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Role of Phosphorylation in Agonist-promoted β_2-Adrenergic Receptor Sequestration

RESCUE OF A SEQUESTRATION-DEFECTIVE MUTANT RECEPTOR BY βARK1*

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The β_2-adrenergic receptor (β_2AR) belongs to the large family of G protein-coupled receptors. Mutation of tyrosine residue 326 to an alanine resulted in a β_2AR mutant (β_2AR-Y326A) that was defective in its ability to sequester and was less well coupled to adenylyl cyclase than the wild-type β_2AR. However, this mutant receptor not only desensitized in response to agonist stimulation but down-regulated normally. In an attempt to understand the basis for the properties of this mutant, we have examined the ability of this regulation-defective mutant to undergo agonist-mediated phosphorylation. When expressed in 293 cells, the maximal response for phosphorylation of the β_2AR-Y326A mutant was impaired by 75%. Further characterization of this phosphorylation, using either forskolin stimulation or phosphorylation site-deficient β_2AR-Y326A mutants, demonstrated that the β_2AR-Y326A mutant can be phosphorylated by cAMP-dependent protein kinase (PKA) but does not serve as a substrate for the β-adrenergic receptor kinase 1 (βARK1). However, overexpression of βARK1 led to the agonist-dependent phosphorylation of the β_2AR-Y326A mutant and rescue of its sequestration. βARK1-mediated rescue of β_2AR-Y326A sequestration could be prevented by mutating putative βARK phosphorylation sites, but not PKA phosphorylation sites. In addition, both sequestration and phosphorylation of the wild-type β_2AR could be attenuated by overexpressing a dominant-negative mutant of βARK1 (C_{20} βARK1-K220M). These findings implicate a role for βARK1-mediated phosphorylation in facilitating wild-type β_2AR sequestration.

The exposure of the β_2-adrenergic receptor (β_2AR)[1] to catecholamines initiates its biological response via coupling to the stimulatory G protein (G_s), which then mediates the stimulation of adenylyl cyclase (1, 2). However, this receptor-mediated adenylyl cyclase response to agonist is followed by a rapid uncoupling of the receptor from its effector system, termed desensitization. The mechanisms of desensitization have been particularly well studied using the β_2AR as a model system (2). Several studies have demonstrated that the functional uncoupling of the β_2AR from G_s is the consequence of its phosphorylation by one of two types of kinases (3–9). Desensitization of agonist-occupied or activated β_2AR involves phosphorylation by a growing family of G protein-coupled receptor kinases, of which β-adrenergic receptor kinase (βARK1) is a member (3, 5, 9). This phosphorylation serves to promote the binding of β-arrestin to the receptor, which when bound further uncouples the receptor (10–12). Moreover, cAMP-dependent protein kinase (PKA) phosphorylation can desensitize the β_2AR in response to elevated intracellular cAMP levels (6–9).

In addition to functional uncoupling of the β_2AR and G_s, agonist-mediated receptor internalization (sequestration) results in spatial uncoupling, such that in response to agonist plasma membrane receptors are removed to an intracellular compartment, probably into endosomes (13). This has led to speculation that sequestration might represent a major mechanism of β_2AR desensitization. However, a large body of experimental evidence suggests that this is not the case, as both pharmacological manipulations and mutant receptors have been used to demonstrate that the β_2AR desensitizes in the absence of receptor sequestration (4, 14–17). In addition, receptor desensitization proceeds much faster than sequestration (18, 19). Thus, sequestration mostly affects receptors that have already been uncoupled from G_s. This has led to the suggestion that receptor sequestration, rather than playing a role in receptor desensitization, might play a more important role in mediating the resensitization of desensitized receptors (14–16, 20).

Sequestered β_2ARs are phosphorylated to a lesser extent than plasma membrane-associated receptors, which prompted the proposal that β_2AR sequestration might be triggered by phosphorylation (20). However, further investigation of this hypothesis using phosphorylation site-deficient β_2AR mutants, as well as various truncated β_2ARs, led to the conclusion that phosphorylation was not a prerequisite of β_2AR sequestration (3, 9, 21, 22). Nonetheless, recent data have renewed interest in the role of βARK phosphorylation in receptor sequestration. Tsuga *et al.* (23) demonstrated that overexpression of βARK1 could facilitate m2 muscarinic acetylcholine receptor sequestration, whereas overexpression of a dominant-negative βARK1 could attenuate it.

Previously, we reported that mutation of tyrosine residue 326 to an alanine residue in the seventh transmembrane domain of the β_2AR resulted in a sequestration-defective receptor

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[1] The abbreviations used are: β_2AR, β_2-adrenergic receptor; PKA, cAMP-dependent protein kinase; βARK1, β-adrenergic receptor kinase 1; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; 293 cells, human embryonic kidney cells; CHW, Chinese hamster fibroblasts; CHO, Chinese hamster ovary cells; IBMX, 1-methyl-3-isobutylxanthine; ISO, isoproterenol.

(β_2AR-Y326A) (15). The β_2AR-Y326A mutant, while unable to sequester, could desensitize, down-regulate, and be phosphorylated in response to agonist and, although its coupling was impaired, was able to maximally stimulate adenylyl cyclase in membranes (15). More importantly, this receptor mutant was impaired in its ability to resensitize. This suggested that the β_2AR-Y326A mutant might provide an excellent tool for directly testing if agonist-promoted receptor sequestration played a role in receptor dephosphorylation. However, further examination of the ability of the β_2AR-Y326A mutant to be phosphorylated revealed that the β_2AR-Y326A mutant could not be used to study receptor dephosphorylation since, while it was a substrate for PKA phosphorylation, it did not serve as a substrate for phosphorylation by G protein-coupled receptor kinases. Thus, the previously described desensitization of this mutant (15) was likely the consequence of PKA- rather than βARK-dependent mechanisms of desensitization. Interestingly though, we have used the β_2AR-Y326A mutant in conjunction with βARK1 overexpression, as well as a dominant-negative βARK1, to demonstrate that βARK phosphorylation can facilitate β_2AR sequestration.

Tsuga *et al.* (23), in a recent report, suggested that the ability of βARK1 to facilitate m2 muscarinic acetylcholine receptor sequestration implied a unique role of phosphorylation for this class of G_i-coupled receptor distinct from the G_s-coupled β_2AR. The present results demonstrate a clear role for βARK phosphorylation in the facilitation of β_2AR sequestration, suggesting that phosphorylation plays a broader role in agonist-promoted G protein-coupled receptor sequestration than previously envisaged.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-Pindolol and [^{32}P]orthophosphate were purchased from DuPont NEN. Isoproterenol, propranolol, forskolin, IBMX, Nonidet P-40, and bovine serum albumin were acquired from Sigma. CGP-12177 was obtained from Boehringer Mannheim. Protein A-Sepharose 4 fast flow was supplied by Pharmacia Biotech Inc. 12CA5 ascites were purchased from Babco. Human embryonic kidney cells (293 cells) were from the American Tissue Culture Collection. Gentamicin, minimal essential medium, phosphate-free Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and fetal bovine serum were provided by Life Technologies, Inc. Restriction enzymes were obtained from Promega. Hot Tub DNA polymerase and ECL Western blotting analysis system were supplied by Amersham Corp. All other chemicals and reagents were purchased from Fisher or VWR.

