

A Central Role for β -Arrestins and Clathrin-coated Vesicle-mediated Endocytosis in β_2 -Adrenergic Receptor Resensitization

DIFFERENTIAL REGULATION OF RECEPTOR RESENSITIZATION IN TWO DISTINCT CELL TYPES*

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G protein-coupled receptor (GPCR) sequestration to endosomes is proposed to be the mechanism by which G protein-coupled receptor kinase (GRK)-phosphorylated receptors are dephosphorylated and resensitized. The identification of β -arrestins as GPCR trafficking molecules suggested that β -arrestins might represent critical determinants for GPCR resensitization. Therefore, we tested whether β_2 -adrenergic receptor (β_2 AR) resensitization was dependent upon β -arrestins and an intact clathrin-coated vesicle endocytic pathway. The overexpression of either the β -arrestin 1-V53D dominant negative inhibitor of β_2 AR sequestration or dynamin I-K44A to block clathrin-coated vesicle-mediated endocytosis impaired both β_2 AR dephosphorylation and resensitization. In contrast, resensitization of a sequestration-impaired β_2 AR mutant (Y326A) was reestablished following the overexpression of either GRK2 or β -arrestin 1. Moreover, β_2 ARs did not resensitize in COS-7 cells as the consequence of impaired sequestration and dephosphorylation. However, β_2 AR resensitization was restored in these cells following the overexpression of β -arrestin 2. These findings demonstrate, using both loss and gain of function paradigms, that β_2 AR dephosphorylation and resensitization are dependent upon an intact sequestration pathway. These studies also indicate that β -arrestins play an integral role in regulating not only the desensitization and intracellular trafficking of GPCRs but their ability to resensitize. β -Arrestin expression levels appear to underlie cell type-specific differences in the regulation of GPCR resensitization.

Reestablishment of the responsiveness of G protein-coupled receptors (GPCRs)¹ after agonist-mediated desensitization is a multifaceted event involving both cellular and biochemical processes (1–3).

Sequestration, the cellular event leading to GPCR endocytosis and recycling, is initiated by the agonist-promoted mobilization of cell surface receptors to an intracellular vesicular compartment, probably endosomes (4–7). Receptors internalized via clathrin-coated vesicles (CCVs) and/or caveolae are thought to be resensitized in endosomes following their dephosphorylation by a membrane-associated phosphatase that exhibits specificity for G protein-coupled receptor kinase (GRK)-phosphorylated GPCRs residing in an acidified endosomal environment (8–10). Subsequently, these dephosphorylated receptors must be mobilized back to the cell surface to reestablish normal receptor signaling (7, 10). However, the molecular mechanisms contributing to each of these events and their relative contributions to the resensitization of GPCR responsiveness are only now becoming recognized.

A role for receptor phosphorylation in the sequestration process was suggested by the observation that overexpression of G protein-coupled receptor kinase 2 (GRK2) in cells expressing the m2 muscarinic acetylcholine receptor could augment agonist-mediated internalization (11). Additionally using a sequestration-defective β_2 -adrenergic receptor (β_2 AR) mutant (Y326A), GRK-mediated phosphorylation, and β -arrestin binding, the same molecular intermediates required for receptor desensitization were demonstrated to initiate β_2 AR endocytosis (12, 13). These studies also demonstrated that GRK phosphorylation was not absolutely required for β_2 AR sequestration (12, 13) but served to increase the affinity of the receptor for β -arrestins, which functioned as β_2 AR trafficking molecules (13). A role of β -arrestin in wild-type β_2 AR sequestration was established using β -arrestin mutants, which specifically impaired wild-type receptor sequestration without altering the ability of the receptor to become phosphorylated or desensitized (13).

Recently, we showed that GPCRs can utilize several distinct endocytic pathways (14). However, in the case of the β_2 AR, a growing body of evidence supports the idea that the endocytic route employed by this receptor is mainly the same CCV-mediated pathway utilized by constitutively recycling transferrin and low density lipoprotein receptors (4, 5, 14–16). An important step in CCV endocytosis is the GTP hydrolysis-dependent pinching off of the vesicles by a large GTPase, dynamin (15, 17–20). Dynamin mutants defective in GTP binding, such as dynamin I-K44A, specifically block clathrin-mediated endocytosis (21–23). We have used this dynamin mutant to demonstrate that agonist-promoted internalization of the β_2 AR occurs via dynamin-dependent CCV-mediated endocytosis (14). Moreover, we demonstrated that β -arrestins serve as adaptor proteins specifically targeting receptors to CCVs (12–14). Biochemical studies have now shown a direct interaction between β -arrestins and clathrin triskelions (24–26). Several recent reports suggest that GRK-mediated phos-

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¹ The abbreviations used are: GPCR, G protein-coupled receptor; β_2 AR, β_2 -adrenergic receptor; CCV, clathrin-coated vesicle; GRK, G protein-coupled receptor kinase; HEK 293 cells, human embryonic kidney cells; PKA, cAMP-dependent protein kinase; Iso, isoproterenol.

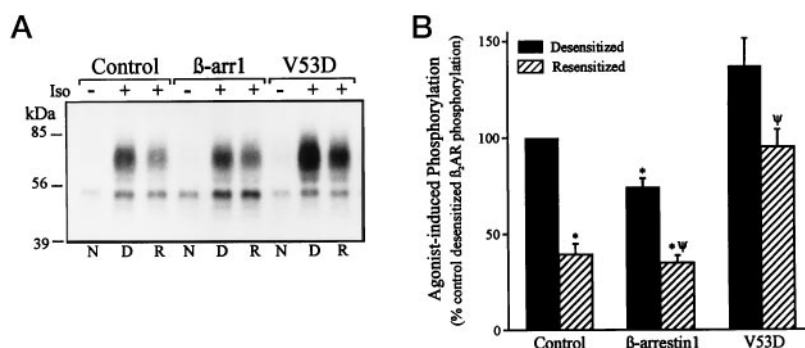


FIG. 1. The effect of β -arrestin 1 and β -arrestin 1-V53D on β_2 AR phosphorylation and dephosphorylation. HEK 293 cells were transiently transfected to overexpress the β_2 AR in the absence (890 ± 150 fmol/mg protein) or presence of either coexpressed β -arrestin 1 (1050 ± 220 fmol/mg protein) or β -arrestin 1-V53D (650 ± 140 fmol/mg protein). A, an autoradiograph from a representative experiment showing the whole cell phosphorylation and dephosphorylation of the β_2 AR. Cells were preincubated 10 min in serum-free medium at 37°C in the absence (naive (N)) or presence (desensitized (D), resensitized (R)) of $10\ \mu\text{M}$ Iso, washed three times on ice, and either allowed to resensitize (R) for 20 min at 37°C or kept on ice (N, D). Each lane was loaded with equivalent amounts of receptor protein as described under "Experimental Procedures". B, the mean \pm S.D. (bars) of four different experiments quantified by PhosphorImager analysis. In these experiments, the data were normalized to the agonist-induced control β_2 AR phosphorylation. *, $p < 0.05$ versus desensitized control β_2 AR phosphorylation; ψ , $p < 0.05$ versus matched desensitized β_2 AR phosphorylation.

phorylation followed by β -arrestin binding may represent a common mechanism required for the sequestration of many other GPCRs (11–14, 27–30).

The identification of β -arrestins as GPCR-specific endocytic trafficking proteins provides the first opportunity to directly assess, using GPCR-interacting proteins, the hypothesis that sequestration and more importantly normal β -arrestin function (as a GPCR-specific adaptor protein) is absolutely required for normal β_2 AR dephosphorylation and resensitization. In addition, we examine, using dynamin I-K44A as an inhibitor of CCV-mediated endocytosis, whether β_2 AR resensitization is dependent upon an intact CCV endocytic pathway. Moreover, we establish that β_2 AR resensitization in different cell types is regulated by differences in the endogenous expression levels of β -arrestin proteins.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney cells (HEK 293) and COS-7 cells were provided by the American Type Culture Collection. Tissue culture media and fetal bovine serum were obtained from Life Technologies, Inc. Isoproterenol (Iso) was purchased from Research Biochemicals Inc. Anti-hemagglutinin 12CA5 monoclonal antibody was from Boehringer Mannheim, anti-FLAG M2 antibody was from Eastman Kodak Co., and fluorescein-secondary antibody was from Sigma. Dithio-bis(succinimidylpropionate) (Lomant's reagent, DSP) was obtained from Pierce. [^{125}I]Pindolol, [^3H]adenine, [^{32}P]ATP, [^3H]ATP, [^{14}C]cAMP, and [^{32}P]orthophosphate were purchased from NEN Life Science Products.

