Characterization of $\beta_2$-Adrenergic Receptor Dephosphorylation: Comparison with the Rate of Resensitization

Tuan M. Tran, Jacqueline Friedman, Faiza Baameur, Brian J. Knoll, Robert H. Moore, and Richard B. Clark

Department of Integrative Biology and Pharmacology, University of Texas Health Science Center, Medical School, Houston, Texas (T.M.T., J.F., F.B., R.B.C.); Department of Pediatrics, Baylor College of Medicine, Houston, Texas (R.H.M.); and Department of Pharmacological and Pharmaceutical Sciences, University of Houston, Houston, Texas (B.J.K.)

Received June 29, 2006; accepted September 28, 2006

ABSTRACT

Dephosphorylation of the cyclic AMP-dependent protein kinase (PKA) site phosphoryserine 262 and the $\beta_2$-adrenergic receptor kinase (GRK) site phosphoryserines 355 and 356 of the $\beta_2$-adrenergic receptor ($\beta_2$AR) were characterized in both intact human embryonic kidney 293 cells and subcellular fractions and were correlated with the rate of resensitization of isoproterenol stimulation of adenylyl cyclase after treatment with isoproterenol and blockade by antagonist. Dephosphorylation of the PKA site after stimulation with 300 pM isoproterenol occurred with a $t_{1/2}$ of 9 min ($k = 0.08 \pm 0.016$ min$^{-1}$) in intact cells in the absence of internalization. Dephosphorylation of the GRK sites in intact cells after treatment with 1.0 $\mu$M isoproterenol for 5 min exhibited a lag phase of $\approx 5$ min, after which dephosphorylation proceeded slowly with a $t_{1/2}$ of 18 min ($k = 0.039 \pm 0.006$ min$^{-1}$). Consistent with the slow rate of GRK site dephosphorylation, the phosphatase inhibitors calyculin A and okadaic acid failed to augment phosphorylation in intact cells during continuous agonist stimulation indicating that GRK site dephosphorylation was minimal. However, both inhibited dephosphorylation of the GRK sites after the addition of antagonist. Slow GRK site dephosphorylation after antagonist treatment was also demonstrated by the relative stability of internalized phosphorylated $\beta_2$AR in cells as observed both by immunofluorescence microscopy using a phospho–site-specific antibody and by studies of the subcellular localization of the GRK-phosphorylated $\beta_2$AR on sucrose gradients that revealed nearly equivalent levels of GRK site phosphorylation in the plasma membrane and vesicular fractions. In addition, dephosphorylation of the GRK sites by intrinsic phosphatase activity occurred only in the heavy vesicle fractions. In contrast to the slow rates of dephosphorylation, the rate of resensitization of isoproterenol stimulation of adenylyl cyclase was 5- and 10-fold faster ($k = 0.43 \pm 0.009$ min$^{-1}$; $t_{1/2} = 1.6$ min), than PKA and GRK site dephosphorylation, respectively, clearly dissociating the rapid phase of resensitization (0–5 min) from dephosphorylation.

In any given cell, the concentration of agonist and the rate constants for phosphorylation and dephosphorylation define the level of PKA and GRK site phosphorylation of the $\beta_2$-adrenergic receptor ($\beta_2$AR) at various times after agonist stimulation, and these factors in turn affect the level of arrestin binding and receptor internalization. During agonist treatment, the rate constants for internalization and recycling control the level of the $\beta_2$ARs in the plasma membrane. To what extent do internalization and dephosphorylation correlate with functional resensitization? This question has been the subject of intense study. Several earlier studies led to the proposal that internalization was required for dephosphorylation and functional resensitization because it appeared that dephosphorylation occurred in endosomes, but not at the plasma membrane (Sibley et al., 1986; Yu et al., 1993; Krueger et al., 1997; Zhang et al., 1997, 1999; Krupnick and Benovic, 1998). Indeed, it is certain that when cells are stimulated with high agonist concentrations, the $\beta_2$AR is rapidly phosphorylated by GRK, and arrestin binds with high affinity (Benovic et al., 1987; Lohse et al., 1989, 1990a,b, 1992; Sohlleman et al., 1995), precluding phosphatase action.

ABBREVIATIONS: PKA, cyclic AMP-dependent protein kinase; GRK, $G$ protein-coupled receptor kinase; $\beta_2$AR, $\beta_2$-adrenergic receptor; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; HEK, human embryonic kidney; PNGase F, peptide $N$-glycosidase F; WT, wild type; DMEM, Dulbecco’s modified Eagle’s medium; AT, ascorbate/thiourea; HE, HEPES/EDTA; ICI-118,551, [1\(-\)]-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[[1-methyllethy]lamino]-2-butanol; PBS, phosphate-buffered saline; CGP-12177, 4-[3-[[1,1-dimethyllethy]lamino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; PAGE, polyacrylamide gel electrophoresis; HV, heavy vesicle; LV, light vesicle.
on the receptor. Upon internalization, it is likely that low affinity agonists, such as epinephrine and isoproterenol, dissociate rapidly from the β2AR, followed by arrestin dissociation, because high affinity arrestin binding requires agonist binding and GRK site dephosphorylation.

Further progress in testing this model was limited by several factors. First was the lack of a method for easily measuring the rate constants for phosphorylation and dephosphorylation of the PKA and GRK sites independently, because early studies were based on 32P-labeling experiments. Second was the general lack of studies of the initial rate of resensitization of functional agonist stimulation after desensitization. A third problem was that the model did not allow for the possible scenario in vivo that agonist stimulation at synapses (and possibly in other physiological situations) is pulsatile, with rapid removal of agonist after stimulation by reuptake, diffusion, and catabolism. That agonist may be removed quickly in vivo, a situation that precludes significant internalization, raises the question of how phosphorylation is reversed.

With the advent of phospho–site-specific antibodies, methods became available to probe the PKA and GRK site phosphorylation and dephosphorylation in a level of detail not previously possible. We were first able to determine the rates of phosphorylation of both the PKA and GRK sites over a range of agonist concentrations because PKA activation is highly amplified and easily dissociated from the relatively unamplified high occupancy-dependent GRK phosphorylation (Tran et al., 2004). Furthermore, we found that at either the low (PKA site) or high (GRK site) range of agonist stimulation, the phosphorylation was remarkably stable (Tran et al., 2004; Vaughan et al., 2006); The ease of following phosphorylation in turn allowed us for the first time to examine dephosphorylation of the PKA and GRK sites independently; we found that dephosphorylation of the PKA site occurred without internalization at the plasma membrane after either washout of agonist or addition of antagonist and that dephosphorylation of the GRK site occurred with blockade of internalization using either sucrose or dominant-negative dynamin (Iyer et al., 2006).

Although our prior study showed that PKA and possibly GRK site dephosphorylations occurred at the plasma membrane, several important questions remained (Kelly, 2006). First, to what extent does functional resensitization of agonist stimulation of the β2AR and adenyl cyclase correlate with the rate constants for dephosphorylation of the PKA and GRK sites? Second, can GRK site dephosphorylation be demonstrated in either plasma membranes or endosomes by biochemical fractionation, obviating the need for internalization blockade? Third, given that plasma membrane dephosphorylation occurs, is it probable that the phosphatases accomplishing PKA and GRK site dephosphorylations are identical? Whereas prior studies suggested that PP2A was the phosphatase involved in GRK site dephosphorylation, PKA site dephosphorylation was not studied. It should be noted that, for the most part, the studies of GRK dephosphorylation were conducted with extracts of a latent PP2A and purified β2AR phosphorylated in vitro (Yang et al., 1988; Pitcher et al., 1995). In addition, there was some evidence that PP2B played a role based on use of FK506 (Shih et al., 1999).