Plasmid Constructions—The putative sites of PKA and βARK phosphorylation (9, 24, 25) were created as follows. The PKA and βARK phosphorylation site-deficient human β_2AR mutants described by Hausdorff *et al.* (3) and the 12CA5 epitope-tagged β_2AR and β_2AR-Y326A in pBC (15) were used as templates for construction of PKA and βARK phosphorylation site-deficient β_2AR-Y326A mutants. In brief, as described previously (3), Ser^{261}, Ser^{262}, Ser^{345}, and Ser^{346} were replaced by an alanine residue to create the PKA phosphorylation site-deficient β_2AR mutant; whereas Ser^{355}, Thr^{360}, Thr^{384}, Thr^{393}, Ser^{401}, Thr^{408}, and Ser^{411} were replaced with an alanine residue and Ser^{356}, Ser^{364}, Ser^{396}, and Ser^{407} were replaced with a glycine residue to create the βARK phosphorylation site-deficient β_2AR mutant. The first mutated PKA site in the third intracellular loop of the PKA site-deficient β_2AR was excised with *Nco*I-*Hpa*I and used to replace the identical cassette of the 12CA5 epitope tagged β_2AR-Y326A cloned into pBC. The second mutated PKA site was created by replacing the *Nco*I-*Fsp*I cassette of the PKA phosphorylation site-deficient β_2AR in pBC with the identical cassette from β_2AR-Y326A containing the first mutated PKA site. A βARK phosphorylation site-deficient 12CA5 epitope-tagged β_2AR-Y326A mutant in pBC was obtained by replacing the *Stu*I-*Acc*I cassette containing all the potential βARK phosphorylation sites with the same cassette from the βARK site-deficient β_2AR in pBC. pcDNA1/Amp constructs were obtained by excising the entire cDNA for each of the mutants from pBC using *Nco*I-*Sal*I, blunting and subcloning them into the *Eco*RV site of pcDNA1/Amp.

A point mutation in bovine βARK1 (K220M) was generated by polymerase chain reaction using a 5′ primer encompassing an *Acc*I (position 614) 5′ of the lysine to be mutated. The codon at positions 658–660, AAG (lysine 220), was mutated to ATG (methionine). A 3′ primer located 3′ to a second *Acc*I site (position 941) was used in conjunction with the 5′ primer to generate the reading frame containing both *Acc*I sites. The DNA was cloned into pCR™II (Invitrogen) according to the specifications of the manufacturer. Positive clones were isolated, and the mutation, as well as the integrity of the coding sequence between both *Acc*I sites, was confirmed by dideoxy DNA sequencing. The *Acc*I fragment was isolated and replaced in C_{20} βARK1 (26) cloned in pBC. An *Msc*I-*Rsr*II cassette in pcDNA1/Amp C_{20} βARK1 was replaced with the same cassette isolated from the pBC construct to create C_{20} βARK1-K220M in pcDNA1/Amp.

Cell Culture and Transfection—293 cells were grown in minimal essential medium with Earle's salts, supplemented with heat-inactivated fetal bovine serum (10% v/v) and gentamicin (100 μg/ml). The cells were seeded at a density of ~2.5×10^6 cells/100-mm dish and transiently transfected using a modified calcium-phosphate method (27). Cells were transfected with either 0.05 μg/dish, for sequestration studies, or with 0.3–0.4 μg/dish, for phosphorylation studies, of human β_2AR receptor cDNA expressed in the pcDNA1/Amp expression vector. In studies where β_2AR receptor cDNA was coexpressed with bovine βARK1 cDNA, the total amount of DNA transfected per dish was kept constant by using appropriate amounts of empty vector. Following transfection (~18 h), the 293 cells were incubated with fresh culture medium and allowed to recover 7–9 h, before being reseeded in either 6- or 12-well dishes (Falcon) and allowed to grow an additional 15–18 h. Transient transfection of COS7 cells was achieved in the same manner as 293 cells except that COS7 cells were glycerol shocked prior to incubation with fresh media. Permanent transfection of Chinese hamster ovary cells (CHO) and Chinese Hamster Fibroblasts (CHW) was performed as described previously (3, 15).

Ligand Binding—Whole cell radioligand binding studies were performed as follows. Cells seeded in 12-well dishes were washed twice with ice-cold PBS and then detached from the wells using ice-cold PBS with 5 mM EDTA. Total receptor binding was measured using saturating concentrations of ^{125}I-pindolol (~1 nM), which, because of its hydrophobicity, can measure both surface and intracellular receptors. Nonspecific binding was measured in the presence of 10 μM propranolol. Binding studies were done at 30 °C for 30 min for the measurement of receptor expression, and bound ligand was separated on glass fiber filters (Whatman, GF/C) by vacuum filtration. The filters were washed four times with 4 ml of cold wash buffer (50 mM Tris, 120 mM NaCl, pH 7.2) and counted in a γ-counter. Protein concentrations were determined using a Bio-Rad assay kit with bovine serum albumin as the standard.

Sequestration—293 cells grown in six-well dishes (Falcon) were washed twice with serum-free culture medium (37 °C). Matching wells were then treated with serum-free culture medium containing 100 μM ascorbate with or without 10 μM isoproterenol (ISO) and incubated 30 min at 37 °C. Receptor sequestration was measured as described previously (3, 15); in brief, the cells were washed and prepared for whole cell binding studies as described above, except that binding was performed at 14 °C for 3 h with ^{125}I-pindolol in the presence or absence of either 100 nM CGP-12177 or 10 μM propranolol. Receptor sequestration was defined as the fraction of specific radioligand binding not competed for by CGP-12177 (a hydrophilic ligand) minus the basal level of sequestration as measured without exposure to agonist.

Whole Cell Phosphorylation—293 cells seeded in six-well dishes were washed twice with phosphate-free DMEM without serum (37 °C) and then labeled for 45 min at 37 °C with 0.5 ml/well of [^{32}P]orthophosphate (100 μCi/ml) in the same medium. Duplicate pairs of matching wells containing labeled cells were then treated with an additional 0.5 ml of serum- and phosphate-free DMEM containing 100 μM ascorbate with or without ISO (10 μM final concentration) and incubated at 37 °C for 15 min or for the indicated times. The cells were washed three times with ice-cold PBS and then scraped in 0.4 ml/well of radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.1 mM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, pH 7.4), following which duplicate wells were combined. The cells were solubilized for 1 h at 4 °C on an inversion wheel. Supernatants were obtained by centrifugation at 436,000 × *g* for 15 min in a TLA 100.2 rotor in a Beckman Optima TL ultracentrifuge, following which aliquots of each sample were taken to determine total protein content in the supernatant of each sample to be immunoprecipitated. Epitope-tagged receptor was immunoprecipitated for 1 h at 4 °C on protein A-Sepharose beads using 12CA5 monoclonal antibody. The Sepharose beads were washed three times with radioimmune precipitation buffer and receptor was eluted from the beads in 50

μl of SDS sample buffer at 65 °C for 10 min. In each experiment, each lane of the SDS-polyacrylamide gel was loaded with equivalent amounts of receptor protein in a 45-μl volume. The amount of receptor in each sample was calculated as the function of receptor expression (pmol/mg of whole cell protein) times the total protein content of the solubilized fraction of each sample subjected to immunoprecipitation (*i.e.* (pmol receptor/mg of whole cell protein) × (mg solubilized protein/sample)= pmol receptor/sample). Receptor expression was determined as described above on cell samples for each transfection. The receptor content of each sample was normalized to the sample with the least receptor content by dilution with sample buffer. Samples were then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The extent of receptor phosphorylation was quantitated using a Molecular Dynamics phosphorimaging system and ImageQuant software.