Cell Culture and Transfection—HEK 293 and COS-7 cells were grown in Eagle's minimal essential medium with Earle's salt and Dulbecco's modified Eagle's medium, respectively, supplemented with heat-inactivated fetal bovine serum (10%, v/v) and gentamicin ($100\ \mu\text{g}/\text{ml}$). The cells were transiently transfected using a modified calcium phosphate method (31) as described previously (12, 13).

Receptor Expression—Receptor expression was measured using saturating concentrations of [^{125}I]pindolol ($\sim 1\ \text{nM}$) at 30°C for 30 min (12). Bound ligand was separated on glass fiber filters (Whatman, GF/C) by vacuum filtration and counted in a γ -counter. Protein concentrations were determined using a Bio-Rad assay kit with bovine serum albumin as the standard.

Sequestration—Receptor sequestration was assessed by flow cytometry as described previously (32). In brief, sequestration was defined as the fraction of total cell surface receptors that, after exposure to agonist, are removed from the plasma membrane and thus are not accessible to antibodies from outside the cell. The cells were exposed to $10\ \mu\text{M}$ isoproterenol before antibody staining.

β -Arrestin Co-immunoprecipitation—HEK 293 cells were transfected with FLAG epitope-tagged β_2 AR with or without either β -arrestin 1 or β -arrestin 1-V53D. Cells were incubated for 10 min with $10\ \mu\text{M}$ Iso at room temperature and then incubated an additional 30 min in the presence of a thio-cleavable chemical cross-linker (Lomant's reagent) at

a final concentration of $2.5\ \text{mM}$ in 10% Me_2SO . Cells were solubilized in radioimmune precipitation buffer with protease inhibitors ($150\ \text{mM}$ NaCl, $50\ \text{mM}$ Tris-HCl, $5\ \text{mM}$ EDTA, $10\ \text{mM}$ NaF, $10\ \text{mM}$ Na_2 -pyrophosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, $0.1\ \text{mM}$ phenylmethylsulfonyl fluoride, $10\ \mu\text{g}/\text{ml}$ leupeptin, $5\ \mu\text{g}/\text{ml}$ aprotinin, $1\ \mu\text{g}/\text{ml}$ pepstatin A, pH 7.4) for 1 h and FLAG epitope-tagged β_2 ARs immunoprecipitated with monoclonal anti-FLAG M2 antibody as described previously (12). Samples were subjected to SDS-polyacrylamide gel electrophoresis followed by electroblotting with a Millipore Milliblot semidry electroblotting system onto nitrocellulose membranes. The membranes were blocked in phosphate-buffered saline with 3% bovine serum albumin, probed with an anti- β -arrestin 1/2 rabbit polyclonal antibody (33), and exposed using the ECL Western blotting analysis system (Amersham Corp.), as described previously (12, 13).

Whole Cell Phosphorylation—Receptor phosphorylation was performed as described previously (12, 13). In brief, the intracellular ATP pool was ^{32}P -labeled by incubating transfected cells seeded in six-well dishes with [^{32}P]orthophosphate ($100\ \mu\text{Ci}/\text{ml}$) in phosphate- and serum-free media at 37°C for 45 min. Cells were then stimulated with or without $10\ \mu\text{M}$ Iso in $100\ \mu\text{M}$ ascorbate for 10 min at 37°C and then washed three times on ice with phosphate-buffered saline. Resensitized cells were allowed to recover at 37°C for 20 min in phosphate-buffered saline. The cells were solubilized in radioimmune precipitation buffer with protease inhibitors and 12CA5 epitope-tagged β_2 AR immunoprecipitated with 12CA5 antibody as described previously (12). In each experiment, equivalent amounts of receptor protein, as determined by receptor expression and the amount of solubilized protein in each lysate, were then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The extent of receptor phosphorylation was quantitated using a Molecular Dynamics PhosphorImager system and ImageQuant software.

Membrane Adenylyl Cyclase Assays—Membranes from cells, treated as described in the legends to Figs. 2, 5, 7, and 9, were prepared by disruption with a Polytron homogenizer for 20 s at $20,000\ \text{rpm}$ followed by centrifugation at $40,000 \times g$ in lysis buffer ($10\ \text{mM}$ Tris-HCl, $5\ \text{mM}$ EDTA, pH 7.4). The cell membrane pellet was resuspended in lysis buffer by Polytron, recentrifuged, and resuspended in cold assay buffer ($75\ \text{mM}$ Tris-HCl, $2\ \text{mM}$ EDTA, $15\ \text{mM}$ MgCl_2 , pH 7.4) to a final concentration of 1 – $2\ \mu\text{g}/\mu\text{l}$ membrane protein. $20\text{-}\mu\text{l}$ aliquots of membrane preparations were assayed for agonist-stimulated adenylyl cyclase activity in a final volume of $50\ \mu\text{l}$ as described previously (34, 35).

Whole Cell Cyclase Assay—Cells were grown in 12-well Falcon dishes at a density of $400,000$ cells/well and were labeled overnight with $1\ \mu\text{Ci}/\text{ml}/\text{well}$ of [^3H]adenine in culture medium. The cells were then washed with fresh medium without serum and treated with varying concentrations of isoproterenol in serum-free medium containing $10\ \text{mM}$ HEPES, $1\ \text{mM}$ isobutylmethylxanthine, and $100\ \mu\text{M}$ ascorbate at pH 7.4 for 10 min at 37°C . The medium was aspirated, and $1\ \text{ml}$ of ice-cold stop solution (2.5% (v/v) perchloric acid, $0.1\ \text{mM}$ cAMP, and $2\ \mu\text{Ci}$ of [^{14}C]cAMP per $500\ \text{ml}$) was added to each well followed by incubation on ice for 20–30 min. The cell lysate was added to tubes containing $100\ \mu\text{l}$ of $4.2\ \text{M}$ KOH, and the cAMP accumulated in the cells was quantitated chromatographically by the method of Salomon (36).