The present work addresses these questions, and demonstrates: 1) a much faster resensitization of agonist stimulation of adenyl cyclase relative to either GRK site or PKA site dephosphorylation; 2) significant GRK site dephosphorylation by intrinsic phosphatase activity in plasma membrane but not endosomal fractions; and 3) a pattern of phosphatase inhibitor effects most consistent with PP1, rather than PP2A, playing the major role in PKA and GRK site dephosphorylation. Furthermore, we conclude, based on the much faster resensitization relative to GRK site dephosphorylation, that the rapid phase of resensitization occurs by the dissociation of arrestin after blockade of agonist stimulation and that the GRK site phosphorylation causes little to no desensitization. This conclusion is consistent with recent fluorescence resonance energy transfer studies demonstrating rapid dissociation of β-arrestin from the β2AR after agonist removal (Krasel et al., 2005), and evidence that GRK phosphorylation alone causes little desensitization (Benovic et al., 1987; Lohse et al., 1990b, 1992; Sohlemann et al., 1995).

Materials and Methods

SuperSignal West Femto maximum sensitivity substrate was purchased from Pierce (Rockford, IL). BA85 nitrocellulose was from Whatman Schleicher and Schuell BioScience (Keene, NH). Cell culture reagents were purchased from Meditech (Hendon, VA). β2AR agonists, antagonists, and calyculin A were purchased from Sigma-Aldrich (St. Louis, MO). Okadaic acid was obtained from Alexis Biochemicals (San Diego, CA). PP1 and PP2A were purchased from Upstate Biotechnology (Lake Placid, NY). Human embryonic kidney (HEK) 293 cells were purchased from American Type Culture Collection (Manassas, VA). Peptide N-glycosidase F (PNGase F) and PP1 inhibitor 2 were purchased from New England Biolabs (Ipswich, MA). Commercially prepared antibodies to the C terminus of the β2AR and to the phosphorylated serines 355 and 356 were from Santa Cruz Biotechnology (Santa Cruz, CA). Custom monoclonal antibodies were obtained from A and G Pharmaceutical, Inc. (Columbia, MD). The antibodies were raised against the peptides C-DRTGHGLRRSpSck-NH2 for the anti-pSer262 PKA site (clone 2G3) and CRAYGNGYpSpSNcN-NH2 for the anti-pS (Ser355,356) (clone 5C3). Monoclonal antibodies were purified using protein A Sepharose. Species-appropriate fluorescent goat anti-rabbit and antimouse secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

Constructs and Cell Lines. HEK 293 cells stably expressing FLAG-WT-β2AR (referred to in the text as WT-β2AR) were used for the majority of the experiments. For some experiments involving PKA site dephosphorylation, we used the N-terminal FLAG-tagged mutant (β2AR-SA3) in which serines 355, 356, and 364 were substituted with alanines (Vaughan et al., 2006). These substitutions were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The mutants were sequenced throughout the entire coding region and epitope tag to ensure accuracy of the mutagenesis. Plasmids were stably transfected into HEK 293 cells using FuGene 6 transfection reagent (Roche Molecular Biochemicals, Basel, Switzerland), and multiple stable clones were selected using 400 µg/ml G418 (Invitrogen) and analyzed with similar results. A431 human epidermoid adenocarcinoma cells were a gift from Dr. Craig Malbon (State University of New York at Stony Brook, Stony Brook, NY).

Cell Culture. HEK 293 cells stably transfected with WT-β2ARs (7–9 pmol/mg) or SA3 (8–10 pmol/mg) mutants and A431 cells were grown in 5% CO2 at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. HEK 293 cells were grown in the same medium as above with the addition of 200 µg/ml G418. When seeding HEK 293 cells for experimentation, dishes were coated with poly-L-lysine to keep the cells attached during washes. One hour before cell treatments, the medium was...
removed and replaced with 40 mM HEPES-buffered, NaHCO₃-free DMEM containing 0.5% fetal bovine serum, and cells were equilibrated without CO₂ at 37°C.

**Intact Cell Phosphorylation and Dephosphorylation Protocols.** HEK 293 cells grown to confluence in 12-well plates were treated at 37°C with β₂AR agonists dissolved in the carrier, 10 mM ascorbate/100 mM thiourea, pH 7 (AT) or AT alone as indicated. These compounds were diluted 100-fold into the cell incubations, and control cells received the appropriate concentration of carrier (0.1 mM ascorbate/1 mM thiourea). For intact cell dephosphorylation, the medium was aspirated and replaced with medium ± 1.0 μM propranolol. To terminate all treatments in preparation for immunoblotting, the medium was removed, and the cells were washed once with 1 ml of 20 mM HEPES and 1.0 mM EDTA, pH 7.7 (HE buffer) at 0–4°C. Cells were solubilized by addition to each well of 200 μl of solubilization buffer at 0–4°C (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.9% dodecyl-β-maltoside, 20 mM tetrasodium pyrophosphate, 10 mM NaF, 0.1 μM okadaic acid, 10 μg/ml benzamidine, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). The cells were scraped into the solubilization buffer, transferred to 1.5-ml micro-centrifuge tubes, and rocked at 4°C for 30 min. The solubilized extract was clarified by centrifugation at 15,000 rpm for 15 min at 4°C, digested with 150 units of PNGase F for 2 h at 37°C, and heated for 15 min at 65°C in SDS sample buffer (2% SDS, 10% glycerol, 100 mM Tris, pH 6.8, bromphenol blue, and 10 mM dithiothreitol). Twenty-microliter aliquots of samples were resolved on 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for immun blotting. The membranes were probed for β₂AR with phosphoryserine-specific antibodies, anti-pS(355,356) for the GRK sites at recommended dilutions, and anti-pS1262 (clone 2G3) for the PKA site phosphorylated Ser residue. Bands were visualized using as primary antibodies anti-phosphoserine–specific monoclonal antibodies and detected by peroxidase at 10 ng/ml for the monoclonal antibodies and visualized using as secondary antibodies species-specific goat anti-rabbit IgG-horseradish peroxidase at 5 ng/ml. The membranes were washed five times and incubated in fresh medium at 37°C, after which 200-μl aliquots were removed at the times indicated, centrifuged at 600g to collect cells, and then solubilized, transferred, and immunoblotted as described above. In control experiments, we demonstrated that GRK site phosphorylation and internalization of the β₂AR were unimpaired by suspending the cells.

**Membrane Preparation and Adenylyl Cyclase Assays.** To prepare membranes for assay, WT-β₂AR cells were treated ± agonist, washed 6 times in ice-cold HE buffer, and scraped into HE buffer containing 10 μg/ml leupeptin and 0.1 μM okadaic acid. The cells were then lysed in a type B Dounce homogenizer with seven strokes. The lysate was placed on a step gradient of 23 and 43% sucrose (w/w) in HE buffer. Membranes at the 23/43 interface were removed and frozen at −80°C.