Western Blotting—Samples of 293 cells resuspended in PBS with 5 mM EDTA were centrifuged at 4000 rpm for 20 min at 4 °C, pellets resuspended in SDS-sample buffer (50 μg of whole cell protein/25 μl), and sonicated 30 s. 25-μl samples were subjected to SDS-polyacrylamide gel electrophoresis followed by electroblotting with a Millipore Milliblot semi-dry electroblotting system onto nitrocellulose membranes. The membranes were blocked using PBS with 3% bovine serum albumin for 1 h at room temperature and then incubated with diluted 1:4000 βARK1/2 specific antiserum (28) in PBS with 3% bovine serum albumin and 0.15% Tween 20 (v/v). After incubation with the antiserum, the membranes were washed three times for 10 min in PBS with 0.2% Tween 20 and then incubated 1 h at room temperature with diluted 1:2000 horseradish peroxidase-conjugated donkey anti-rabbit IgG supplied with the ECL Western blotting analysis system in PBS with 3% bovine serum albumin and 0.15% Tween 20. After removal of the secondary antiserum, the membranes were washed three times in PBS with 0.2% Tween 20 and then were exposed using the ECL Western blotting analysis system.

Data Analysis—Mean and either the standard deviation or standard error of the mean are expressed for values obtained on the number of separate experiments indicated. Statistical significance was determine by both ANOVA and multiple comparisons between groups using a two-tailed *t* test for independent samples and found to be equivalent. The statistical significance values for the multiple comparisons made are shown in the figure legends. *p* values were not corrected for multiple comparisons. Phosphorylation time course data was fit to the curve $Y = (R_0 \times k_1/(k_1 + k_2)) \times (1 - \exp(-(k_1 + k_2)) \times X)$, where R_0 = maximal response, k_1 = forward rate constant, and k_2 = reverse rate constant, and analyzed using GraphPad Prism. Dose-response data were analyzed using GraphPad Prism.

RESULTS

Comparison of Wild-type β_2AR and β_2AR-Y326A Mutant Phosphorylation—Initial experiments tested the time course for the whole cell phosphorylation of wild-type β_2AR and β_2AR-Y326A expressed at similar levels in 293 cells, in response to 10 μM ISO stimulation (Fig. 1*A*). This resulted in a maximum increase in phosphorylation of 5.2 ± 1.0-fold over basal for the β_2AR, but only 2.6 ± 0.7-fold over basal for the β_2AR-Y326A mutant (a similar result was obtained for permanently transfected CHO cells, data not shown). Maximal phosphorylation of each receptor was observed within 3–5 min of agonist stimulation ($t_{1/2}$ = 41 ± 14 s for the β_2AR *versus* 76 ± 13 for the β_2AR-Y326A, $p < 0.05$).

Although the wild-type β_2AR and β_2AR-Y326A mutant phosphorylate at somewhat different rates, this could not account for the large differences in the extent of phosphorylation observed for these two receptors. The β_2AR serves as substrate for phosphorylation by both PKA and βARK (3, 4). Therefore, the β_2AR-Y326A mutant was tested for its ability to be phosphorylated by these two types of kinases. This was tested in two ways. First, wild-type β_2ARs and β_2AR-Y326A mutants, expressed in 293 cells, were challenged for 15 min with either 10 μM ISO or 25 μM forskolin (in the presence of 1 mM IBMX) to stimulate adenylyl cyclase and raise intracellular cAMP levels in the presence or absence of receptor activation, respectively. These results are illustrated in Fig. 1*B* and are normalized to agonist-induced stimulation of wild-type β_2AR phosphorylation. Under these conditions, the magnitude of agonist- and forskolin-induced phosphorylation of the β_2Y326A mutant was identical (26 ± 5.7% and 23 ± 7.6% of wild-type β_2AR phosphorylation, respectively). This was equivalent to forskolin-induced phosphorylation of the wild-type β_2AR (21 ± 7.6%). Second, wild-type β_2AR and β_2AR-Y326A mutants were prepared in which their putative sites of PKA and βARK phosphorylation were removed. The removal of the putative PKA and

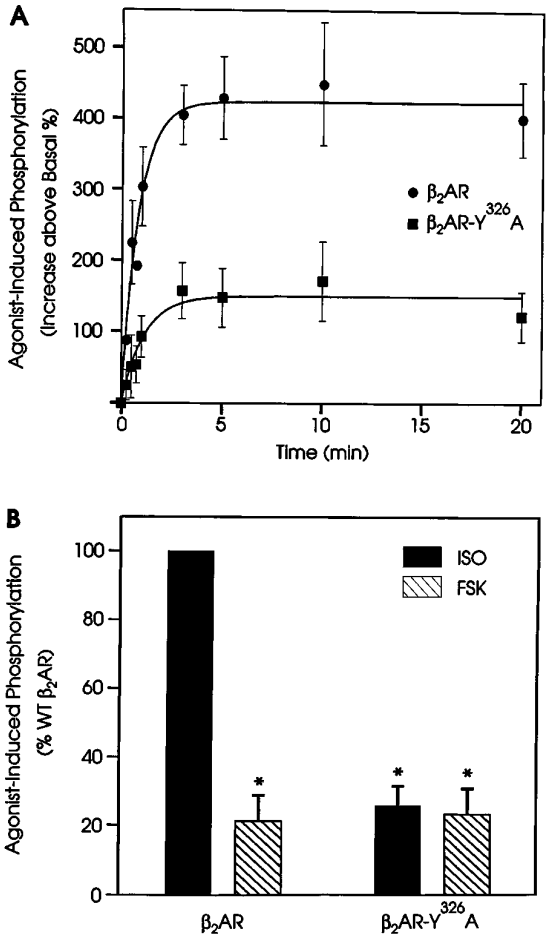

FIG. 1. **Comparison of the whole cell phosphorylation of wild-type β_2ARs and β_2AR-Y326A mutants.** 293 cells were transfected with pcDNA1/Amp cDNA encoding β_2AR and β_2AR-Y326A and assayed for whole cell phosphorylation as described under "Experimental Procedures." *A*, time course for the phosphorylation of wild-type β_2AR (725 ± 261 fmol/mg of whole cell protein) and β_2AR-Y326A mutant (646 ± 175 fmol/mg of whole cell protein) in response to stimulation with 10 μM ISO (15 s to 20 min). The *R* values for the curve fits for β_2AR and β_2AR-Y326A mutant phosphorylation time courses were 0.97 and 0.98, respectively. *B*, the comparison of agonist-induced (10 μM ISO) phosphorylation with forskolin (*FSK*)-mediated (25 μM in the presence of 1 mM IBMX) phosphorylation of the wild-type β_2AR (772 ± 457 fmol/mg of whole cell protein) and β_2AR-Y326A mutants (763 ± 385 fmol/mg of whole cell protein). In each experiment, the basal phosphorylation for each receptor type was subtracted from that seen in the presence of agonist to give the agonist-stimulated increase in the level of phosphorylation. This value, in turn, was compared with that seen with wild-type β_2AR in the same experiment and expressed as a percentage of the agonist-induced wild-type β_2AR phosphorylation, which was increased 5.7 ± 1.0-fold above basal, following 15-min stimulation with 10 μM ISO. The data from the autoradiographs were analyzed using a Molecular Dynamics phosphorimaging system. Basal phosphorylation (agonist-independent phosphorylation) of the β_2AR and β_2AR-Y326A mutant in *A* and *B* was identical as quantitated using the Molecular Dynamics phosphorimaging system. The data in each panel represent the mean ± S.D. (*bars*) values from four different experiments; where no error bar is shown, the S.D. was smaller than the symbol. In *B*, the *asterisk* indicates $p < 0.001$ *versus* wild-type β_2AR phosphorylation in response to agonist.