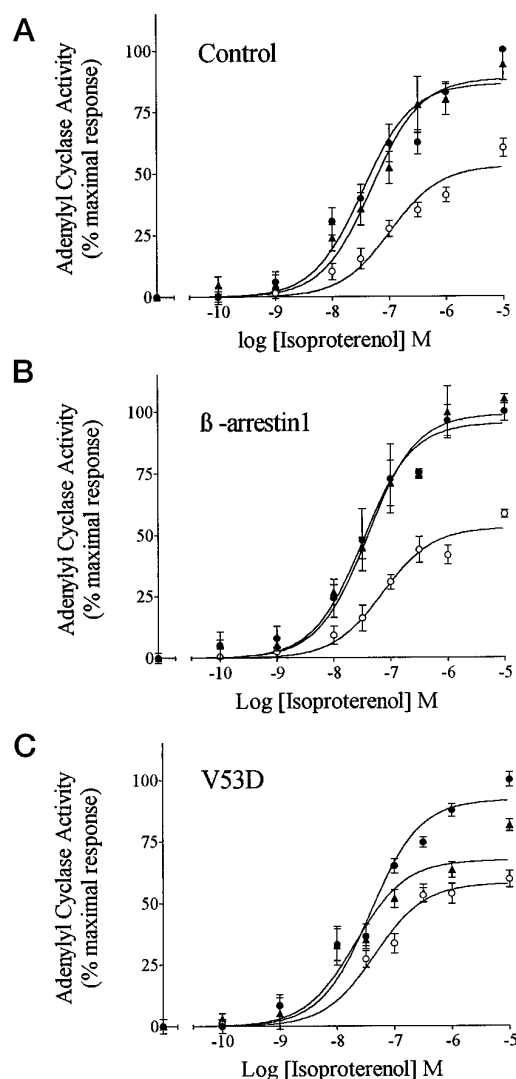


FIG. 2. Effect of β -arrestin 1 and β -arrestin 1-V53D on the desensitization and resensitization of β_2 AR-stimulated adenylyl cyclase activity. HEK 293 cells were transfected to overexpress the β_2 AR in the absence (1120 ± 290 fmol/mg protein) (A) or presence of either coexpressed β -arrestin 1 (1280 ± 730 fmol/mg protein) (B) or β -arrestin 1-V53D (1170 ± 340 fmol/mg protein) (C). Cells were preincubated for 15 min in serum-free media at 37°C in the absence (●) or presence (○, ▲) of a desensitizing stimulus, $10\ \mu\text{M}$ Iso, washed three times, and either allowed to resensitize for 30 min at 37°C (▲) or kept on ice (○, ●). Cell membranes were prepared, and adenylyl cyclase activity was determined under basal conditions as well as in the presence of increasing concentrations of Iso (10^{-10} to 10^{-5} M) and $1\ \mu\text{M}$ forskolin as described under "Experimental Procedures." Adenylyl cyclase activity is expressed as the percentage of the maximal response of control cells (●) to $10\ \mu\text{M}$ Iso. The V_{max} and EC_{50} values for β_2 AR-mediated adenylyl cyclase activation were 30 ± 3 pmol/min/mg protein and 31 ± 2 nM, respectively and were not affected by the overexpression of either β -arrestin 1 or β -arrestin 1-V53D. Forskolin-stimulated adenylyl cyclase activity was 257 ± 22 pmol/min/mg protein. The data represent the mean \pm S.E. of 4–6 different experiments done in duplicate.

Data Analysis—Statistical significance was determined by analysis of variance when appropriate and multiple comparisons between groups were made using a two-tailed t test for independent samples. Dose response data were analyzed using GraphPad Prism.

RESULTS

Effect of β -Arrestins on β_2 AR Dephosphorylation—We have reported that overexpression of β -arrestin 1-V53D, a dominant negative inhibitor of β_2 AR sequestration, increased the extent of agonist-induced β_2 AR phosphorylation, suggesting that β -

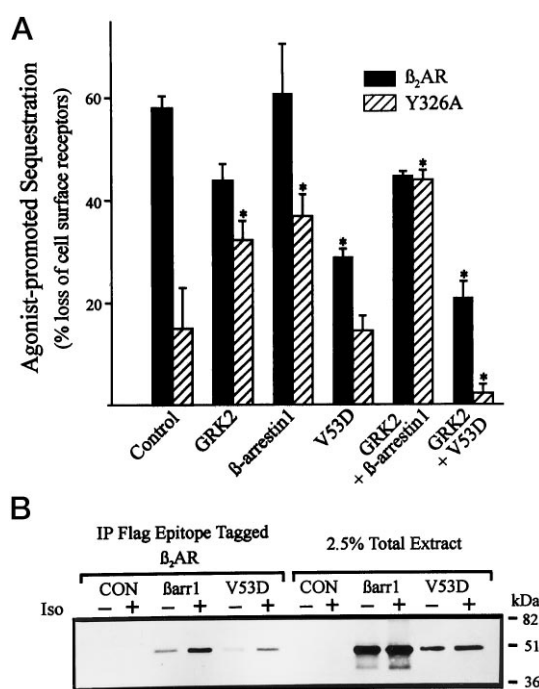


FIG. 3. The effect of GRK2, β -arrestin 1, and the β -arrestin 1-V53D dominant negative mutant on β_2 AR and β_2 AR-Y326A mutant sequestration and the co-immunoprecipitation of β -arrestin 1 and β -arrestin 1-V53D with the β_2 AR. A, 12CA5 epitope-tagged wild-type and Y326A mutant β_2 ARs were transiently expressed in HEK 293 cells in a pcDNA1-Amp expression vector together with $10\ \mu\text{g}$ of empty vector (control), $5\ \mu\text{g}$ of pcDNA1-Amp GRK2, $10\ \mu\text{g}$ of pcMV5 β -arrestin 1, $10\ \mu\text{g}$ of pcDNA1-Amp β -arrestin 1-V53D, or $5\ \mu\text{g}$ of GRK2 with $10\ \mu\text{g}$ of either β -arrestin 1 or β -arrestin 1-V53D. Cell surface receptors were assessed by flow cytometry using 12CA5 monoclonal antibody, and receptor sequestration was expressed as a loss of cell surface immunofluorescence following a 30-min exposure to $10\ \mu\text{M}$ Iso at 37°C . The data represent the mean \pm S.E. of three independent experiments. *, $p < 0.05$ compared with control values. B, polyclonal β -arrestin 1 antibody immunoblot of the agonist-dependent co-immunoprecipitation of β -arrestins with FLAG epitope-tagged β_2 AR in the absence (CON) or presence of either overexpressed β -arrestin 1 (β arr1) or β -arrestin 1-V53D (V53D) following chemical cross-linking as described under "Experimental Procedures."

arrestin might play a role in β_2 AR dephosphorylation and resensitization (13). To begin to test this question, the effect of overexpressing either wild-type β -arrestin or β -arrestin 1-V53D on β_2 AR dephosphorylation was examined in HEK 293 cells. In the absence of overexpressed β -arrestins, agonist stimulation with $10\ \mu\text{M}$ isoproterenol for 10 min increased the extent of β_2 AR phosphorylation 3.1 ± 0.3 -fold over basal level (Fig. 1A). When cells were allowed to recover for 20 min following the removal of agonist, β_2 AR dephosphorylation ($61 \pm 6\%$) was observed (Fig. 1, A and B). Overexpression of β -arrestin 1 led to a reduction in agonist-induced β_2 AR phosphorylation ($75 \pm 4\%$ of control β_2 AR phosphorylation) without significantly affecting the ability of the receptor to be dephosphorylated ($54 \pm 3\%$ of matched control) (Fig. 1, A and B). In contrast, β -arrestin 1-V53D not only increased the extent of agonist-induced β_2 AR phosphorylation ($137 \pm 14\%$ of control β_2 AR phosphorylation) but significantly impaired the ability of the receptor to be dephosphorylated ($28 \pm 12\%$ of matched control) (Fig. 1, A and B). While complete inhibition of β_2 AR dephosphorylation was not observed in the presence of β -arrestin 1-V53D, the absolute value of β_2 AR phosphorylation remained $95 \pm 9\%$ of control β_2 AR phosphorylation. Taken together, these data indicate that normal β -arrestin function contributes to the regulation of β_2 AR dephosphorylation.

Effect of β -Arrestins on β_2 AR Resensitization—To determine

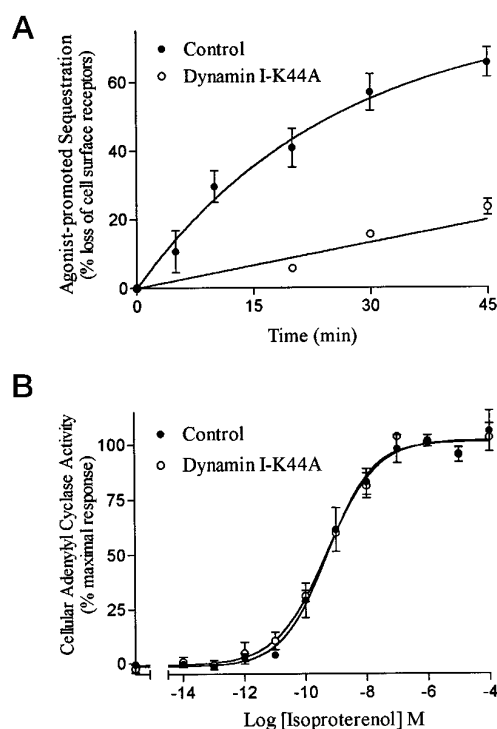


FIG. 4. The effect of dynamin I-K44A on β_2 AR sequestration and β_2 AR-stimulated whole cell adenylyl cyclase activity in HEK 293 cells. 12CA5 epitope-tagged β_2 ARs were transiently expressed in HEK 293 cells in a pcDNA1-Amp expression vector together with 3 μ g of empty pCMV5 vector (control) (984 ± 191 fmol receptor/mg protein) or 3 μ g of pCB1 rat dynamin I-K44A (1317 ± 223 fmol receptor/mg protein). Expression of dynamin I-K44A was monitored by immunoblot using an antibody for dynamin I (49). A, β_2 AR sequestration was assessed by flow cytometry as described previously (14, 32). B, the maximal β_2 AR-stimulated percentage conversion of [3 H]cAMP was 2.03 ± 0.09 in the absence and 2.38 ± 0.03 in the presence of dynamin I-K44A. The data represent the mean \pm S.E. of three independent experiments.

