 mutations (56% reduction in the rate of internalization for SD3 versus 87% reduction for SA3; Fig. 2). Third, the SA3 mutant showed no detectable translocation of EGF-p-β-arrestin 2 to the plasma membrane, indicating impaired receptor-β-arrestin binding, whereas the SD3 mutants supported the translocation of EGF-p-β-arrestin 2 in an agonist-dependent manner, consistent with its partial internalization (Fig. 3).

In this study overexpression of wild-type β-arrestin 1 did not increase the internalization of the β2-AR-SA3 mutant (Fig. 8). This result supports the model of two independent arrestin binding sites on the β2AR that are individually of low affinity for arrestin, and with our SA3 mutant, overexpression of β-arrestin was not sufficient to overcome this low affinity interaction. This finding is in contrast to two reports in which β-arrestin overexpression was found to rescue the internalization of phosphorylation-defective receptors (18, 41). However, these mutants differed from ours, one being truncated at cysteine 341 (Thr-341) and lacking the carboxyl terminus, whereas the other receptor (phos-) lacked all 11 potential serine/threonine residues in the carboxyl tail (including Ser-355, -356, and -364) and the consensus PKA sites. The rescued internalization of T341 after β-arrestin overexpression suggests that in the absence of phosphorylation, the carboxyl terminus sterically inhibits β-arrestin interaction with the β2AR at other sites. This prediction is consistent with the multisite model of β-arrestin-receptor interaction and our studies of the β2AR-SA3 mutant. Based on previous studies of the phos- mutant, we would have expected that β-arrestin 1 overexpression similarly would rescue the internalization of the β2AR-SA3 mutant. However, recent published data based on fluorescence resonance energy transfer directly show that GRK site

---

**FIGURE 7. Effect of β-arrestin 1 overexpression on GRK site phosphorylation.** HEK293 cells were transfected to transiently co-express wild-type β2ARs and either vector alone, wild-type β-arrestin 1, or the β-arrestin 1-(R169E) mutant. Forty-eight hours after the transfection, cells were treated with vehicle or ISO (10 μM) for 5 min then immediately chilled and lysed. Cellular extracts were probed with antibodies against phospho-serines (pS) 355 and 356 to determine GRK site phosphorylation, the β2AR cytoplasmic tail to determine total receptor load, or β-arrestin 1 to determine the extent of overexpression. Representative Western blots from one experiment. Endogenous β-arrestin 1 was barely detectable with the short exposure times required to detect overexpression. The extents of phosphorylation were normalized to total β2AR load for each experiment. The extents of phosphorylation were compared with that of ISO-treated vector-transfected cells, which was set to 1.0. Results represent the means ± S.D. for three independent experiments. The asterisk signifies p < 0.05 as compared with agonist treated, vector-transfected cells. **WT**, wild type.

---

**FIGURE 8. Effect of β-arrestin 1 overexpression of β2AR internalization.** HEK293 cells were transfected to transiently co-express the indicated β2ARs and vector alone as a control, wild-type β-arrestin 1, or β-arrestin 1-(R169E). Twenty-four hours later cells were split into 24-well clusters and incubated in media for an additional 24 h. Cells then were treated with vehicle or ISO (10 μM) for 30 min. β2AR internalization was measured using [3H]CGP12177 as described. The resulting charts represent the mean ± S.E. from three independent experiments performed in triplicate. The asterisk signifies p < 0.05 compared with wild-type vector transfectants, and the double asterisk signifies p < 0.05 compared with SA3 vector. The expression of the β-arrestin 1-(R169E) mutant significantly increased the internalization of β2AR-SA3. The extent of overexpression of wild-type β-arrestin 1 and the β-arrestin 1-(R169E) mutant could not be determined due to the low signal of endogenous β-arrestin 1, but their expression levels were similar to that observed in Fig. 7A and did not vary among receptor types (data not shown).
phosphorylation is required for β-arrestin binding to β_{2}ARs and that phosphorylation-deficient (phos-) receptors are unable to support this interaction (43). Our data are consistent with these published results and localize Ser-355–364 as key residues for receptor interaction with β-arrestin.

Recently, β-arrestin mutants were discovered that bind GPCRs in an agonist-dependent, phosphorylation-independent manner. Studies of these β-arrestin mutants indicate that β-arrestin undergoes a conformational shift, exposing a polar core in its N-domain cavity, thus allowing interaction with phosphorylated residues of GPCRs. A key residue in this core of visual arrestin, Arg-175 (or its cognate in β-arrestin 1 (Arg-169)) directly binds phosphate groups attached to receptors (44–46). Charge reversal of this arginine creates a mutant that can interact with agonist-activated receptors in a phosphorylation-independent manner (45). We utilized the remarkable properties of this β-arrestin mutant to further probe the nature and specificity of the receptor-β-arrestin interaction. Previously published data indicate that the expression of β-arrestin 1–(R169E) significantly reduces total agonist-mediated β_{2}AR phosphorylation as determined by nonselective 32P incorporation, probably by competitively inhibiting GRK-receptor interactions (32).