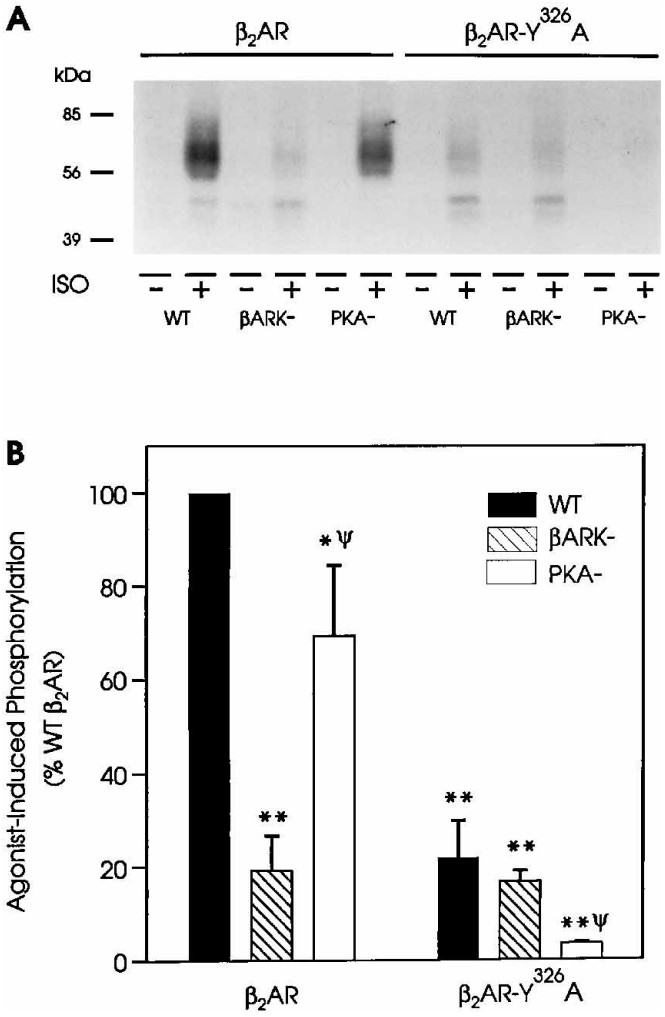

FIG. 2. **Phosphorylation of wild-type β_2AR and β_2AR-Y326A phosphorylation site-deficient mutants.** 293 cells were transfected with pcDNA1/Amp cDNA encoding wild-type (*WT*), βARK phosphorylation site-deficient (*βARK-*) or PKA phosphorylation site-deficient (*PKA-*) β_2AR and β_2AR-Y326A and assayed for whole cell phosphorylation as described under "Experimental Procedures." *A*, autoradiograph from a representative experiment showing the whole cell phosphorylation of each receptor following a 15-min incubation in the absence (−) or presence (+) of 10 μM ISO. Expression of each of the mutant receptors was equivalent in these experiments, and each lane was loaded with equivalent amounts of receptor protein as described under "Experimental procedures." *B*, The mean ± S.D. (*bars*) for the quantitative analysis of four different experiments. In these experiments, the data were normalized to the agonist-induced wild-type β_2AR phosphorylation which was increased 6.6 ± 1.2-fold above basal (see legend to Fig. 1). The expression levels for each receptor (fmol/mg of whole cell protein) were as follows β_2AR-WT = 1725 ± 455, β_2AR-βARK$^-$ = 1729 ± 122, β_2AR-PKA$^-$ = 1995 ± 183, β_2AR-Y326A-WT = 1665 ± 268, β_2AR-Y326A-βARK$^-$ = 1786 ± 128 and β_2AR-Y326A-PKA$^-$ = 1879 ± 283. As seen in the autoradiograph there was no difference in the basal phosphorylation for any of the receptors. *Asterisk*, $p < 0.05$; *double asterisks*, $p < 0.0005$ *versus* agonist-stimulated wild-type β_2AR phosphorylation. ψ, $p < 0.01$ *versus* agonist-stimulated wild-type β_2AR-Y326A phosphorylation.

βARK phosphorylation sites in either the wild-type β_2AR or β_2AR-Y326A did not prevent the mobilization of an adenylyl cyclase response by any of the phosphorylation site-deficient mutants (Ref. 3; data not shown). The agonist induced-phosphorylation of these phosphorylation site-deficient mutants is illustrated in Fig. 2, *A* and *B*. In 293 cells, the wild-type β_2AR was phosphorylated predominantly at βARK phosphorylation sites (69.3 ± 15.3%) rather than PKA phosphorylation sites (22 ± 7.9%). However, the β_2AR-Y326A served solely as a PKA substrate, as the level of its phosphorylation was unchanged by the removal of βARK phosphorylation sites (20 ± 7.5% *versus* 17 ± 2.3%). β_2AR-Y326A mutant phosphorylation was essentially inhibited upon the removal of its PKA phosphorylation sites (Fig. 2).

Rescue of β_2AR-Y326A Mutant Phosphorylation and Sequestration—It was clear that the β_2AR-Y326A mutant, under the experimental conditions tested, did not serve as a substrate for phosphorylation at βARK phosphorylation sites and served only as a substrate for PKA in 293 cells. However, the possibility existed that the Y326A mutation reduced the effectiveness of the activated mutant receptor as a substrate for βARK phosphorylation. Therefore, we tested whether βARK1 overexpression could overcome the presumed impairment as a substrate for βARK phosphorylation of the β_2AR-Y326A mutant. Transfection of 293 cells with increasing amounts of βARK1 cDNA in a pcDNA1 expression vector resulted in increased βARK1 expression levels (Fig. 3*A*). As shown in Fig. 3*B*, βARK1, when overexpressed, rescued the phosphorylation of the β_2AR-Y326A mutant, such that the extent of phosphorylation of the mutant receptor was equivalent to wild-type β_2AR phosphorylation if both receptors where coexpressed with 10 μg of βARK1 cDNA. The level of agonist-induced β_2AR-Y326A mutant phosphorylation was increased >5-fold from 26.9 ± 2.6% to a maximum of 146 ± 36% of wild-type β_2AR phosphorylation (Fig. 3*C*). Therefore, the Y326A mutation appears to decrease the effectiveness of β_2AR as a substrate for βARK phosphorylation. Overexpressed βARK1 also increased wild-type β_2AR phosphorylation by 50–70% above control phosphorylation levels, indicating that the availability of βARK in these cells may be limiting in some fashion.