For resensitization experiments, cells were treated for 15 min with AT or isoproterenol, then washed three times (20 s) in warm medium. Cells were then incubated for various times with 100 μM metoprolol, and membranes were prepared as described above. Adenylyl cyclase activity was determined as described previously (Seibold et al., 2000). In brief, 5 μg of membrane protein was incubated at 30°C for 10 min in 100 μl of buffer containing 40 mM HEPES, pH 7.7, 6 mM MgCl₂, 1 mM EDTA, 100 μM ATP, 1 μM GTP, 0.1 mM 3-isobutyl-1-methylxanthine, 8 mM creatine phosphate, 16 U/ml creatine phosphokinase, and 2 μCi of [α-32P]ATP (30 Ci/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Adenylyl cyclase activities for six to eight concentrations of agonists were assayed in triplicate, and the EC₅₀ and Vₘₐₓ values were determined using Prism software (GraphPad Software, San Diego, CA).

**Internalization.** The procedure for measuring internalization of the β₂AR has been described in detail previously (Seibold et al., 2000) with the following modifications. Cells in 12-well plates were treated with various concentrations of isoproterenol or AT for the times indicated in Fig. 2B. Cells were then washed two times with DMEM (37°C) and three times with ice-cold DMEM, placed on ice, and then incubated with 10 nM [3H]CGP-12177 with or without 1 μM alprenolol in DMEM. Dishes were then incubated for 1 h on ice and washed twice with ice-cold PBS to remove unbound [3H]CGP-12177; cells released by trypsin were transferred to scintillation vials for counting.

**Immunofluorescence Microscopy.** WT-β₂AR cells growing on poly-1-lysine-coated coverslips in six-well clusters were treated with AT or 1 μM isoproterenol for 5 min. Cells were immediately fixed with 4% paraformaldehyde in PBS containing 1.2% sucrose at 4°C for 10 min or washed five times and incubated in fresh medium at 37°C for varying times up to 20 min to allow receptor recycling before fixation. Fixed cells were permeabilized with 0.2% Triton X-100, blocked using 10% heat-inactivated goat serum, and labeled using as primary a phospho–site-specific monoclonal antibody directed against pS355,356 (clone 5C3) at 1 μg/ml, and a polyclonal anti-β₂AR C terminus antibody at 2 μg/ml, followed by species-specific Alexa594-anti-mouse IgG and Alexa488-anti-rabbit IgG, both at a 1:400 dilution. The coverslips were mounted in Mowiol and viewed using a DeltaVision deconvolution microscopy system (Applied Precision Inc., Issaquah, WA) equipped with a Zeiss Axiovert microscope. Imaging was performed using a Zeiss 100× (1.4 numerical aperture) oil immersion lens, and sections were collected at an optical depth of 150 nm in the z-plane. Images were optimized using DeltaVision deconvolution software and transferred to Adobe Photos hop 6.0 (Adobe Systems, Mountain View, CA) for the production of final figures.
Cell-Free Dephosphorylation Protocols. For assessing dephosphorylation in a cell-free assay, HEK 293 cells stably expressing the WT-β2AR were grown to confluence in 100-mm dishes. To induce phosphorylation, cells were treated with isoproterenol or vehicle (AT) at the concentrations and for the times indicated in the figure legends. To stop the stimulation, cells were rapidly washed three times with ice-cold HE buffer and scraped into 3 ml of buffer A (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 50 mM β-mercaptoethanol, 40 μg/ml bovine serum albumin, 10 μg/ml leupeptin, 1.0 mM benzamidine, and 1.0 mM phenylmethylsulfonyl fluoride). Cells were homogenized with seven strokes in a Dounce homogenizer, transferred to microcentrifuge tubes, and centrifuged at 600 g for 5 min; the supernatants pooled (all steps at 0–4°C). For the time course of dephosphorylation, the pooled post-600g supernatants were incubated at 37°C in a water bath, and 100-μl aliquots were removed for the times indicated in the figure legends and mixed with 2× solubilization buffer. For experiments with phosphatase inhibitors, the pooled supernatant (0.5–1.0 ml) was distributed into tubes containing inhibitors and incubated at 37°C; 100-μl aliquots were removed at the times indicated in the figure legends. The dephosphorylation assay was stopped by addition of 100 μl of ice-cold 2× solubilization buffer. Samples were treated with 150 units of PNGaseF for 2 h at 37°C, and then SDS sample buffer was added. SDS-PAGE, transfer, and Western blotting were as described above.

To determine whether the GRK dephosphorylation was retained in the particulate fraction, two extensions of the cell-free protocol were used. First, to measure the dephosphorylation in the crude particulate fraction, cells were prepared as discussed above in the cell-free protocol. The post-600g supernatant fractions were pooled, and 500 μl was centrifuged at 21,000 g for 10 min at 0–4°C. The supernatant was removed, and the pellet was suspended in 500 μl of buffer A. Aliquots were transferred to tubes for incubation at 37°C, and samples were removed at the indicated times for measurement of GRK site dephosphorylation. Second, to measure GRK site dephosphorylation in heavy and light vesicle fractions, cells were pretreated for 20 min with AT or 1.0 μM isoproterenol, washed twice with PBS (0–4°C), incubated with concanavalin A (0.25 mg/ml in PBS) for 15 min on ice, lysed in HE buffer (containing 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine), and fractionated on a step gradient of 18, 28, and 43% sucrose (w/w) in HE buffer by ultracentrifugation at 25,000 rpm for 35 min at 4° in a Beckman SW28 rotor (Beckman Coulter, Fullerton, CA). These gradient procedures allow separation of the “heavy vesicle” plasma membrane fraction from the “light vesicle” endosomal fraction after agonist stimulation of the β2AR (Clark et al., 1985; Krueger et al., 1997). Light vesicles (LV) at the 18/28 boundary and Heavy Vesicles (HV) at the 28/43 interface were collected (2.5–3.5 ml), and 400-μl aliquots were placed in microcentrifuge tubes containing a final concentration of 50 mM β-mercaptoethanol. To assess the action of purified phosphatases, 0.05 units of either PP1 or PP2A were added to select tubes. Aliquots were incubated for 20 min at 37°C and then mixed with 2× solubilization buffer. The solubilized extracts were processed for Western blot analysis as described above. In some experiments, full sucrose gradients were prepared as described previously (Clark et al., 1985). In brief, gradients were formed by layering 18, 23, 27, 31, 35, and 43% sucrose (w/w) in HE buffer. The sucrose gradient was prepared 12 h before use and stored at 4°C to allow a continuous gradient to form. Samples prepared as given above were added to the gradient tubes and centrifuged at 25,000 rpm for 90 min at 4°C. Fractions (1 ml) were collected, and aliquots were solubilized and processed for either SDS-PAGE and Western blotting with C-tail and GRK site antibodies, or for determination of isoproterenol-stimulated adenyl cyclase activity as described above.

Standardization of Okadaic Acid, Calyculin A, and PP1-Inhibitor 2 IC50 Values by Fluorescent Assay. To determine whether the phosphatase inhibitors were fully active, we measured their IC50 values using the fluorescence-based assay RediPlate 96 EnzChek Serine/Threonine Phosphatase Assay Kit from Invitrogen. The IC50 values for okadaic acid against PP1 and PP2A were determined using the assay as directed with purified PP1 and PP2A. The IC50 values for okadaic acid and calyculin inhibitions of PP2A were 0.43 and 0.4 nM, respectively, and for PP1, 23 and 0.4 nM, respectively. For PP1-inhibitor 2, the IC50 for PP1 was 2 nM. These IC50 values are in excellent agreement with published data for these phosphatase inhibitors.