whether normal β -arrestin function and receptor dephosphorylation were absolutely required for β_2 AR resensitization, the ability of the β_2 AR to both desensitize and resensitize in the presence of either wild-type or V53D mutant β -arrestin was tested in HEK 293 cells. When expressed alone, the β_2 AR desensitized in response to a desensitizing stimulus (10 μ M isoproterenol for 15 min), which was measured as a 2-fold rightward shift in the half-maximal effective concentration (EC_{50}) and a $43 \pm 3\%$ reduction in the maximal velocity (V_{max}) for agonist-stimulated β_2 AR-responsive adenylyl cyclase activity (Fig. 2A). When cells were allowed to recover for 30 min in agonist-free media, following the desensitizing stimulus, complete resensitization of β_2 AR-responsive adenylyl cyclase activity was observed (Fig. 2A). While the overexpression of either wild-type or V53D mutant β -arrestins did not affect the ability of the β_2 AR to desensitize (48 ± 4 and $36 \pm 1\%$ decrease in V_{max} , respectively; 2.3- and 1.3-fold rightward shift in the EC_{50} , respectively) (Fig. 2, B and C), overexpression of β -arrestin 1-V53D impaired the recovery of the V_{max} for β_2 AR-mediated adenylyl cyclase activation by 70% (Fig. 2C). Consequently, by virtue of its ability to impair β_2 AR endocytosis, β -arrestin 1-V53D acts as a dominant negative inhibitor of both β_2 AR dephosphorylation and resensitization.

Effect of β -Arrestins on β_2 AR Sequestration—To confirm that the effects of β -arrestin 1-V53D on β_2 AR dephosphorylation and resensitization were the consequence of its ability to block β_2 AR endocytosis, we reexamined the effects of GRK and β -arrestin regulatory proteins on both wild-type β_2 AR and Y326A mutant receptor sequestration. As expected (12, 13), GRK2 and

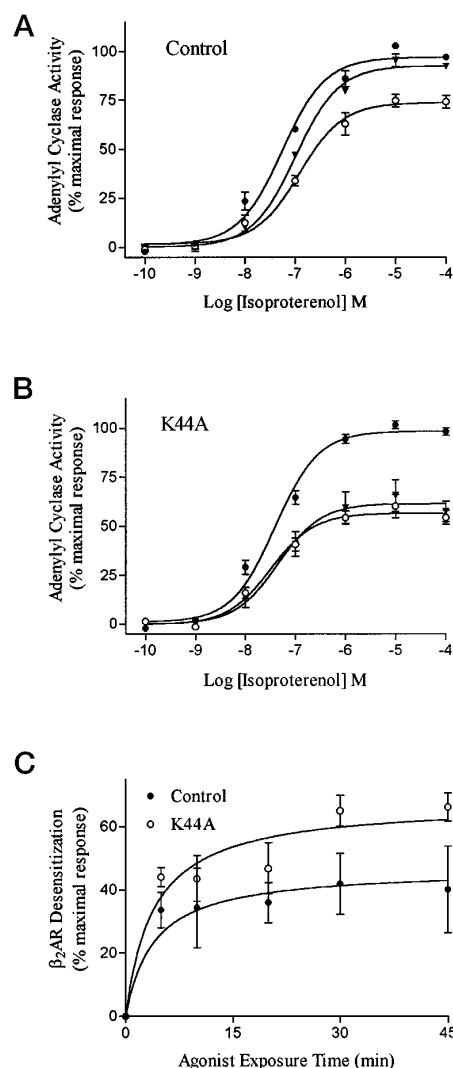


FIG. 5. The effect of dynamin I-K44A on the desensitization and resensitization of β_2 AR-stimulated adenylyl cyclase activity in HEK 293 cells. HEK 293 cells were transiently transfected to overexpress β_2 ARs in the absence (803 ± 109 fmol/mg protein) (A) or presence (1206 ± 156 fmol/mg protein) (B) of 3 μ g of cotransfected dynamin I-K44A in pCB1. Cells were preincubated for 10 min in serum-free media at 37 $^{\circ}$ C in the absence (naive (●)) or presence (desensitized (○); resensitized (▼)) of a desensitizing stimulus, 2 μ M isoproterenol, washed three times, and either allowed to resensitize for 20 min at 37 $^{\circ}$ C (▼) or kept on ice (○, ●). Cell membranes were prepared, and adenylyl cyclase activity was determined under basal conditions as well as in the presence of increasing concentrations of isoproterenol (10^{-10} to 10^{-4} M) or 10 μ M forskolin as described under "Experimental Procedures." Adenylyl cyclase activity was normalized according to forskolin-stimulated cyclase response and was expressed as the percentage of the maximal response of naive cells (●). The EC_{50} values for β_2 AR-mediated adenylyl cyclase activation were 54 ± 10 and 39 ± 7 nM, respectively, in the absence and presence of overexpressed dynamin I-K44A. The data represent the mean \pm S.E. of four different experiments done in triplicate. C, cells transfected with β_2 ARs in the absence (●) or presence (○) of dynamin I-K44A were desensitized with 2 μ M isoproterenol for different periods of time (0–45 min) at 37 $^{\circ}$ C in serum-free media and washed. Cell membranes were prepared, and then adenylyl cyclase activity was determined in the presence of 10 μ M isoproterenol or 10 μ M forskolin. Adenylyl cyclase activity was normalized according to forskolin-stimulated cyclase response, and desensitization was expressed as the percentage of adenylyl cyclase activity in cells that had not been preincubated with agonist (*i.e.* $t = 0$). The data represent the mean \pm S.E. of three different experiments done in triplicate.

β -arrestin 1 overexpression rescued the sequestration phenotype of the Y326A mutant receptor, whereas overexpression of β -arrestin 1-V53D not only impaired wild-type β_2 AR sequestration but blocked GRK2 phosphorylation-mediated rescue of

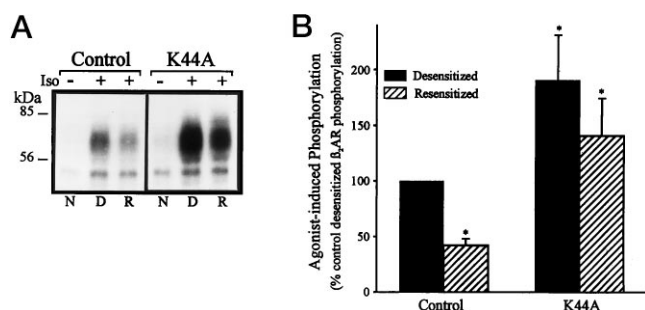


FIG. 6. The effect of dynamin I-K44A on β_2 AR phosphorylation and dephosphorylation in HEK 293 cells. 12CA5 epitope-tagged β_2 ARs were transfected into HEK 293 cells with (1070 ± 140 fmol/mg protein) or without (890 ± 150 fmol/mg protein) dynamin I-K44A. **A**, an autoradiograph from a representative experiment showing the whole cell phosphorylation and dephosphorylation of the β_2 AR. Cells were preincubated for 10 min in serum-free medium at 37°C in the absence (naive (N)) or presence (desensitized (D); resensitized (R)) of $10\ \mu\text{M}$ isoproterenol, washed three times on ice, and either allowed to resensitize (R) for 15 min at 37°C or kept on ice (N, D). Each lane was loaded with equivalent amounts of receptor protein as described under "Experimental Procedures." **B**, the mean \pm S.E. of four different experiments quantified by PhosphorImager analysis. The data were normalized to the agonist-induced control β_2 AR phosphorylation. *, $p < 0.05$ versus desensitized control β_2 AR phosphorylation.