Our data employing a phosphosine site-specific antibody (Fig. 7) showed an ~60% reduction in GRK site phosphorylation, thus extending previous results and selectively localizing this inhibition, at least in part, to Ser-355 and -356. Furthermore, mutant β-arrestin specifically increased the internalization of β_{2}AR-SA3 mutants (Fig. 8) but not wild-type receptors or SD3 mutants. These results indicate that the β-arrestin mutant binds to the activation recognition site of the β_{2}AR in the absence of phosphorylation of Ser-355, -356, and -364 and suggest that phosphate groups on these serines directly interact with residues within the polar core of β-arrestin.

Given that the β_{2}AR-SD3 mutant recruits EGFP-β-arrestin 2 to the plasma membrane and undergoes substantial agonist-mediated internalization, albeit at a reduced rate, it is unclear why receptor-level desensitization of this mutant after high agonist concentrations was not greater. The most plausible explanation for this apparent discrepancy in β-arrestin translocation and β_{2}AR desensitization is that the aspartic acid substitutions allow β-arrestin binding to the receptor cytoplasmic tail that is sufficient to promote receptor internalization but not the high affinity binding that may be required for the uncoupling of receptors from G_{s}, a defect that can be overcome in part by the overexpression of β-arrestin 1. Such a mechanistic distinction between β-arrestin-dependent receptor internalization and desensitization has recently been described for the chemokine receptor CCR5 (42). We cannot exclude the possibility that aspartic acid substitutions of Ser-355, -356, and -364 confers a receptor state that interferes with β-arrestin interaction by nonspecific, delocalized effects on other receptor domains.

One of the interesting possibilities regarding GRK-PKA phosphorylation site cross-talk is that there is hierarchy. We previously have shown that the elimination of the PKA consensus sites does not alter the concentration dependence, kinetics, or extent of GRK site phosphorylation (1). In the present work (Fig. 1B) we now show that the GRK site mutants show an initial rate of PKA site (Ser-262) phosphorylation that is similar to wild-type receptors. Interestingly, with prolonged agonist treatments mutant receptors actually show sustained PKA site phosphorylation, whereas that of wild-type receptors decays over time. We speculate that this decay in wild-type receptors may be caused by β-arrestin binding and internalization of the receptor, thus inhibiting PKA phosphorylation of Ser-262.

Prior studies have suggested that receptor phosphorylation may regulate the endocytic pathway and cellular trafficking of GPCRs (12, 24, 28). In this study we addressed the role of the GRK sites on the endocytosis and endosome sorting of β_{2}ARs. We found that the endocytosis of mutant receptors, although reduced, proceeds via a dynamin-dependent manner through clathrin-coated vesicles, similar to wild-type receptors (Fig. 5A). Similarly, the lack of effect of filipin on wild-type and mutant receptor internalization indicate that GRK site mutations do not induce a shift of receptors to a caveoleae-mediated pathway (Fig. 5, B and C). Furthermore, GRK site mutations have no apparent effect on the colocalization of receptors and transferrin in early endosomes or on their endosome sorting to the rapid recycling pathway (Fig. 4). Together, these data indicate that GRK site phosphorylation does not regulate the intracellular trafficking of β_{2}ARs.

In summary, by studying β_{2}ARs with both alanine and aspartic acid substitutions of the GRK sites, we have provided insights into the role of these sites in promoting receptor-β-arrestin interactions. Our results support the hypothesis that Ser-355, -356, and -364 serve as the phosphorylation recognition domain for β-arrestin binding. Furthermore, our data suggest that phosphate groups on one of these sites directly interact with the polar core of the N-domain of β-arrestin and that negatively charged aspartic acids may be sufficient to at least weakly promote this interaction. Finally, our data raise the possibility that β-arrestin desensitization requires a higher affinity interaction with β-arrestin than is necessary for receptor internalization.