Since the β_2AR-Y326A mutant phosphorylation deficit could be reversed by overexpressing βARK1, we reasoned that agonist-promoted sequestration might also be influenced by the overexpression of βARK1. Under control conditions, 42 ± 3.1% of wild-type β_2ARs and 5 ± 1.9% of β_2AR-Y326A mutants sequestered in response to 10 μM ISO stimulation (Fig. 4). However, as shown in Fig. 4, the increase in βARK1 expression (Fig. 3*A*) produced a progressive rescue of β_2AR-Y326A mutant sequestration essentially back to wild-type β_2AR levels. Overexpression of βARK1 had no effect on the sequestration of the wild-type β_2AR.

In addition to 293 cells, the ability of the β_2AR-Y326A mutant to sequester was also tested in CHO, CHW, and COS7 cells. In each case, the sequestration of the β_2AR-Y326A mutant was impaired to an equivalent or greater extent than reported here for its sequestration in 293 cells, indicating that its inability to sequester in response to agonist was not the consequence of the cellular environment in which it was tested (data not shown).

Role of Phosphorylation in Sequestration—βARK1 rescued both the phosphorylation and sequestration of the β_2AR-Y326A mutant. The idea that βARK-mediated receptor phosphorylation might facilitate agonist-promoted sequestration was confirmed by two approaches. First, we tested the ability of βARK1 overexpression to rescue the sequestration of phosphorylation site-deficient β_2AR-Y326A mutants. Second, a dominant-negative βARK1 was assessed for its ability to inhibit both phosphorylation and sequestration of wild-type β_2AR.

For these experiments, geranylgeranylated (C_{20} isoprenylated) versions of βARK1 or βARK1-K220M (C_{20} βARK1) in pcDNA1/Amp were used. Previously, Kong *et al.* (29) demonstrated that βARK1-K220R did not inhibit β_2AR sequestration. However, this dominant-negative βARK1 mutant appeared to be impaired in its interaction with G protein βγ subunits (29). Isoprenylation of βARK1 directly targets the cytosolic kinase to the membrane, without the need for coupling of the receptor to

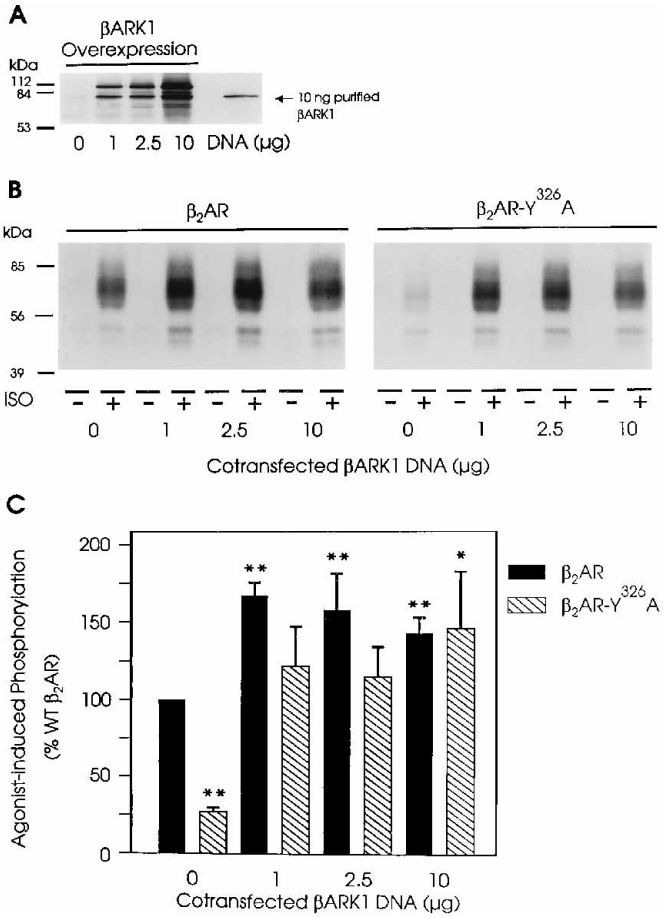

FIG. 3. **Effect of the overexpression of βARK1 on β_2AR and β_2AR-Y326A mutant phosphorylation.** 293 cells were transfected with 0, 1, 2.5, or 10 μg of βARK1 cDNA in a pcDNA1 expression vector along with pcDNA1/Amp cDNA either coding for wild-type β_2AR (1237 ± 142 fmol/mg of whole cell protein) or β_2AR-Y326A (2071 ± 250 fmol/mg of whole cell protein) and assayed for whole cell phosphorylation as described under "Experimental Procedures." *A*, immunoblot of the overexpression of βARK1 protein with increasing amounts of transfected βARK1/2 cDNA. The *far right lane* shows the appropriate migration of 10 ng of purified βARK1. Two major molecular weight species were detected with βARK1/2 ½ antibody, when βARK1 was overexpressed using the pcDNA1/Amp expression vector. The *lower band* migrates at the same molecular weight as the purified βARK1. The *upper band* is presumed to represent improperly processed βARK1 resulting from its overexpression in 293 cells. Nontransfected 293 cells express both βARK1 and βARK2 but cannot be easily visualized in the immunoblot without saturating the lanes containing samples of cells overexpressing βARK1. *B*, autoradiograph showing a representative whole cell phosphorylation of wild-type β_2AR and β_2AR-Y326A following coexpression with increasing amounts of βARK1 following incubation for 15 min in the absence (−) or presence (+) of 10 μM ISO. Each lane was loaded with equivalent amounts of wild-type and Y326A mutant receptor protein as described under "Experimental procedures." The major species of the β_2AR expressed in 293 cells is a glycoprotein of a molecular mass ranging from 56 to 85 kDa. Immunoblots with biotinylated 12CA5 identified each of the major phosphorylated bands (data not shown). *C*, the mean ± S.D. (*bars*) of the quantitative analysis of three different experiments. In these experiments, the data were normalized to the agonist-induced wild-type β_2AR phosphorylation, which was increased 4.2 ± 1.4-fold above basal (see legend to Fig. 1). The basal phosphorylation of either receptor was unaffected by the coexpression of increasing amounts of βARK1. $p < 0.05$ (*asterisk*) or $p < 0.001$ (*double asterisks*) *versus* control agonist-stimulated β_2AR phosphorylation.

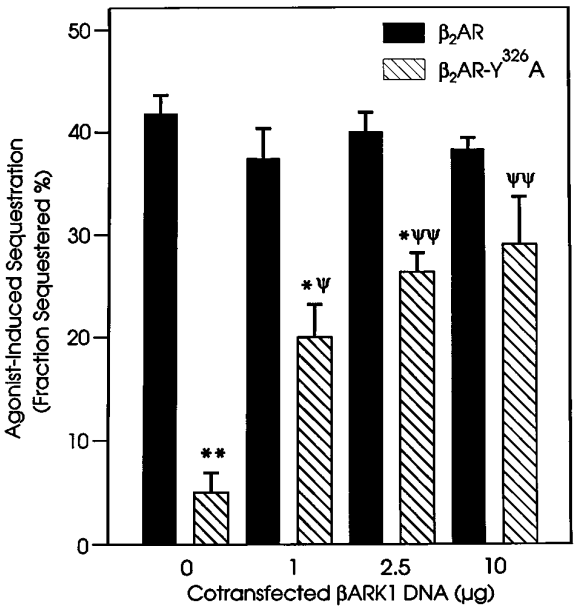

FIG. 4. **Effect of the overexpression of βARK1 on β_2AR and β_2AR-Y326A mutant sequestration.** 293 cells were transfected with 0, 1, 2.5, or 10 μg of βARK1 cDNA in a pcDNA1 expression vector along with pcDNA1/Amp cDNA either coding for wild-type β_2AR (967 ± 85 fmol/mg of whole cell protein) or β_2AR-Y326A mutant (812 ± 94 fmol/mg of whole cell protein) and assayed for agonist-promoted sequestration as described under "Experimental Procedures." Basal sequestration of both the β_2AR and β_2AR-Y326A mutant were unaffected by coexpression with increasing amounts of βARK1. Basally sequestered receptors represented 27 ± 4% and 31 ± 2.4% of total cellular β_2AR and β_2AR-Y326A, respectively, in these experiments. The data represents the mean ± S.E. (*bars*) for three different experiments. $p < 0.01$ (*asterisk*) or $p < 0.0001$ (*double asterisks*) *versus* control agonist-promoted β_2AR sequestration. $p < 0.005$ (ψ) or $p < 0.0005$ (ψψ) *versus* control agonist-promoted β_2AR-Y326A mutant sequestration.