Y326A receptor sequestration (Fig. 3A). The lack of complete blockade of β_2 AR endocytosis by β -arrestin 1-V53D probably underlies the observation that some receptor dephosphorylation and resensitization occurs in its presence (see Figs. 1 and 2C).

To test whether the mutant β -arrestin bound the β_2 AR, we examined whether both wild-type β -arrestin 1 and β -arrestin 1-V53D could be co-immunoprecipitated in an agonist-dependent manner with a FLAG epitope-tagged β_2 AR. As shown in Fig. 3B, both β -arrestins were co-immunoprecipitated in an agonist-dependent manner with FLAG-tagged β_2 AR. Consistent with results obtained using purified β_2 AR and *in vitro* translated β -arrestins (37), co-immunoprecipitation of both β -arrestin 1 and β -arrestin 1-V53D was observed in the absence of agonist stimulation, but the amount of each β -arrestin immunoprecipitated was substantially increased following agonist stimulation (Fig. 3B). These results indicate that β -arrestin 1-V53D exhibits the capacity to interact with the β_2 AR, albeit less effectively than wild type β -arrestin. The mutant β -arrestin probably acts as a sequestration dominant negative by failing to mediate appropriate interactions with downstream proteins required for β_2 AR endocytosis, such as clathrin (24–26), rather than being constitutively bound to cellular components required for clathrin-coated vesicle-mediated receptor internalization.

Effect of Dynamin I-K44A on β_2 AR Function—Because we have demonstrated that β -arrestins target GPCRs to CCVs (14), we tested whether β_2 AR dephosphorylation and resensitization were dependent upon an intact CCV pathway. To do this, we examined the effects of the overexpression of dynamin I-K44A on β_2 AR signaling, sequestration, dephosphorylation, and resensitization. Dynamin I-K44A not only inhibited the maximum extent of agonist-promoted β_2 AR internalization by $64 \pm 4\%$ ($t = 45$ min) in HEK 293 cells but reduced the rate of β_2 AR sequestration as judged by the slope of the curves at $t = 0$ (Fig. 4A). Moreover, dynamin I-K44A had no effect on the capacity of the β_2 AR to signal through adenylyl cyclase, indicating that the effect of dynamin I-K44A on β_2 AR internalization is independent of receptor signaling (Fig. 4B).

When expressed alone, the β_2 AR desensitized in response to a short exposure to agonist ($2\ \mu\text{M}$ isoproterenol for 10 min) and then resensitized when allowed to recover 20 min in agonist-

free medium (Fig. 5A). In contrast, overexpression of dynamin I-K44A not only enhanced β_2 AR desensitization (42 ± 2 versus $24 \pm 2\%$ decrease in maximal response in control cells) but also abolished the resensitization of β_2 AR-mediated adenylyl cyclase activation as measured by the recovery in V_{max} (Fig. 5B). Overexpression of dynamin I-K44A increased the extent of β_2 AR desensitization at all time points tested (Fig. 5C).

To further characterize whether the inhibition of β_2 AR resensitization by dynamin I-K44A was the consequence of impaired β_2 AR dephosphorylation, we examined the effect of dynamin I-K44A overexpression on both the phosphorylation and dephosphorylation of β_2 ARs expressed in HEK 293 cells. In the absence of dynamin I-K44A, phosphorylation of the β_2 AR was observed following agonist stimulation with $10\ \mu\text{M}$ isoproterenol for 10 min (Fig. 6A). Following a 15-min recovery period in the absence of agonist, β_2 ARs were dephosphorylated to $42 \pm 5\%$ of control levels (Fig. 6, A and B). Consistent with the observed increase in β_2 AR desensitization, overexpression of dynamin I-K44A with the β_2 AR significantly increased the maximal extent of β_2 AR phosphorylation ($191 \pm 40\%$ of control β_2 AR phosphorylation) (Fig. 6, A and B). In contrast, dynamin I-K44A significantly blocked β_2 AR dephosphorylation ($27 \pm 6\%$ dephosphorylation of matched control) (Fig. 6, A and B). The maximal extent of β_2 AR phosphorylation in the presence of dynamin I-K44A remained higher ($141 \pm 33\%$) than control β_2 AR phosphorylation even when the cells were allowed to recover for 15 min in the absence of agonist (Fig. 6, A and B).

Effect of β -Arrestins and GRK2 on β_2 AR-Y326A Mutant Resensitization—The work implicating GRK-mediated phosphorylation and β -arrestin binding in β_2 AR sequestration has stemmed from the characterization of a β_2 AR mutant (Y326A), which desensitizes but neither sequesters nor resensitizes following agonist exposure (12, 13, 32, 38). While this mutant does not serve as a substrate for GRK-mediated phosphorylation (12, 13), agonist-dependent desensitization of this mutant still occurs as the consequence of cAMP-dependent protein kinase (PKA)-mediated phosphorylation (12). Therefore, to determine whether impaired GRK-phosphorylation and β -arrestin binding also underlie the resensitization defect of the Y326A receptor mutant, we tested whether overexpression of GRK2 and/or β -arrestin 1 would result in a gain of function with respect to the ability of this mutant receptor to resensitize.

The β_2 AR-Y326A mutant maximally stimulated adenylyl cyclase activity in membranes prepared from HEK 293 cells when compared with the wild-type β_2 AR ($11 \pm 1\%$ of forskolin-stimulated adenylyl cyclase activity for both receptors). However, the EC_{50} for agonist-stimulated adenylyl cyclase activity was shifted 2-fold to the right when compared with the wild-type β_2 AR (see legends to Figs. 2 and 7). When expressed alone, the Y326A receptor desensitized ($19 \pm 3\%$ decrease in V_{max}) but did not resensitize (Fig. 7A). Interestingly, overexpression of GRK2 and β -arrestin 1 either alone or together not only enhanced Y326A receptor desensitization (33 ± 3 , 31 ± 5 , and $41 \pm 4\%$ decrease in V_{max} , respectively) but also rescued the ability of the receptor mutant to resensitize (Fig. 7, B, C, and D). However, while overexpression of β -arrestin 1-V53D either without or with GRK2 enhanced Y326A receptor desensitization (33 ± 3 and $33 \pm 1\%$ decrease in V_{max} , respectively), the β -arrestin dominant negative mutant did not support Y326A receptor resensitization and blocked GRK2-mediated rescue of Y326A mutant receptor resensitization (Fig. 7, E and F). Therefore, the Y326A mutant resensitization phenotype is probably the consequence of impaired interactions with GRKs and β -arrestins (12–14, 38). Consequently, these experiments provide direct evidence that GRK-mediated receptor phosphorylation