Statistical Analysis. The mean ± S.E. was determined for each treatment group in the individual experiments. Apparent rate constants were calculated using Prism software and nonlinear regression assuming a one-phase exponential decay. Comparisons between treatments were performed using Prism one-way analysis of variance.

Results

Dephosphorylation of the PKA Site in Intact Cells. We have shown previously that phosphorylation of the β2AR PKA consensus site occurred rapidly with an EC50 of approximately 30 pM in HEK 293 cells stably expressing β2AR to levels of 3 to 5 pmol/mg of membrane protein (Tran et al., 2004). With 300 pM isoproterenol, the level of phosphorylation reaches a maximum within 2 min and remains constant for up to 20 min. This allows evaluation of the phosphorylation and dephosphorylation of the β2AR under pretreatment concentrations of agonist that do not promote internalization or PKA site phosphorylation (Tran et al., 2004; Iyer et al., 2006). To determine the rate of dephosphorylation of the PKA site, WT-β2AR or β2AR-SA3 cells were stimulated with 300 pM isoproterenol for 10 min, and then medium was aspirated and immediately replaced with medium with or without 1.0 μM propranolol. Addition of propranolol at this concentration causes the removal of agonist stimulation within 10 sec (Stickler and Barber, 1989, 1991; Krasel et al., 2005). Dephosphorylation of the PKA site, serine 262, was monitored using a phospho–site-specific monoclonal antibody and the anti-C-tail antibody to control for receptor levels. Because similar rates of dephosphorylation were found in the two cell lines, the data were combined and plotted as shown in Fig. 1. After 90 min, the level of phosphorylation approached that measured in controls, representing approximately a 90% decay of stimulated levels. The rate of dephosphorylation of the PKA site calculated using Prism software was 0.080 ± 0.016/min.

Kinetics of the Dephosphorylation of the GRK Sites in Intact Cells and in Cell-Free Assays. To examine the dephosphorylation of the GRK site serines 355 and 356, HEK 293 cells expressing the WT-β2AR were treated with 1.0 μM isoproterenol for 2, 5, and 20 min. In previous studies, we demonstrated that this concentration of agonist results in a rapid (t1/2 = 40 s), 15- to 20-fold increase in GRK site phosphorylation relative to basal levels, followed by only a slight decline in phosphorylation after 20 to 30 min of stimulation (Tran et al., 2004). After the various times of agonist treatment, the medium was removed and replaced with medium containing 1.0 μM propranolol. The data shown in Fig. 2A are the results from cells stimulated for 5 min with isoproterenol. Dephosphorylation of the GRK site was characterized by a brief 5-min lag phase followed by an increased rate of dephosphorylation (Fig. 2A). After 90 min of dephosphorylation, the level of GRK site phosphorylation was indistinguishable from control levels. The rate constant calculated for the data from 5 to 90 min was 0.039 ± 0.006/min. Fig. 2A,
inset, shows the decay of the GRK sites for 30 min after addition of propranolol for the 2-, 5-, and 20-min treatment times with isoproterenol. Whereas the decay curves varied slightly after the various times of stimulation, the differences were not statistically significant. The lag in the dephosphorylation of the GRK site indicated that time-dependent activation of phosphatase activity was initiated by the addition of antagonist that was independent of the time of agonist stimulation. Because 1.0 μM isoproterenol stimulated marked internalization over this time period (Morrison et al., 1996; January et al. 1997, 1998; Seibold et al., 1998, 2000; Tran et al., 2004; Iyer et al., 2006; Vaughan et al., 2006), we determined the extent of β2AR internalization during a 20-min isoproterenol stimulation (Fig. 2B). The loss of surface receptor was 31, 54, and 71% after 2-, 5-, and 20-min stimulation, respectively. Because the lag and rates of GRK site dephosphorylations were similar with either 2, 5, or 20 min of agonist stimulation, there seems to have been no significant effect of internalization on the rate of dephosphorylation.

Because cell-free dephosphorylation of the β2AR GRK site had not previously been reported, we explored whether dephosphorylation of the GRK sites could be observed in lysates. WT-β2AR cells were treated with 1.0 μM isoproterenol for 2, 5, or 20 min, the post-600g supernatant was prepared in buffer A, and the dephosphorylation kinetics of the GRK sites was immediately assessed by incubation of the lysate at 37°C. Our data show that the GRK sites are indeed dephosphorylated in lysates; however, in contrast to the intact cells, no lag was observed (Fig. 2C), and the initial rate of dephosphorylation was estimated to be $\approx 0.06$ to 0.08/min, which is significantly faster than in the intact cells. The rate of dephosphorylation from 0 to 30 min was similar for the 2-, 5-, and 20-min pretreatment times, although after 20 min of stimulation, the extent of cell-free dephosphorylation was somewhat attenuated relative to the earlier treatment times.

Because the GRK site dephosphorylation was studied primarily with overexpressed β2AR, we also examined the GRK site dephosphorylation in A431 cells that express endogenous β2AR at approximately 400 fmol/mg. We determined previously that the kinetics of phosphorylation of this site resembled closely that of HEK 293 cells (Tran et al., 2004). In Fig. 2D, we show that dephosphorylation of the GRK site in A431 cells after a 5-min stimulation with 1.0 μM isoproterenol and addition of antagonist was $\approx 60\%$ after 60 min ($p = 0.005$). The rate of dephosphorylation of the GRK site in A431 seemed even slower than in HEK 293 cells, indicating that the slow rate of GRK site dephosphorylation observed in HEK 293 cells was not a function of receptor overexpression but might vary with cell type.

**Immunolocalization of GRK-Phosphorylated β2AR after Agonist Treatment and Addition of Propranolol.** To monitor the intracellular phosphorylation and dephosphorylation of the GRK sites in WT-β2AR, we employed immunofluorescence deconvolution microscopy to determine the subcellular localization of GRK phosphorylated receptors (Fig. 3). To accomplish this we used a monoclonal antibody (5C3) that we generated against the GRK site serines 355 and 356 that showed specific binding to the GRK-phosphorylated receptor, but not to the dephosphorylated receptor or the SA3 mutant (data not shown). The movement of the β2ARs was followed simultaneously with an antibody directed against the receptor’s carboxyl terminus. Cells were stimulated with 1.0 μM isoproterenol for 5 min, medium was removed, and the cells were washed and incubated without agonist for 20 min. In agreement with the intact cell kinetics of phosphorylation and internalization, the receptor was phosphorylated by 5 min and, predictably, had moved to endosomal vesicles. Although quantitation was not possible, the phosphorylation state of the β2AR in vesicles was initially stable after agonist removal, and receptors seemed to recycle to the plasma membrane, at least in part, in the phosphorylated states. After 10 min, significant dephosphorylation was observed, a time at which most β2ARs recycled to the plasma membrane. Some phosphorylated receptors were observed at the plasma membrane even after 20 min of recycling. Similar results were obtained using 1.0 μM propranolol for blockade of agonist action rather than washout (data not shown).