REFERENCES
1. Tran, T. M., Friedman, J., Qunailti, E., Raameur, F., Moore, R. H., and Clark, R. B. (2004) Mol. Pharmacol. 65, 196–206
2. Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680
3. Lefkowitz, R. J. (1993) Cell 74, 409–412
4. Krupnick, J. G., and Benovic, J. L. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 289–319
5. Lin, F. T., Krueger, K. M., Kendall, H. E., Daaka, Y., Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1998) J. Biol. Chem. 272, 31051–31057
6. Goodman, O. B. Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
7. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3712–3717
8. Hausdorf, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2979–2983
9. Seibold, A., Williams, B., Huang, Z. F., Friedman, J., Moore, R. H., Knoll, B. L., and Clark, R. B. (2000) Mol. Pharmacol. 58, 1162–1173
10. Millim, E. E., Rosenfeld, J. L., Vaughan, D. J., Nguyen, J., Dai, W., Alpizar-Foster, E., Clark, R. B., Knoll, B. L., and Moore, R. H. (2004) Br. J. Pharmacol. 141, 277–284
11. Trester-Zelditz, M., Burlingame, A., Kobikia, B., and von Zastrow, M. (2005) Biochemistry 44, 6133–6143
12. Cao, T. T., Deacon, H. W., Reczek, D., Bretsch, A., and von Zastrow, M. (1999) Nature 401, 286–289
13. January, B., Seibold, A., Allal, C., Whaley, R. S., Knoll, B. L., Moore, R. H., Dickey, B. F., Barber, R., and Clark, R. B. (1998) Br. J. Pharmacol. 123, 701–711
14. Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1442–1446
15. Whaley, B. S., Yuan, N., Birnbaumer, L., Clark, R. B., and Barber, R. (1994) Mol. Pharmacol. 45, 481–489
16. Morrison, K. J., Moore, R. H., Carsrud, N. D. V., Millman, E., Trial, J., Clark, R. B., Barber, R., Tuvin, M., Dickey, B. F., and Knoll, B. J. (1996) Mol. Pharmacol. 50, 692–699
17. Moore, R. H., Millman, E. E., Alpizar-Foster, E., Dai, W., and Knoll, B. J. (2004) J. Cell Sci. 117, 3107–3117
18. Ménard, L., Ferguson, S. S. G., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) Mol. Pharmacol. 51, 800–808
19. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1601–1606
20. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) J. Biol. Chem. 275, 23120–23126
21. Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1997) J. Biol. Chem. 272, 32507–32512
22. von Zastrow, M., and Kobikia, B. K. (1992) J. Biol. Chem. 267, 3530–3538
23. Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angeles, K., Trial, J., Carsrud, N. D. V., Dickey, B. F., and Knoll, B. J. (1995) J. Cell. Sci. 108, 2983–2991
24. Innamorati, G., Sadegh, H., Tran, N. T., and Birnbaumer, M. (1998) Proc. Natl. Acad. Sci.
25. Zhang, J., Ferguson, S. S. G., Barak, L. S., Menard, L., Caron, M. G., and Ferguson, S. G. (1996) J. Biol. Chem. 271, 18302–18305
26. Gagnon, A. W., Kallal, L., and Benovic, J. L. (1998) J. Biol. Chem. 273, 6976–6981
27. von Zastrow, M., and Kobilka, B. K. (1994) J. Biol. Chem. 269, 18448–18452
28. Rapacciuolo, A., Suvarna, S., Barki-Harrington, L., Luttrell, L. M., Cong, M., Letowski, R. J., and Rockman, H. A. (2003) J. Biol. Chem. 278, 35403–35411
29. Henley, J. R., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (1998) J. Cell Biol. 141, 85–99
30. Celver, J., Vishnivetskiy, S. A., Chavkin, C., and Gurevich, V. V. (2002) J. Biol. Chem. 277, 9043–9048
31. Kovoor, A., Celver, J., Abdryashitov, R. I., Chavkin, C., and Gurevich, V. V. (1999) J. Biol. Chem. 274, 6831–6834
32. Pan, L., Gurevich, E. V., and Gurevich, V. V. (2003) J. Biol. Chem. 278, 11623–11632
33. Gabilondo, A. M., Hegler, J., Krasel, C., Boivin-Jahns, V., Hein, L., and Lobse, M. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12285–12290
34. Gurevich, V. V., and Benovic, J. L. (1993) J. Biol. Chem. 268, 11628–11638
35. Gurevich, V. V., Dion, S. B., Onorato, J. P., Ptasinski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M., and Benovic, J. L. (1995) J. Biol. Chem. 270, 720–731
36. Krupnick, J. G., Gurevich, V. V., Scheppers, T., Hamm, H. E., and Benovic, J. L. (1994) J. Biol. Chem. 269, 3226–3232
37. Gurevich, V. V., and Gurevich, E. V. (2004) Trends Pharmacol. Sci. 25, 105–111
38. Vishnivetskiy, S. A., Schubert, C., Clísimo, G. C., Gurevich, Y. V., Velez, M. G., and Gurevich, V. V. (2000) J. Biol. Chem. 275, 41049–41057
39. Raman, D., Osawa, S., Gurevich, V. V., and Weiss, E. R. (2003) J. Neurochem. 84, 1040–1050
40. Jala, V. R., Shao, W. H., and Haribabu, B. (2005) J. Biol. Chem. 280, 4880–4887
41. Ferguson, S. S., Downey, W. E., III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–366
42. Huttenrauch, F., Nitzki, A., Lin, F. T., Honing, S., and Oppermann, M. (2002) J. Biol. Chem. 277, 30769–30777
43. Krasel, C., Bünemann, M., Lorenz, K., and Lohse, M. J. (2005) J. Biol. Chem. 280, 9528–9535
44. Granzin, J., Wilden, U., Choe, H. W., Labahn, J., Krafft, B., and Buldt, G. (1998) Nature 391, 918–921
45. Gurevich, V. V., and Benovic, J. L. (1997) Mol. Pharmacol. 51, 161–169
46. Hirsch, J. A., Schubert, C., Gurevich, V. V., and Sigler, P. B. (1999) Cell 97, 257–269