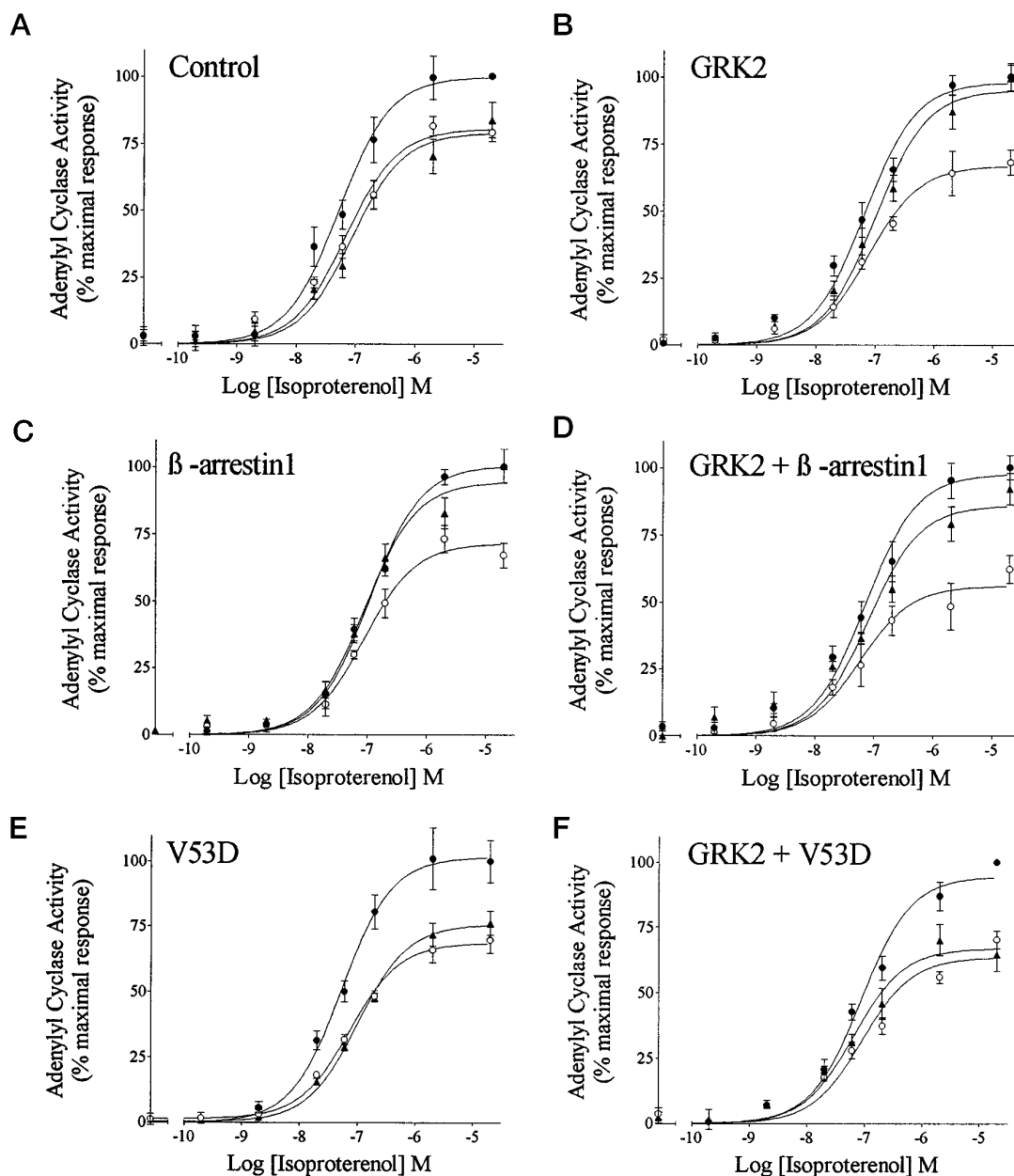


FIG. 7. Effect of GRK2, β -arrestin 1, and β -arrestin 1-V53D on the desensitization and resensitization of β_2 AR-Y326A mutant-stimulated adenylyl cyclase activity. HEK 293 cells were transiently transfected to overexpress the β_2 AR-Y326A mutant in the absence (2220 ± 870 fmol/mg protein) (A) or the presence of cotransfected GRK2 (2450 ± 390 fmol/mg protein) (B), β -arrestin 1 cDNA (2040 ± 100 fmol/mg protein) (C), β -arrestin 1-V53D cDNA (1910 ± 120 fmol/mg protein) (D), GRK2 and β -arrestin 1 cDNA (1470 ± 220 fmol/mg protein) (E) or GRK2 and β -arrestin 1-V53D (1490 ± 340 fmol/mg protein) (F). Cells were treated as described in the legend to Fig. 2, and adenylyl cyclase activity in response to Iso ($10^{-9.7}$ to $10^{-4.5}$ M) was determined. \bullet , naive cells; \circ , desensitized cells; \blacktriangle , resensitized cells. Adenylyl cyclase activity is expressed as the percentage of the maximal response of control cells (\bullet) to 30μ M Iso. The V_{max} and EC_{50} values for β_2 AR-Y326A mutant-mediated adenylyl cyclase activation were 62 ± 6 pmol/min/mg protein and 72 ± 9 nM, respectively, and were not affected by the co-expression of GRK and β -arrestin regulatory proteins. Forskolin-stimulated adenylyl cyclase activity was 533 ± 40 pmol/min/mg protein. The data represent the mean \pm S.E. of three different experiments done in duplicate.

followed by β -arrestin binding serves as the signal triggering β_2 AR resensitization.

Cell Type-specific β_2 AR Resensitization—Compared with HEK 293 cells, β_2 AR sequestration was significantly blunted in COS-7 cells (Fig. 8A). This impairment of β_2 AR sequestration is the consequence of low endogenous expression levels of GRK and β -arrestin proteins in this cell line (39). β -Arrestin overexpression not only rescued the sequestration phenotype but also increased the rate of β_2 AR internalization in COS-7 cells (Fig. 8A). The low β_2 AR sequestration in COS-7 cells provided a unique opportunity to examine the relationship among receptor sequestration, dephosphorylation, and resensitization in a

natural cellular environment. As observed in HEK 293 cells, the β_2 AR in COS-7 cells was effectively phosphorylated upon agonist stimulation, indicating that GRK expression was not limiting for β_2 AR phosphorylation (Fig. 8B). However, the extent of receptor dephosphorylation ($34 \pm 4\%$) following a 15-min recovery of the COS cells after the removal of agonist was significantly lower than that observed in HEK 293 cells (Fig. 8, B and C versus Fig. 1, A and B). Overexpression of a β -arrestin 2 wild-type construct not only enhanced β_2 AR sequestration in COS-7 cells (Fig. 8A) but restored receptor dephosphorylation to a level ($58 \pm 4\%$ dephosphorylation of matched control) comparable with that observed in HEK 293 cells (Fig. 8, B and

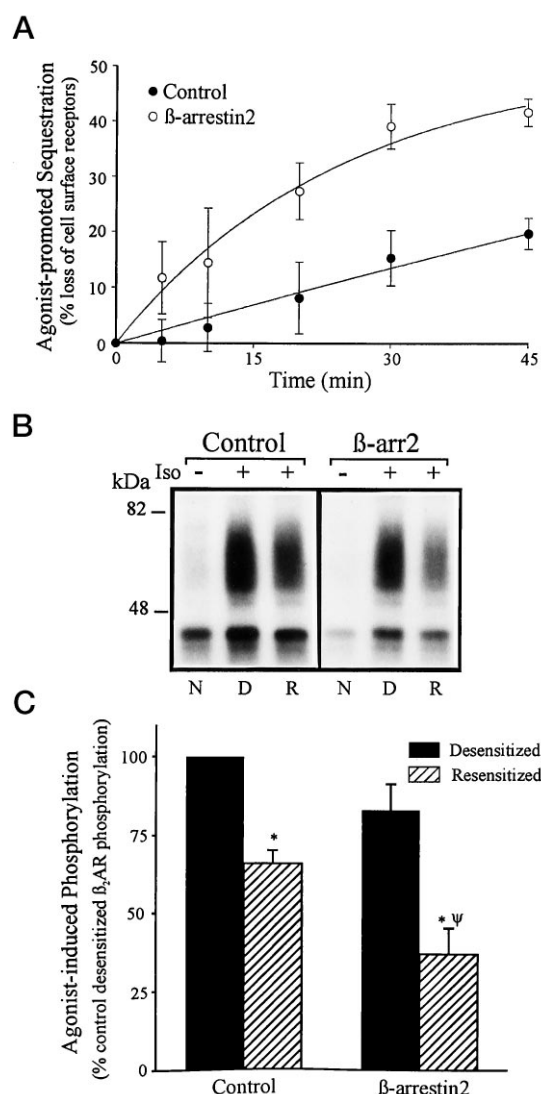


FIG. 8. β_2 AR sequestration, phosphorylation, and dephosphorylation in COS-7 cells in the absence and presence of overexpressing β -arrestin 2. 12CA5 epitope-tagged β_2 ARs were transiently expressed in COS-7 cells in a pcDNA1-Amp expression vector together with 8 μ g of empty pCMV5 vector or 8 μ g of pCMV5 β -arrestin 2. **A**, time course of β_2 AR sequestration in the presence or absence of β -arrestin 2. The data represent the mean \pm S.E. of three independent experiments. **B**, an autoradiograph from a representative experiment showing the whole cell phosphorylation and dephosphorylation of the β_2 AR in COS-7 cells in the absence (1300 ± 200 fmol/mg protein) and presence (1490 ± 300 fmol/mg protein) of overexpressing β -arrestin 2. The experiments were performed as described in the legend to Fig. 6 and under "Experimental Procedures." **C**, the mean \pm S.E. of three different experiments quantified by PhosphorImager analysis. The data were normalized to the agonist-induced control β_2 AR phosphorylation. *, $p < 0.05$ versus desensitized control β_2 AR phosphorylation; ψ , $p < 0.05$ versus matched desensitized β_2 AR phosphorylation.