**Effect of Phosphatase Inhibitors on GRK and PKA Site Dephosphorylation.** The time course of dephosphorylation of the GRK sites was examined in the presence of calyculin A and okadaic acid to probe the nature of the phosphatases involved. Calyculin A is a nonselective PP1 and PP2A inhibitor, whereas okadaic acid is approximately 20- to
30-fold more potent as an inhibitor of PP2A versus PP1, as we confirmed using a fluorescence-based assay as described under Materials and Methods. In the experiment shown in Fig. 4A, WT-β_{2}AR cells were stimulated with 100 nM isoprote-nerol, washed, suspended, and then incubated with 100 nM calyculin A. This treatment completely blocked dephosphor-

![Image](https://example.com/image.png)

**Fig. 2.** Dephosphorylation of the β_{2}AR GRK site in intact cells and cell-free assays. HEK 293 cells expressing the WT-β_{2}AR were grown to confluence in either 12-well plates for intact cell dephosphorylation or 100-mm dishes for cell-free experiments. Cells were pretreated with 1.0 μM isoproterenol or carrier AT for 2, 5, and 20 min. A, intact cells. After agonist treatment, the medium was aspirated and replaced with medium plus 1 μM propranolol as indicated by the arrows. Cells were solubilized at the times indicated, treated with PNGase F, and Western blots were performed with Santa Cruz anti-pS(355,356) and anti-C-tail antibodies. The values from the 5 min isoproterenol treatment are shown in the main panel (n = 7–8 for the 0–30-min data and n = 3 for the 60- and 90-min data). The inset shows the 0–30-min dephosphorylation after the 2-, 5-, and 20-min treatments with isoproterenol. A typical Western blot is shown below the figure. The rate of dephosphorylation for the 5-min isoproterenol treatments of intact cells was k = 0.039 ± 0.006/min. Because of the lag phase, the rates were determined with the 5- to 25-min values using a nonlinear regression curve and a one phase exponential decay equation (GraphPad Prism), setting the 5-min value as the zero time. B, isoproterenol-induced internalization of the β_{2}AR. Cells expressing the WT-β_{2}AR were stimulated with 1.0 μM isoproterenol for the times indicated, and surface receptors were then measured with [H]CGP-12177 as described under Materials and Methods. Data presented are the mean ± S.E. of four experiments, each performed in triplicate. C, cell-free dephosphorylation. After isoproterenol treatment, cells were washed three times and lysed in buffer A. Lysates were homogenized, the post-600g supernatants were pooled and aliquoted into microcentrifuge tubes, and the incubations and Western blots were performed as discussed under Materials and Methods. For both intact cell and cell-free experiments, Western blots were normalized as described above to the C-tail antibody and to the control phosphorylation after 2-, 5-, and 20-min isoproterenol treatments before the start of dephosphorylation. Data shown are the mean ± S.E. of three experiments, each performed in duplicate. D, A431 dephosphorylation. The cells were treated with AT or 1 μM isoproterenol for 5 min, washed, and then incubated with 0.1 μM ICI 118,551 in medium at 37°C and solubilized at the times indicated. Purification and Western blots were performed as discussed under Materials and Methods. Data shown are means ± S.E. of four or five experiments. P values for the 30- and 60-min dephosphorylations were 0.02 and 0.005, respectively.
ylation of the GRK site in intact cells (the arrow indicates the time of the wash and addition of calyculin). Consistent with the lag and the slow rate of GRK dephosphorylation in intact cells, there was little augmentation of GRK site phosphorylation after addition of calyculin A. To examine this in more detail calyculin A was added to the intact cells 30 min before agonist stimulation, and even this more prolonged pretreatment failed to augment GRK site phosphorylation significantly (data not shown). Calyculin A was also found to block dephosphorylation in the post-600g cell-free assay (Fig. 4B).

Similar results were obtained with okadaic acid (1.0 μM) added 60 min before agonist stimulation; that is, it failed to significantly augment isoproterenol-stimulated GRK site phosphorylation before addition of antagonist, although it blocked GRK site dephosphorylation (Fig. 4C). It was surprising that 0.1 μM okadaic acid was ineffective in blocking GRK site dephosphorylation (data not shown). The PKA site dephosphorylation was also examined in intact cells after stimulation with 300 pM isoproterenol. In contrast to the GRK site, 1.0 μM okadaic acid failed to inhibit PKA site dephosphorylation (Fig. 4C), whereas 100 nM calyculin A blocked it as effectively as that seen with the GRK site (data not shown).

The cell-free assay in the post-600g lysate also was used to determine the IC_{50} values for inhibition of both GRK and the PKA site dephosphorylation to circumvent the problem of cell penetration. For the GRK site, WT-β_{2}AR cells were stimulated for 2 min with 1.0 μM isoproterenol, whereas for the PKA site (pS262), cells were stimulated for 10 min with 300 pM isoproterenol. After stimulation, the post-600g lysate was incubated with or without various concentrations of either calyculin A (Fig. 5A) or okadaic acid (Fig. 5B) for 20 min. Both inhibitors significantly blocked receptor dephosphorylation; the IC_{50} values for calyculin A and okadaic acid were ≈10 and 100 nM, respectively, for both the GRK and PKA sites.

The high IC_{50} for okadaic acid inhibition of GRK and PKA site dephosphorylation in the cell-free system suggested that PP1 activity was involved and that perhaps PP2A was not the primary phosphatase. To further examine a possible role for PP1 activity, the inhibition of GRK and PKA site dephosphorylation by PP1 inhibitor 2 was monitored in the post-600g supernatant. GRK site dephosphorylation was inhibited approximately 35% by 100 nM and 70% by 500 nM inhibitor 2 (Fig. 5C). Although there was a slight inhibition of PKA dephosphorylation, it was not significant. These data suggest that PP1 activity is involved in the GRK site dephosphorylation. In addition, the IC_{50} for okadaic acid (100 nM) was more consistent with inhibition of PP1 (i.e., 5-fold higher than the control fluorescence-based assays versus 250-fold higher than expected against PP2A). Although the calyculin A data suggested that either PP1 or PP2A was involved, they do not discriminate between the two phosphatases, and the 10 nM IC_{50} that we observed is 25-fold higher than in the fluorescence-based assay for either phosphatase. Overall, our data from both intact cell and cell-free experiments with inhibitors of GRK site dephosphorylation more strongly support a role for PP1, although previous cell-free studies have suggested that a “latent PP2A” was involved (Yang et al., 1988; Pitcher et al., 1995).

For PKA site dephosphorylation, a role for PP2A also remains questionable given that the cell-free IC_{50} for okadaic acid was 250-fold greater than that in the fluorescence-based assay, where penetration of the inhibitor is not a problem, and it was ineffective in intact cells. Whereas calyculin A was approximately 10-fold more potent relative to okadaic acid in the cell-free assay and in intact cells, it is nonselective for PP2A. The reason for the reduced potency of both calyculin A and okadaic acid in our cell-free assays relative to the fluorescence-based assay is unknown, but it is perhaps unsurprising given the disruption of possible localization of the phosphatase activity in cell-free assays and/or the presence of factors that inhibit their activity.

**Dephosphorylation of the GRK Site after Subcellular Fractionation.** To determine the subcellular fraction in which the cell-free GRK site dephosphorylation occurred, we...
first stimulated WT-β₂AR cells with 1.0 μM isoproterenol for 2 min, homogenized the cells, separated the post-600g lysate into crude membrane (21,000g) and cytosolic fractions, and compared the rates of dephosphorylation of the two fractions with that of the lysate. Within experimental error, the rate of dephosphorylation seemed similar in the lysate and the membrane fraction (Fig. 6A), and no activity or receptor was observed in the cytosolic fraction.