G_s, resulting in the subsequent dissociation of $G_{\beta\gamma}$ which then mediates translocation of βARK to the plasma membrane (5, 26, 30). This suggested that a C_{20} isoprenylated dominant-negative βARK1 might be more effective at inhibiting β_2AR phosphorylation and sequestration. When tested, C_{20} BARK1, like wild-type βARK1, could rescue both wild-type β_2AR-Y326A mutant sequestration (compare Figs. 4 and 5) and phosphorylation (data not shown). C_{20} βARK1 was used to test the rescue of phosphorylation site-deficient β_2AR and β_2AR-Y326A mutants. Removal of PKA phosphorylation sites had no effect on the sequestration of the wild-type β_2AR, but removal of βARK phosphorylation sites reduced agonist-induced sequestration by 50%, again hinting toward a potential role for phosphorylation in the process. Overexpression of C_{20} βARK1 was unable to rescue the sequestration of the βARK phosphorylation-deficient β_2AR. In the absence of C_{20} βARK1 overexpression, all of the β_2AR-Y326A mutants were impaired in their ability to sequester. However, overexpression of C_{20} βARK1 rescued the sequestration of the wild-type β_2AR-Y326A and PKA phosphorylation site-deficient β_2AR-Y326A mutants, but was unable to rescue the sequestration of the βARK phosphorylation site-deficient β_2AR-Y326A mutant.

A kinase mutant with a methionine residue substituted for lysine 220 in the catalytic domain of C_{20} βARK1 (C_{20} βARK1-K220M) was overexpressed using a pcDNA1/Amp expression vector (Fig. 6*A*) and tested for its ability to inhibit agonist-induced wild-type β_2AR phosphorylation (Fig. 6*B*) and sequestration (Fig. 7). Agonist-induced phosphorylation of the β_2AR was inhibited significantly following cotransfection with either 1 μg (31 ± 11%, $p < 0.01$) or 2.5 μg (40 ± 6.8%, $p < 0.001$) of dominant-negative kinase DNA, but β_2AR-Y326A mutant phosphorylation was unaffected (Fig. 6*C*). Cotransfection with larger amounts of DNA did not further enhance the inhibition of wild-type β_2AR phosphorylation by the dominant-negative kinase. Shown in Fig. 7 is a dose-response curve for agonist-promoted wild-type β_2AR sequestration in either the presence or absence of 1 μg of cotransfected C_{20} βARK1-K220M cDNA.

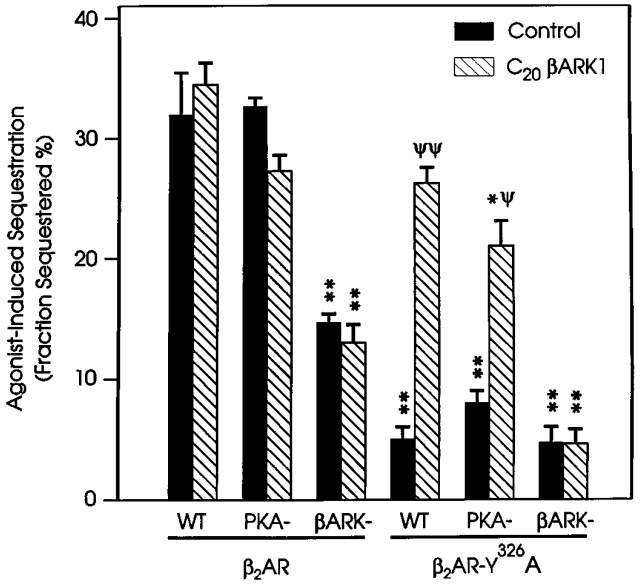

FIG. 5. **Effect of C_{20} βARK1 on the sequestration of wild-type β_2AR and β_2AR-Y326A phosphorylation site-deficient mutants.** 293 cells were transfected with 1 μg of pcDNA1/Amp C_{20} βARK1 cDNA along with pcDNA1/Amp cDNA encoding one of the following: wild-type (*WT*), βARK phosphorylation site-deficient (*βARK-*), or PKA phosphorylation site-deficient (*PKA-*) β_2AR and β_2AR-Y326A and assayed for agonist-promoted sequestration as described under "Experimental Procedures." The basal sequestration of each of the receptors was equivalent and was not affected by coexpression of 1 μg of C_{20} βARK1 cDNA. Basally sequestered receptors represented 30 ± 1.8% of total cellular receptors in these experiments. The expression levels for each receptor (fmol/mg of whole cell protein) were as follows β_2AR-WT = 1102 ± 173, β_2AR-βARK$^-$ = 1108 ± 243, β_2AR-PKA$^-$ = 1440 ± 252, β_2AR-Y326A-WT = 757 ± 112, β_2AR-Y326A-βARK$^-$ = 795 ± 161 and β_2AR-Y326A-PKA$^-$ = 951 ± 161. The data represent the mean ± S.E. (*bars*) for three to five different experiments. $p < 0.05$ (*asterisk*) or $p < 0.005$ (*double asterisks*) *versus* agonist-promoted wild-type β_2AR sequestration. $p < 0.05$ (ψ) or $p < 0.005$ ($\psi\psi$) *versus* agonist-promoted wild-type β_2AR-Y326A mutant sequestration.