C, versus Fig. 1, A and B). In addition, probably due to increased receptor dephosphorylation, agonist-induced β_2 AR phosphorylation in COS-7 cells overexpressing β -arrestin 2 was reduced by $17 \pm 8\%$ compared with control cells (Fig. 8, B and C).

Consistent with the agonist-stimulated β_2 AR phosphorylation observed in COS-7 cells, β_2 AR-responsive adenylyl cyclase activity desensitized following a 10-min exposure to 2μ M isoproterenol, as demonstrated by both a rightward shift in the agonist dose response and a $14 \pm 3\%$ decrease in V_{\max} (Fig. 9A). In addition, little resensitization of the desensitized β_2 ARs was observed when COS-7 cells were allowed to recover 20 min in

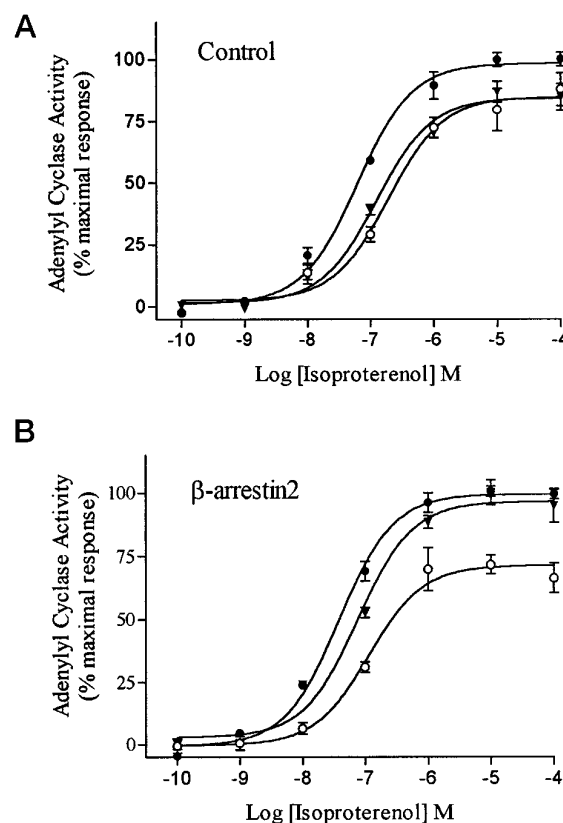


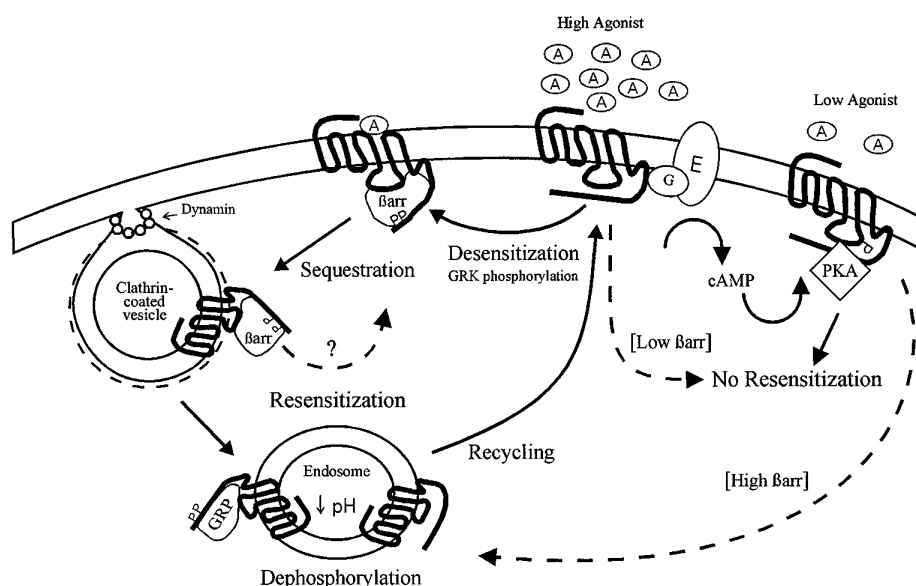
FIG. 9. Desensitization and resensitization of β_2 AR-stimulated adenylyl cyclase activity in COS-7 cells in the absence and presence of overexpressing β -arrestin 2. COS-7 cells were transiently transfected to overexpress β_2 ARs in the absence (1444 ± 277 fmol/mg protein) (**A**) and presence (2182 ± 350 fmol/mg protein) (**B**) of cotransfected β -arrestin 2. Cells were treated as described in the legend of Fig. 5, and adenylyl cyclase activity in response to isoproterenol (10^{-10} to 10^{-4}) was determined. ●, naive cells; ○, desensitized cells; ▼, resensitized cells. Adenylyl cyclase activity was expressed as the percentage of the maximal response of naive cells (●). The EC_{50} values for β_2 AR-mediated adenylyl cyclase activation were 62 ± 10 and 38 ± 5 nM, respectively, in the absence and presence of overexpressed β -arrestin 2. The data represent the mean \pm S.E. of three different experiments done in triplicate.

the absence of agonist (Fig. 9A). The relatively low level of β_2 AR desensitization and the absence of receptor resensitization in COS-7 cells corresponded well with the low endogenous β -arrestin expression levels found in these cells (39). Therefore, to test whether this was the case, the effect of β -arrestin 2 overexpression on β_2 AR desensitization and resensitization was examined in COS-7 cells. Overexpression of β -arrestin 2 not only increased agonist-induced receptor desensitization to $28 \pm 3\%$ but also completely restored β_2 AR resensitization (Fig. 9B). Taken together, these data demonstrated an intimate dependence of β_2 AR dephosphorylation and resensitization on β -arrestin expression levels and receptor sequestration.

DISCUSSION

Our findings provide direct biochemical evidence using GPCR-specific endocytic inhibitors that sequestration plays an intimate role in both β_2 AR dephosphorylation and resensitization. Furthermore, the data reveal that GRK-mediated phosphorylation and β -arrestin binding are obligatory events leading to normal β_2 AR resensitization. This role is underscored by experiments demonstrating both a loss and a gain of function with respect to the resensitization phenomenon. Not only could wild-type β_2 AR dephosphorylation and resensitization be inhibited by a sequestration dominant negative β -arrestin, but Y326A receptor mutant resensitization could be rescued by the

FIG. 10. **Model for β_2 AR resensitization.** Details of the model are outlined under "Discussion." A, agonist; P, phosphorylation site; β arr, β -arrestin; GRP, G protein-coupled receptor phosphatase; E, effector; G, G protein.



overexpression of GRK2 and/or β -arrestin 1. Moreover, our findings provide evidence supporting an essential role for CCVs in mediating β_2 AR dephosphorylation and resensitization in endosomes. In addition, our results reveal that β -arrestins are not only important determinants of β_2 AR desensitization, sequestration, and resensitization but also that the differential expression of β -arrestin proteins may confer cell- and tissue-specific regulation of GPCR resensitization.

The capacity of the β -arrestin 1-V53D mutant to inhibit β_2 AR resensitization as well as to enhance Y326A receptor desensitization without supporting its resensitization indicates that the desensitization and resensitization functions of β -arrestin are dissociable. Moreover, the role of GRK2-mediated phosphorylation in GPCR sequestration and resensitization is probably independent of its role in receptor desensitization, considering that co-expression of β -arrestin 1-V53D with GRK2 restored phosphorylation of the Y326A receptor mutant (12, 13) and enhanced its desensitization but did not rescue its resensitization (Fig. 7F). This idea is further supported by the observation that β -arrestin expression can reestablish Y326A mutant receptor resensitization in the absence of GRK-mediated phosphorylation (Fig. 7C).