To further probe the subcellular localization of the particulate fraction phosphatase activity, cells were pretreated for 20 min with carrier or 1.0 μM isoproterenol, and the plasma membrane (HV) and endosomal (LV) fractions prepared using either continuous gradients or sucrose step gradients. Figure 6, B–D, shows a typical profile of the continuous gradients or sucrose step gradients. membrane (HV) and endosomal (LV) fractions prepared using either continuous gradients or sucrose step gradients.

For further examination of dephosphorylation, experiments were performed using the step gradients to allow more rapid and efficient handling of the LV and HV fractions (Fig. 6E). As with the continuous gradients, we found that the levels of GRK site phosphorylation in the LV and HV fractions were nearly comparable when the GRK site phosphorylation was normalized to the total β₂AR (C-tail antibody), and that only the HV fraction showed significant dephosphorylation with intrinsic phosphatase activity ($p < 0.01$). To determine whether the phosphorylated β₂ARs in the LV were subject to dephosphorylation, purified PP2A and PP1 were added to the LV fraction. This resulted in an extent of dephosphorylation of the LV fraction for both phosphatases that was comparable with that observed in the HV (Fig. 6E), although only the PP1-stimulated dephosphorylation was significantly different from the 20-min dephosphorylation control cells ($p < 0.05$). Addition of the phosphatases to the HV fraction did not further increase the rate of dephosphorylation (data not shown). Furthermore, we found that reconstitution of the LV fraction with the supernatant fraction phosphorylation of the receptor in the LV and HV fractions (Fig. 6D). When phosphorylation was normalized to the total receptor level, the profile was essentially similar to that in Fig. 6D.

![Fig. 4. Inhibition of GRK and PKA site dephosphorylation of the β₂AR with calyculin A and okadaic acid in intact cells and cell-free assay. A, for the intact cell experiment, WT-β₂AR cells were grown to confluence in 100-mm dishes and stimulated with either 100 nM isoproterenol or carrier (AT) for 10 min. After agonist treatment cells were washed, suspended, and incubated with or without 100 nM calyculin A (CL-A) as indicated by the arrows. Data shown are means ± S.E. of four experiments. The augmentation of GRK phosphorylation by calyculin relative to the control (10 min) was not significant at any time point. B, for the cell-free experiments, the post-600g supernatants were incubated with or without calyculin A and solubilized at the times indicated, and Western blots were performed as discussed under Materials and Methods. Data were normalized to the C-tail antibody and then to the maximum level of GRK site phosphorylation in each experiment. Data shown are means ± S.E. of three experiments. C, for the effect of okadaic acid on intact cell dephosphorylations, WT-β₂AR cells in 12-well dishes were preincubated ≤ 1 μM okadaic acid for 60 min. Cells were then stimulated with either 300 μM isoproterenol for either 10 min for the PKA site dephosphorylation, or 2 min with 1.0 μM isoproterenol for the GRK site dephosphorylation. The medium was removed and replaced with medium plus 1 μM propranolol, and cells were incubated for 30 min. The extent of dephosphorylation was determined by Western blots as given above. Data shown are the means ± S.E. of three experiments. The effect of inhibitor 2 was significantly different from control cells at only the 500 nM level for the GRK site phosphorylation ($p < 0.001$).
from the top of the gradient also caused dephosphorylation of the LV fraction (data not shown), indicating that cytosolic phosphatase activity does have access to the GRK site in the endosomal fraction.

**Time Course of Resensitization of Adenylyl Cyclase Activity after Addition of Antagonist.** Whereas we previously have determined the rate of agonist-induced desensitization of adenylyl cyclase activity and the rates of $\beta_2$AR recycling to the plasma membrane (Morrison et al., 1996; Seibold et al., 1998, 2000), we had not determined the kinetics of resensitization of adenylyl cyclase after agonist stimulation. Determination of the rate of resensitization necessitated a modification of the protocol used for the dephosphorylation assays. Propranolol cannot be used for the blockade of agonist stimulation, because its rate of dissociation is too slow. To circumvent this problem we used 100 $\mu$M metoprolol, a low affinity (240 nM $K_\text{d}$) pan-blocker of agonist stimulation of $\beta_2$ARs and $\beta_2$ARs (Contreras et al., 1986; January et al., 1997, 1998). Cells were stimulated for 15 min with or without 1.0 $\mu$M isoproterenol, rapidly washed three times with medium at 37°C, and subsequently incubated for 2 to 30 min with medium containing 100 $\mu$M metoprolol. To stop resensitization, cells were washed and lysed, and membranes were prepared on sucrose step gradients for assay of isoproterenol stimulation of adenylyl cyclase activity. The incubation with metoprolol had no effect on adenylyl cyclase activity in membranes from controls (AT-treated), demonstrating effective removal of the antagonist with the wash procedure (data not shown). The data shown (Fig. 7, A and B) are normalized to the $V_{\text{max}}$ to emphasize the receptor level desensitization/resensitization, because we have demonstrated previously that the receptor-level desensitization in $\beta_2$AR-overexpressing cells is almost exclusively reflected in the $EC_{50}$ shift, and the decrease in $V_{\text{max}}$ is downstream of the receptor (Seibold et al., 2000). In these experiments, we observed a 6-fold increase in the $EC_{50}$ for isoproterenol stimulation of adenylyl cyclase after the isoproterenol treatment. After washout of agonist, we found that ~55% and 80% of activity was restored in just 2 and 5 min of resensitization, respectively, as assessed by the decrease in the $EC_{50}$ for isoproterenol stimulation ($p < 0.001$ values for the decrease in $EC_{50}$ values for all time points relative to control cells). Longer incubations (20–30 min) were required to fully restore activity, consistent with the rates of recycling (Morrison et al., 1996; Seibold et al., 2000) and dephosphorylation. A similar rapid phase of resensitization was observed after

![Fig. 5](https://i.imgur.com/3Q5Q5.png)