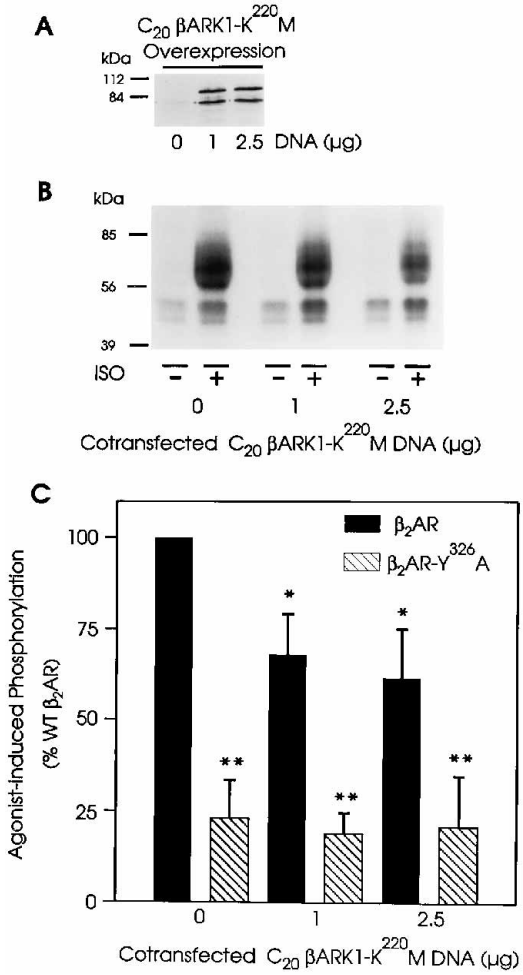

FIG. 6. **The effect of C_{20} βARK1-K220M overexpression on the phosphorylation of wild-type β_2AR and β_2AR-Y326A mutant.** 293 cells were transfected with 0, 1, or 2.5 μg of pcDNA1/Amp C_{20} βARK1-K220M cDNA along with pcDNA1/Amp cDNA coding for wild-type β_2AR (1409 ± 104 fmol/mg of whole cell protein) or β_2AR-Y326A (994 ± 164 fmol/mg of whole cell protein) and assayed for whole cell phosphorylation as described under "Experimental Procedures." *A*, immunoblot of the overexpression of C_{20} βARK1-K220M protein with increasing amounts of transfected cDNA. Two major molecular weight species were also detected with βARK1/2 antibody, when C_{20} βARK1-K220M was overexpressed using the pcDNA1/Amp expression vector (see Fig. 3 legend). *B*, autoradiograph showing a representative whole cell phosphorylation of wild-type β_2AR following coexpression with increasing amounts of C_{20} βARK1-K220M incubated for 15 min in the absence (−) or presence (+) of 10 μM ISO. Each lane was loaded with equivalent amounts of receptor protein as described under "Experimental procedures." *C*, the mean ± S.D. (*bars*) of the quantitative analysis of four different experiments. In these experiments, the data were normalized to the agonist-induced wild-type β_2AR phosphorylation which was increased 5.1 ± 1.9-fold over basal (see legend to Fig. 1). The basal phosphorylation of either receptor was unaffected by the coexpression of increasing amounts of C_{20} βARK1-K220M. $p < 0.01$ (*asterisk*) or $p < 0.001$ (*double asterisks*) *versus* control agonist-stimulated β_2AR phosphorylation.

Overexpression of C_{20} βARK1-K220M significantly reduced the maximal response for sequestration, 25 ± 5.8% ($p < 0.05$). Expression of the dominant-negative kinase had no effect on the EC_{50} for sequestration. The EC_{50} values in the presence and absence of C_{20} βARK1-K220M were 11 ± 2.9 nM and 12 ± 1.7 nM, respectively. Under similar conditions, β_2AR-Y326A mutant sequestration was unaffected by C_{20} βARK1-K220M, which is presumably consistent with the inability of C_{20} βARK1-K220M to rescue phosphorylation of the mutant receptor (data not shown).

DISCUSSION

The present experiments clearly demonstrate a role for βARK-mediated phosphorylation in facilitating β_2AR sequestration. This idea is supported by three observations. First, βARK1, when overexpressed, rescues both the phosphorylation and the sequestration of the β_2AR-Y326A mutant which was defective in its ability to be phosphorylated by βARK and to sequester in response to agonist stimulation. Second, overexpressed βARK1 can rescue the sequestration of PKA phosphorylation site-deficient, but not βARK phosphorylation site-deficient, β_2AR-Y326A mutants. Third, sequestration of the wild-type β_2AR can be attenuated by overexpressing a dominant-negative βARK1, which also diminishes agonist-induced phosphorylation of the receptor to a similar extent. These results are in agreement with the work of Tsuga *et al.* (23) where they describe the ability of βARK1 to augment m2 muscarinic acetylcholine receptor sequestration.

The conclusion that βARK1 phosphorylation is involved in β_2AR sequestration evolved from experiments testing the role of sequestration in receptor dephosphorylation using the β_2AR-Y326A mutant. Upon investigation, we found that the phosphorylation of the receptor mutant was reduced and that it served predominantly as a substrate for PKA-mediated phosphorylation, when expressed in 293 cells. This result indicated that the β_2AR-Y326A mutant could not be used to study receptor dephosphorylation. Nonetheless, these results suggested that mutation of tyrosine residue 326 to an alanine not only inhibits the ability of the β_2AR to sequester but also abolished its ability to act as a substrate for βARK phosphorylation.

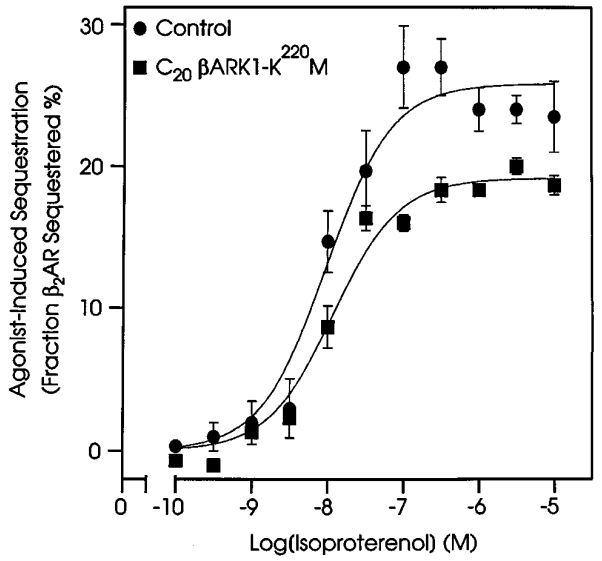

FIG. 7. **Dose response for the inhibition of wild-type β_2AR sequestration by C_{20} βARK1-K220M.** 293 cells were transfected with pcDNA1/Amp cDNA either coding wild-type β_2AR with (1435 ± 319 fmol/mg of whole cell protein) or without (1345 ± 150 fmol/mg of whole cell protein) 1 μg of pcDNA1/Amp C_{20} βARK1-K220M cDNA and assayed for agonist-promoted sequestration as described under "Experimental Procedures" except that sequestration was measured following 30-min stimulation with 10^{-10} to 10^{-5} M ISO. The basal sequestration of the β_2AR in these experiments in the presence or absence C_{20} βARK1-K220M cDNA was 33 ± 1.2% and 30 ± 0.8%, respectively. The data represent the mean ± S.E. (*bars*) for three different experiments. The *R* values for the curve fits in the presence and absence of C_{20} βARK1-K220M were 0.96 and 0.98, respectively.

Therefore, the desensitization of the β_2AR-Y326A mutant previously reported (15) was likely the consequence of PKA- rather than βARK-mediated phosphorylation of the receptor, since both mechanisms have been demonstrated to effectively desensitize the wild-type β_2AR (3, 4).