Using dynamin 1-K44A, the present study implicates CCVs and consequently receptor internalization as a critical step in mediating β_2 AR resensitization. Dynamin plays a role in GPCR internalization by acting as part of the cellular clathrin-mediated endocytic machinery (14) analogous to its role in mediating the endocytosis of a variety of other receptors, including transferrin and epidermal growth factor receptors (15, 17–23). However, unlike β -arrestin, which functions by interacting directly with the GPCRs, dynamin influences cellular signaling mainly as a direct result of its role in CCV-mediated endocytosis (23). Consequently, the ability to assess the function of proteins involved at different stages in the β_2 AR resensitization process, *i.e.* β -arrestins at the level of the receptor and dynamin in CCV endocytosis, provides the framework from which to begin to dissect the contribution of other proteins involved in the compartmentalization and/or resensitization of β_2 AR responsiveness.

A central role for β -arrestins in β_2 AR sequestration and resensitization is further corroborated by our observations in COS-7 cells. The low levels of endogenous β -arrestin expression in these cells has been correlated with reduced β_2 AR sequestration (14, 39). However, while robust β_2 AR phosphorylation was observed in COS-7 cells, indicating that GRK expression

levels were not limiting, both β_2 AR dephosphorylation and resensitization were low. In these cells, the overexpression of β -arrestin 2 not only reestablished β_2 AR sequestration but rescued the ability of the receptor to be dephosphorylated and resensitized. Furthermore, the enhancement of β_2 AR desensitization in COS-7 cells in the presence of overexpressed β -arrestin further substantiates the role of β -arrestin as a molecular intermediate required for receptor desensitization (40, 41). The observation that GRK overexpression rescued β_2 AR-Y326A mutant phosphorylation and reestablished its resensitization in the absence of supplemented β -arrestins suggests that differences in the endogenous expression levels of GRKs may also influence the relative ability of receptors to sequester and resensitize. Moreover, differences in the ability of the β_2 AR to resensitize in two distinct cell lines suggest that in tissues expressing relatively little β -arrestin and/or GRK proteins, receptor resensitization may be impaired or delayed. In contrast, high levels of GRK and β -arrestin proteins are found in the brain and highly innervated tissues (33, 42), specifically at synaptic locations, where continuous neuronal responses probably require rapid resensitization of neurotransmitter receptors for the maintenance of normal synaptic transmission.

While direct experimental evidence is lacking, the present experiments raise the possibility that PKA-phosphorylated receptors remain desensitized in the absence of GRK phosphorylation and/or β -arrestin binding. This hypothesis arises from the observation that over the time period being tested (30 min) the β_2 AR-Y326A mutant, which does not serve as a substrate for GRK-mediated phosphorylation (12, 13), still desensitizes as the consequence of PKA phosphorylation (12) but does not resensitize. Second messenger-dependent protein kinases, such as PKA, contribute to both agonist-dependent (homologous) and agonist-independent (heterologous) receptor desensitization, whereas GRKs contribute mainly to agonist-dependent desensitization (1–3). Consequently, if heterologously (PKA) desensitized receptors do not resensitize until sequestered, this might provide an additional means by which these receptors remain quiescent following their desensitization through the activation of a parallel signal transduction pathway. Such a mechanism could serve to differentially modulate the temporal sensitivity of both signal transduction pathways. However, further experimentation will be required to test the relevance and validity of this hypothesis to GPCR signal transduction.

The data presented in this paper suggest the following model for GPCR receptor resensitization (Fig. 10). Agonist activation

leads to receptor phosphorylation by both PKA- and GRK-mediated mechanisms, resulting in receptor desensitization. GRK-mediated phosphorylation promotes the binding of β -arrestin to the receptor, leading not only to further receptor desensitization (33, 40, 41) but also to the initiation of receptor endocytosis via CCVs (12–14, 24–26). Moreover, endocytosis via CCVs is crucial for β_2 AR resensitization. Receptors proceed from CCVs to early endosomes, where they are dephosphorylated and resensitized by a mechanism that is proposed to involve a conformational change in the receptor brought about by acidification in the endosomal compartment (9). This putative conformational change is proposed to enhance dephosphorylation of GRK phosphorylation sites by a membrane-associated G protein-coupled receptor phosphatase (9). It is unknown whether this event contributes to the dissociation of β -arrestin from the receptor. However, β -arrestin dissociation may be particularly important considering the evidence that arrestin binding to rhodopsin prevents dephosphorylation of the visual pigment (43). Potentially, this same conformational change in the receptor may be required for resensitization of the PKA-phosphorylated Y326A mutant receptors. Unfortunately, it was not possible to determine whether sequestration was required for the dephosphorylation of PKA-phosphorylated receptors due to the extremely low level of PKA-mediated β_2 AR-Y326A phosphorylation in HEK 293 cells (Refs. 12 and 13; data not shown). We postulate that PKA-phosphorylated receptors, either as the consequence of the heterologous activation of the kinase or exposure to low concentrations of agonist, may neither sequester nor resensitize and remain desensitized until β -arrestin can bind. In addition, under conditions of low endogenous β -arrestin expression, such as those found in COS-7 cells, receptor sequestration and resensitization of GRK-phosphorylated receptors are also impaired.

Recently, it was reported that GRK2 co-localizes with vesicles following β_2 AR stimulation, and while there is as yet no biochemical evidence to support the hypothesis, the authors suggested that GRK2 may play a role in GPCR endocytosis that is independent of the β -arrestin adaptor function (44). Nonetheless, evidence presented here and in previous publications (13, 39) indicates that β -arrestin can serve as a β_2 AR adaptor in the absence of GRK-mediated receptor phosphorylation and that GRK-mediated receptor phosphorylation is not absolutely required for β_2 AR endocytosis and resensitization (12–14, 35). Rather, it seems more likely that the reported presence of GRK2 immunoreactivity in internalized vesicles is functionally related to the subcellular localization of GRK2 to microsomal membrane fractions (45). Evidence also suggests that β -arrestin can associate with membrane-bound receptors in response to agonist-stimulation (Fig. 3B) and that following agonist exposure a small fraction of β -arrestin may redistribute to the light vesicular pool with the receptor (39). With respect to the cellular partners with which β -arrestin might interact to mediate its adaptor function, clathrin was recently shown to interact with purified β -arrestins and β -arrestin colocalized with clathrin in cells (24). If this interaction turns out to mediate the cellular trafficking of GPCRs and their resensitization, then understanding the cellular events controlling the onset of the interaction and the dissociation of the complex will be of interest.

In conclusion, the data clearly indicate that GPCR desensitization and resensitization are distinct yet intimately related processes, since the same molecular components are either shared by both processes or indirectly influence each other's activity. For instance, overexpression of β -arrestin in COS-7 cells not only potentiated β_2 AR desensitization but increased receptor sequestration and resensitization as well. In contrast,

overexpression of a dynamin dominant negative mutant increased the extent of agonist-mediated β_2 AR phosphorylation and desensitization as a consequence of the blockade of CCV-mediated receptor endocytosis and resensitization. These findings highlight the intimate relationship between mechanisms contributing to receptor desensitization and resensitization and suggest that biochemical alteration of one process may have significant impact upon the other. This may be particularly relevant to the understanding of the pathophysiology of diseases such as congestive heart failure and drug tolerance, which are associated with a loss of normal GPCR function, potentially as the consequence of elevated GRK expression and enhanced desensitization (46–48). The present study predicts that manipulation of β -arrestin expression levels might either accelerate or retard receptor resensitization without adversely influencing receptor desensitization. Moreover, our results establish a clear role for both β -arrestins and CCV-mediated endocytosis in β_2 AR resensitization. However, not all GPCRs utilize CCVs for endocytosis (14). Therefore, it will be of interest in the future to determine whether receptor resensitization is particular to the CCV-mediated endocytic pathway or whether other endocytic pathways subserve this function as well.

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