**Fig. 5.** Inhibition of GRK and PKA site dephosphorylation of the $\beta_2$AR with calyculin A, okadaic acid, and PP1-inhibitor 2 in cell-free assays. To determine the effective concentrations of calyculin A (A), okadaic acid (B), and PP1-inhibitor 2 (C), cells in 100-mm dishes were stimulated for either 10 min with 300 pM (PKA site) or 2 min with 1.0 $\mu$M (GRK site) isoproterenol, and the post-600 g supernatant was incubated for 20 or 30 min with or without the various concentrations of inhibitors. The dephosphorylation was then followed in the post-600 g lysate, and Western blots were performed as described in Fig. 4. Results were normalized to the respective control isoproterenol levels and to the C-tail antibody. Data are the means ± S.E. of three experiments for A and B, each performed in triplicate ($A, p < 0.01$ for both PKA and GRK at 100 nM; $B, p < 0.01$ for 100 nM for GRK, and $p < 0.001$ for 1000 nM for both PKA and GRK). For C, GRK data, $n = 4$ for 100 nM ($p > 0.05$) and $n = 3$ for 500 nM inhibitor 2 ($p < 0.05$); for PKA data, $n = 3$, and the $p$ values were not significant.
Fig. 6. Dephosphorylation of GRK site of WT-β2ARs in the crude particulate fraction and in heavy and light vesicle fractions. A, WT-β2AR were grown to confluence in 100-mm dishes and stimulated with 1.0 μM isoproterenol or carrier AT for 2 min. Cells were then washed, lysed, and homogenized in buffer A as described under Materials and Methods for the cell-free preparation. The post-600g supernatant (lysate) was then centrifuged at 21,000g, the pellet was suspended in 500 μL of buffer A (membrane), and aliquots were taken for incubation at 37°C for the dephosphorylation. Samples were removed at the times indicated and solubilized, and Western blots were performed sequentially using anti-pS(355,356) and anti-C tail antibodies. The post-600g fraction was also incubated in parallel for comparison. B–D, continuous sucrose gradient fractionation. Cells were grown to confluence in 150-mm dishes, stimulated with 1.0 μM isoproterenol or carrier for 20 min, washed twice in ice-cold PBS, and incubated on ice with concanavalin A for 15 min. Cells were washed three times in HE buffer, homogenized, and added to the continuous gradients (18, 23, 27, 31, 35, and 43% sucrose) as described under Materials and Methods. One-milliliter fractions were collected starting with the top of the gradient, and 400-μL aliquots were either processed for determination of isoproterenol-stimulated adenylyl cyclase activity (B) or solubilized for Western blots using either the C-tail antibody (C), or GRK site antibody (D). For the Western blots, data were normalized to fraction 6. For figures C and D, the controls (AT) are shown as solid lines and the isoproterenol-stimulated samples as open bars. E, for measurement of dephosphorylation, samples treated with AT or isoproterenol as given above were placed on step gradients (18, 28 and 43% sucrose) and centrifuged. Light vesicles at the 18/28 boundary (LV) and heavy vesicles (HV) at the 28/43 interface were removed and aliquoted into microcentrifuge tubes containing 50 mM β-mercaptoethanol with or without 0.05 units of either PP1 or PP2A. Western blotting was performed with anti-pS(355,356) and C-tail antibodies as described under Materials and Methods. Western blots were first normalized to the C-tail antibody and then further normalized to the maximum phosphorylation after 20 min of isoproterenol treatment (CTRL ISO). Data shown are mean ± S.E. of three experiments. Comparison of the values for dephosphorylation of the LV fraction relative to the 20-min dephosphorylation control was significant for PP1 only (p < 0.05). For the HV fraction, the 20-min dephosphorylation relative to control was significant (p < 0.05).
1.0 μM isoproterenol stimulation for either 5 or 30 min (data not shown).

To compare the rates of dephosphorylation with the rate of resensitization of adenylyl cyclase activity, the plot shown in Fig. 8 was constructed using the intact cell data from Figs. 1A, 2A (5-min isoproterenol stimulation only) and 7. The rates of dephosphorylation of the GRK and PKA sites in intact cells (≈0.039/min and 0.08/min, respectively) after the lag occurred far more slowly than the rate of resensitization (≈0.43/min).

**Discussion**

Previous studies of the phosphorylation and dephosphorylation of the β2AR have been limited by the lack of methods for examining quantitatively and independently the dephosphorylation of the two key domains that we have shown are crucial for agonist-induced desensitization (Tran et al., 2004; Iyer et al., 2006; Vaughan et al., 2006). This limitation also precluded a thorough correlation of the resensitization of agonist stimulation of adenylyl cyclase activity with dephosphorylation of the two sites. Through the use of a panel of phospho–site-specific antibodies, we have been able to characterize the phosphorylation and dephosphorylation of the two domains that we have shown are the sites of PKA and GRK inhibition of β2AR stimulation (Tran et al., 2004) and to show that the translocation of arrestin was dependent on the intact GRK site (Vaughan et al., 2006). We have also shown that the phosphorylation of the PKA site serine 262 was exquisitely sensitive to the concentration of epinephrine and was phosphorylated rapidly with an EC50 of 30 μM in cells overexpressing the WT receptor. In contrast, the EC50 for agonist stimulation of GRK site (serines 355 and 356) phosphorylation, was 30 nM, approximately 1000-fold higher (January et al., 1997; Seibold et al., 1998; Tran et al., 2004). Using the profound differences in the agonist dependence of PKA and GRK phosphorylation, we were able to demonstrate that dephosphorylation of the PKA site occurs in the plasma membrane after rapid removal of agonist stimulation under conditions (300 μM isoproterenol) that precluded agonist-induced GRK site phosphorylation, β-arrestin binding, or internalization (Iyer et al., 2006). We also found that blockade of internalization by two independent methods after high agonist stimulation had little effect on GRK dephosphorylation. The present work was initiated with the goals of determining the rates of dephosphorylation of the two sites, their relationship to resensitization of the receptor, and further defining the locale and nature of the phosphatase activity.

**Comparison of the Rates of Dephosphorylation of the PKA and GRK Sites with Resensitization.** In studies presented here, we found that PKA site dephosphorylation proceeded without a lag with a t1/2 of ≈9 min, and that the GRK site dephosphorylation proceeded with a relatively slower t1/2 of ≈18 min after a lag phase of ≈5-min duration. The lag phase in GRK site dephosphorylation was not appreciably affected by the time of pretreatment of cells with isoproterenol in the range of 2 to 20 min, despite significant differences in the extent of internalization at these time points. In contrast, resensitization of adenylyl cyclase activity occurred far more rapidly (t1/2 = 1.6 min) than expected from the rates of dephosphorylation of the GRK or PKA sites. Thus, within 2 min of antagonist addition, there was a 2-fold decrease in the EC50 for isoproterenol stimulation and, after 5 min, a 4-fold reduction in the EC50; these are times at which little dephosphorylation was observed, equivalent to restoration of ≈50% and 80% of activity, respectively (Whaley et al., 1994). The rate of receptor resensitization was approximately 10-fold faster than even the maximum rate of dephosphorylation of the GRK sites, regardless of whether agonist stimulation of cells was for 5, 15, or 30 min. It was also approximately 5-fold faster than PKA site dephosphorylation. The data clearly dissociate the initial rapid rate of resensitization from dephosphorylation of the GRK site and demonstrate that recycling of internalized GRK site phosphorylated β2AR is not required for this phase of resensitization. Whereas previous studies of resensitization suggested that internalization and dephosphorylation were
required for resensitization (Sibley et al., 1986; Yu et al., 1993; Pippig et al., 1995; Zhang et al., 1997), the rapid kinetics of adenyl cyclase resensitization and dephosphorylation of the GRK and PKA sites individually after cessation of agonist action were not examined.