Historically, most studies have found that receptor phosphorylation was not essential for β_2AR sequestration. In particular, Hausdorff *et al.* (3) demonstrated that neither βARK nor PKA phosphorylation sites were required for sequestration of β_2AR stably expressed by CHW cells. In addition, truncation of the carboxyl tail of the hamster β_2AR, which removes its putative βARK phosphorylation sites, resulted in normal sequestration when expressed in mouse L cells (22), although further truncation of this receptor did result in some impairment of sequestration (31). Finally, Lohse *et al.* (4), using permeabilized A431 cells, demonstrated that sequestration was unaffected by inhibitors of either PKA and βARK phosphorylation. Indeed, in the present study, β_2ARs lacking putative PKA and βARK phosphorylation sites, when expressed in 293 cells, also sequestered in response to agonist exposure, although the β_2ARs lacking putative βARK phosphorylation sites were somewhat impaired in their sequestration (50% of control). The observed impairment in the sequestration of β_2ARs lacking putative βARK phosphorylation sites might be related to the fact that we have used transient transfections in the present study to examine their sequestration, whereas Hausdorff *et al.* (3) selected permanently transfected clonal cell lines which might vary in their sequestration properties depending upon the clone selected. Nonetheless, these mutant receptors do sequester in response to agonist stimulation in 293 cells in the absence of βARK-mediated phosphorylation which is consistent with previously described work. We have tested the ability of the β_2AR-Y326A mutant to sequester in four different cell lines (CHO, 293, CHW, and COS7) and in each case the mutant was impaired in its ability to sequester. This suggests that the inability of this receptor to sequester and be phosphorylated by βARK is likely an intrinsic property of the β_2AR-Y326A mutant receptor rather than the consequence of the cell type in which it has been expressed

It is likely that the intrinsic properties of the β_2AR-Y326A mutant have allowed us to uncover a previously unappreciated role for phosphorylation in the β_2AR sequestration process. The results suggest that phosphorylation of the β_2AR by βARK is facilitory rather than required for sequestration. βARK1 phosphorylation of the β_2AR-Y326A mutant effectively rescues its complete lack of sequestration, whereas removal of the βARK phosphorylation sites in the wild-type β_2AR only reduces sequestration by 50% in 293 cells. This indicates that the basis for the sequestration impairment of the β_2AR-Y326A mutant goes beyond a simple lack of phosphorylation. We suggest that mutation of tyrosine residue 326 to an alanine alters the ability of the agonist-occupied receptor to achieve and/or maintain a conformational state required for receptor phosphorylation by βARK as well as agonist-promoted sequestration. In fact, a smaller proportion of β_2AR-Y326A mutant receptors exhibit high-affinity agonist binding (15). The isomerization of the receptor from its low- to high-affinity state ($R \rightarrow R^*$) might serve to trigger such a change in receptor conformation. Initiation of β_2AR sequestration requires the occupancy of the receptor with agonist; antagonist occupancy is not sufficient. Previous results with a cyc^- variant line of S49 lymphoma cells, which lack functional G_s, provide support for the idea that agonist occupancy is sufficient for both homologous desensitization (βARK-dependent phosphorylation) and sequestration in the absence of coupling to adenylyl cyclase (32). Certainly, constitutively active receptors which achieve R^* in the absence of agonist occupancy are both constitutively phosphorylated and desensitized (33). Thus, an impairment in the active conformation of the β_2AR-Y326A mutant may explain a lack of phosphorylation by the normal endogenous complement of G protein-coupled receptor kinase in 293 cells, but that overexpression of βARK1 in these cells can overcome this deficit. In fact, Ungerer *et al.* (34) have suggested that in the heart βARK might be the limiting component in β-adrenergic receptor desensitization. This might explain why overexpression of βARK leads to increased phosphorylation of the wild-type β_2AR in several cell lines.

The observation that β_2ARs lacking putative βARK phosphorylation sites can sequester in response to agonist stimulation clearly indicates that βARK-mediated phosphorylation neither serves as the signal initiating the sequestration process nor is it an absolute requirement. Instead, we hypothesize that βARK phosphorylation either stabilizes the conformation of the receptor or promotes the interaction of the receptor with some as yet unidentified cellular element that mediates β_2AR internalization, even in the absence of βARK phosphorylation. Since arrestins appear to be required for desensitization, and phosphorylation leads to increased affinity of rhodopsin, β_2AR, and m2 muscarinic receptor for members of the arrestin family (11, 35–38), it is tempting to speculate that arrestins might also play a role in sequestration as they have also been shown to interact with agonist-occupied nonphosphorylated receptor, albeit less effectively (35–38).

The dominant-negative βARK1-K220M, while able to inhibit both the phosphorylation and sequestration of β_2AR to equivalent extents in 293 cells, was not overwhelmingly effective at inhibiting either process, 31 ± 11% and 25 ± 6%, respectively. However, a modest effect of βARK1-K220M on the sequestration of the wild-type β_2AR might be expected, since complete

blockade of βARK phosphorylation should lead at most to a 50% decrease in sequestration (see Fig. 5, βARK phosphorylation site-deficient β_2AR mutant). In two recent studies (23, 29), dominant-negative βARKs have been tested for their ability to affect the sequestration and phosphorylation of β_2AR and m2 muscarinic acetylcholine receptors. Kong *et al.* (29) reported that βARK-K220R, while able to inhibit *in vitro* β_2AR phosphorylation, was impaired in its $G_{\beta\gamma}$ targeting and had no effect on sequestration. However, Tsuga *et al.* (23) demonstrated that βARK1-K220W could inhibit both the phosphorylation and sequestration of the m2 muscarinic acetylcholine receptor subtype, but this was dependent upon the level of endogenously expressed kinase. In the present study, a similar result was obtained for C_{20} βARK1-K220M, which attenuated both whole cell phosphorylation and sequestration of the β_2AR. The apparent discordance in the efficacy of a particular dominant-negative βARK mutant to inhibit sequestration might be dependent on the nature of the residue substituted for lysine 220 or the cellular background in which they are tested. In the work by Tsuga *et al.* (23), the authors concluded that βARK-facilitated sequestration was unique to the G_i-coupled m2 muscarinic receptor, since putative βARK phosphorylation sites are likely found in the third intracellular loop of this receptor rather than in the short cytoplasmic tail (39). Our findings indicate that, in contrast to what is described in the literature for the β_2AR (3, 4, 21, 22), a role for βARK phosphorylation in facilitating receptor sequestration might be more general.

Other studies also support the idea that phosphorylation might be important for sequestration of the β_2AR. For example, the β_3AR does not sequester or phosphorylate in response to agonist stimulation, yet, replacing its carboxyl tail with the tail of the β_2AR rescues both sequestration and phosphorylation of the chimeric β_3/β_2AR (40). In addition, the sequestration of other G protein-coupled receptors, such as the angiotensin II, neurotensin, and α_{1B}-adrenergic receptors, as well as the receptor for parathyroid hormone and parathyroid hormone-related protein, are inhibited by the truncation of their carboxyl tails (41–44). However, the possibility exists that specific sequestration motifs might exist in the tails of these receptors. Interestingly though, a regulatory sequence identified in the tail of the receptor for parathyroid hormone and parathyroid hormone-related protein contains several serine and threonine residues that might act as potential βARK phosphorylation sites (42).

In summary, the present studies demonstrate a clear role for βARK1-mediated phosphorylation in the facilitation of β_2AR sequestration. It will be of interest to determine whether this property is unique to βARK1 or if phosphorylation by other members of the G protein-coupled receptor kinase family can promote β_2AR sequestration as well. We suggest that βARK phosphorylation facilitates, rather than initiates, sequestration as a βARK phosphorylation site-deficient β_2AR mutant can sequester, albeit not normally. βARK phosphorylation has now been shown to facilitate the sequestration of two different G protein-coupled receptors, indicating that phosphorylation plays a broader role in agonist-promoted receptor sequestration than originally envisaged.

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