**The Mechanism of the Rapid Resensitization of Agonist Stimulation.** Our data raise the question as to the mechanism of rapid receptor resensitization. The most plausible explanation is that β-arrestin dissociation, not GRK site dephosphorylation, is the major determinant of the rapid phase of resensitization (0–5 min). In this regard, previous in vitro work with reconstituted β2ARs demonstrated that in response to high agonist concentrations, arrestin binding to GRK-phosphorylated receptor was required for the major desensitization in subsequent assays with reconstituted Gs (Benovic et al., 1987; Lohse et al., 1992). Phosphorylation of the GRK sites, β-arrestin translocation and binding to the β2AR, and receptor internalization all occur rapidly (January et al., 1997; Zhang et al., 1997; Krupnick and Benovic, 1998; Clark et al., 1999; Seibold et al., 2000; Tran et al., 2004; Krasel et al., 2005), and high-affinity arrestin binding is dependent on the synergy between the “activation-recognition domain” and the “phosphorylation-recognition domain” (Gurevich and Gurevich, 2004). Therefore, if agonist binding is abruptly terminated, β-arrestin should rapidly dissociate from the β2AR even if the receptor is phosphorylated. It was previously demonstrated that the $t_{1/2}$ for the dissociation of GFP-arrestin from the β2AR in HEK 293 cells was $\sim6–7$ s, during which time there was little decrease in total receptor phosphorylation (2 min after removal of agonist). In that study, a perfusion system was used to rapidly remove agonist, and β-arrestin interaction with the β2AR was measured by fluorescence resonance energy transfer analysis (Krasel et al., 2005). With the caveat that the fluorescent C-terminal tags (enhanced cyan fluorescent protein or yellow fluorescent protein) may alter β-arrestin affinity and the rate of internalization (McLean and Milligan, 2000), the kinetics of β-arrestin dissociation support the proposal that rapid (0–5 min) resensitization is caused by this event.

The mechanisms for β2AR resensitization are obviously complicated by the fact that recycling of the receptor must contribute to this process and probably becomes more important with time (2–20 min) after removal of agonist. The β2AR recycles with a $t_{1/2}$ of 7 to 8 min (Morrison et al., 1996; Seibold et al., 2000); thus, recycling would contribute additional receptor to the plasma membrane with 5 min of resensitization, although because of the lag in GRK site dephosphorylation, the β2AR would retain the GRK site phosphorylation.

**On the Nature and Locale of GRK Dephosphorylation.** In addition to our findings of a lag and subsequent very slow rate of GRK site dephosphorylation, a number of our other findings support the relative stability of the GRK phosphorylation. First, we found little augmentation of GRK site dephosphorylation when dephosphorylation was inhibited by calyculin A or okadaic acid. Second, the rate of GRK phosphorylation ($k = 1–2$/min) exceeded that of dephosphorylation ($k = 0.04$/min) by 30-fold, predicting that steady-state GRK site phosphorylation should be near saturation, as was demonstrated by mass spectrometry of the β2AR (Trester-Zedlitz et al., 2005). In addition, we find a near 1:1 ratio of GRK site phosphorylation to C-tail antibody reactivity using saturating antibodies (data not shown). Third, the levels of GRK site phosphorylation in the endosomal fractions were equivalent to those in the plasma membrane fraction. Fourth, the β2AR in the heavy vesicle plasma membrane fraction, but not the endosomal receptor, was dephosphorylated by intrinsic phosphatase activity. Fifth, immunofluorescence of the phosphorylated β2AR in endosomes suggested that it is initially removed slowly. These data, along with our previous report (Iyer et al., 2006), clearly do not support the proposal that dephosphorylation of the GRK site requires internalization. If that were the case, then the endosomal level of GRK-phosphorylated receptor should be much reduced relative to that in the plasma membrane. Our data do not rule out the possibility that receptor dephosphorylation also may occur in the endosomal fraction, because the addition of purified phosphatases to the endosomal fraction caused GRK site dephosphorylation (Pippig et al., 1995; Krueger et al., 1997). Although the acidification of endosomes may be required for GRK site dephosphorylation, because GRK site dephosphorylation in our endosomal fractions was observed upon addition of purified PP1 and PP2A in a buffered milieu, we did not investigate this possibility further.

Our data raise the question of what causes the lag in dephosphorylation of the GRK site. The explanation most consistent with our findings is that the lag in GRK site dephosphorylation is caused by the necessity of internalized receptor ($\sim31$ and 71% after 2- and 20-min stimulation with agonist, respectively) to recycle to the plasma membrane for dephosphorylation. The 5-min lag time is consistent with recycling, because we and others have determined previously that the $t_{1/2}$ for recycling is 7 to 8 min (Morrison et al., 1996; Seibold et al., 2000; Liang et al., 2004). There are other possible explanations for the lag. One is that there is a time-dependent translocation of a phosphatase and association with the β2AR, as was shown for the CXCR2 receptor (Fan et al., 2001), that could include second-messenger regulation of phosphatase and β2AR scaffolding on AKAPs (Shih et al., 1999; Malbon et al., 2004). Second, it has been well...
established that PKA phosphorylation of PP1 inhibitor blocks PP1 activity (Huang et al., 1999; Gupta et al., 2002; Sike and Shenolikar, 2005). The lag phase in this scenario could reflect the time required for the rapid hydrolysis of cAMP and relief of PKA inhibition. A third possibility is regulation of phosphatase activity by ubiquitination (P. H. Fishman, personal communication) (Liang and Fishman, 2004; Shenoy and Lefkowitz, 2005). These possibilities are currently being explored. A related question is the nature of the phosphatase activities. Our work thus far with the phosphatase inhibitors indicates that PP1 plays a role in GRK site dephosphorylation but does not rule out a possible role for PP2A in both PKA and GRK site dephosphorylation, although the high IC50 in cell-free or intact cell assays for okadaic acid is much higher than expected if PP2A is the major activity. Resolution of the role or roles of PP1 and PP2A or perhaps other phosphatases for both sites in intact cell dephosphorylation, and the control of their activity and localization, will require further study beyond the scope of the present work.

To summarize, we demonstrate that the relatively slow kinetics of dephosphorylation of the β2AR PKA and GRK sites (20 and 10% of the resensitization rate, respectively), compared with the rapid kinetics of resensitization of agonist stimulation of adenyl cyclase, clearly indicate that dephosphorylation in itself cannot explain the rapid phase of resensitization. Rather, we propose that the key event in the rapid restoration of adenyl cyclase activity after removal of agonist is the fast dissociation of β-arrestin from membrane-bound βAR coupled with recycling of the receptor to the plasma membrane, where any remaining GRK or PKA sites can be dephosphorylated. Our data support the possibility that there is a memory of phosphorylation of the GRK sites that persists even as much activity is restored and that this in turn could cause more rapid desensitization upon a second stimulation by agonist, consistent with previous data (Krasel et al., 2005). Finally, our data support the conclusion that dephosphorylation of not only the PKA site but also the GRK site occurs in the plasma membrane, although receptor GRK site dephosphorylation in early endosomes also may contribute, particularly if rapid dissociation of agonist and β-arrestin is not achieved by perfusion or the addition of antago- nist. As noted above, in the continued presence of agonist, it is possible that internalization of the receptor is required for removal of agonist and β-arrestin intracellularly and the subsequent reintroduction of the βAR to the plasma membrane, as has been proposed (Yu et al., 1993; Pippig et al., 1995; Krueger et al., 1997; Zhang et al., 1997), although even in this scheme, our data would support the possibility that dephosphorylation can occur in the plasma membrane.

Acknowledgments

We thank Dr. Peter H. Fishman, National Institutes of Health, for many useful discussions and suggestions during the performance of these experiments and in the preparation of the manuscript.

References


Address correspondence to: Richard B. Clark, Dept. of Integrative Biology and Pharmacology, University of Texas Health Science Center Houston, Medical School, 6431 Fannin, Houston, TX. E-mail: richard.b.clark@uth.tmc.edu