

Characterization of β_2 -Adrenergic Receptor Dephosphorylation: Comparison with the Rate of Resensitization

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Received June 29, 2006; accepted September 28, 2006

ABSTRACT

Dephosphorylation of the cyclic AMP-dependent protein kinase (PKA) site phosphoserine 262 and the G protein-coupled receptor kinase (GRK) site phosphoserines 355 and 356 of the β_2 -adrenergic receptor (β_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 ($k = 0.08 \pm 0.016/\text{min}$) in intact cells in the absence of internalization. Dephosphorylation of the GRK sites in intact cells after treatment with 1.0 μM isoproterenol for 5 min exhibited a lag phase of ≈ 5 min, after which dephosphorylation proceeded slowly with a $t_{1/2}$ of 18 min ($k = 0.039 \pm 0.006/\text{min}$). 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 de-

phosphorylation 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 β_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 β_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 ($k = 0.43 \pm 0.009/\text{min}$; $t_{1/2} = 1.6$ 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 β_2 -adrenergic receptor (β_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 recy-

cling control the level of the β_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 β_2 AR is rapidly phosphorylated by GRK, and arrestin binds with high affinity (Benovic et al., 1987; Lohse et al., 1989, 1990a,b, 1992; Sohlemann et al., 1995), precluding phosphatase action

This work was supported by National Institutes of Health grants GM031208 (to R.B.C.), HL64934 (to R.H.M.), and 0455072Y (to B.J.K.).

This work has been presented previously in abstract form at the Keystone Meeting on G Protein-Coupled Receptors: Evolving Concepts and New Techniques: Feb 12–17, 2006; Keystone, CO.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.028456.

ABBREVIATIONS: PKA, cyclic AMP-dependent protein kinase; GRK, G protein-coupled receptor kinase; β_2 AR, β_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, (\pm)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; PBS, phosphate-buffered saline; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-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 β_2 AR, followed by arrestin dissociation, because high affinity arrestin binding requires agonist binding and GRK site phosphorylation.

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 ^{32}P -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 β_2 AR and adenylyl 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 β_2 AR 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 stimula-

tion of adenylyl 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 β_2 AR 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; Sohlmann 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 Mediatech (Herndon, VA). β_2 AR 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 β_2 AR 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-DRTGHGLRRSpSKF-NH₂ for the anti-pSer262 PKA site (clone 2G3) and CKAYGNGYpSpSNGN-NH₂ for the anti-pS (Ser355,356) (clone 5C3). Monoclonal antibodies were purified using protein A Sepharose. Species-appropriate fluorescent goat anti-rabbit and anti-mouse secondary antibodies were obtained from Invitrogen (Carlsbad, CA).

Constructs and Cell Lines. HEK 293 cells stably expressing FLAG-WT- β_2 AR (referred to in the text as WT- β_2 AR) were used for the majority of the experiments. For some experiments involving PKA site dephosphorylation, we used the N-terminal FLAG-tagged mutant (β_2 AR-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 $\mu\text{g}/\text{ml}$ 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- β_2 ARs (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 $\mu\text{g}/\text{ml}$ 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, NaHCO_3 -free DMEM containing 0.5% fetal bovine serum, and cells were equilibrated without CO_2 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 $\beta_2\text{AR}$ 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 \pm 1.0 μM propranolol. To terminate all treatments in preparation for immunoblotting, the medium was removed, and the cells were washed once rapidly 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 $\mu\text{g/ml}$ benzamidine, 10 $\mu\text{g/ml}$ trypsin inhibitor, and 10 $\mu\text{g/ml}$ leupeptin). The cells were scraped into the solubilization buffer, transferred to 1.5-ml microcentrifuge 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, bromophenol blue, and 10 mM dithiothreitol). Twenty-microliter aliquots of samples were resolved on 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for immunoblotting. The membranes were probed for $\beta_2\text{AR}$ with phosphoserine-specific antibodies, anti-pS(355,356) for the GRK sites at recommended dilutions, and anti-pS262 (clone 2G3) for the PKA site at 1.0 $\mu\text{g/ml}$. Membranes were then washed twice and incubated with either goat anti-rabbit IgG-horseradish peroxidase at 5 ng/ml for the polyclonal antibodies or goat anti-mouse IgG-horseradish peroxidase at 10 ng/ml for the monoclonal antibodies and detected by SuperSignal reagent. After quantification of $\beta_2\text{AR}$ phosphorylation, the immunoblots were stripped and reprobed with a $\beta_2\text{AR}$ -specific rabbit anti-C-tail antibody to determine $\beta_2\text{AR}$ levels. The blots were imaged using a charge-coupled device camera system (GeneGnome; Syngene, Frederick, MD), and bands quantified using Syngene software. All results with the anti-phosphoserine-specific antibodies were normalized to the corresponding levels of $\beta_2\text{AR}$ measured with the anti-C-tail antibody. For averaging data of separate dephosphorylation experiments, the results were first expressed as a fraction of the control isoproterenol treatment, and then the means \pm S.E.M., where $n \geq 3$ or the range ($n = 2$) were calculated and shown in the figures.

Dephosphorylation experiments with A431 cells required purification of the solubilized extracts using wheat germ agglutinin affinity resin (Vector Laboratories, Burlingame, CA). A431 cells, cultured in DMEM supplemented with 10% fetal bovine serum, were grown to confluence in 100-mm dishes. The growth medium was removed and replaced with 40 mM NaHCO_3 -free HEPES-buffered DMEM for 1 h. The cells were treated with AT or 1 μM isoproterenol for 5 min, washed once with 5 ml of medium, and incubated for various times with medium containing a 0.1 μM concentration of the antagonist ICI-118,551. To end the incubation, the medium was removed, and cells were washed twice with HE and scraped in 500 μl of solubilization buffer. The samples were rocked at 4° for 30 min, then centrifuged for 15 min at 15,000 rpm. For purification, 50 μl of packed wheat germ agglutinin affinity resin was added to each of the solubilized samples. The tubes were rocked for 90 min at 4° and centrifuged briefly to pellet the resin. The pellets were washed twice in solubilization buffer, and the receptor was eluted with 2 \times SDS sample buffer for 15 min at 65°C. Eluates were run on SDS-PAGE, transferred, and immunoblotted with pS(355,356) and C-tail antibodies.

For the GRK site dephosphorylation experiments with intact cells involving the phosphatase inhibitor calyculin A, we used suspended

cells because preliminary studies showed that calyculin A caused release of HEK 293 cells from the surface similar to that reported previously for A431 cells (Shih et al., 1999). Cells grown on 100-mm dishes were stimulated with agonist, washed three times in PBS, and then washed with 0.02 mM EDTA for 1 min to initiate cell release, followed by suspension of the cells in 3 ml of HEPES-buffered NaHCO_3 -free DMEM. Aliquots of 1 ml were placed in tubes with or without 100 nM calyculin A and incubated with rocking 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 $\beta_2\text{AR}$ were unimpaired by suspending the cells.

Membrane Preparation and Adenylyl Cyclase Assays. To prepare membranes for assay, WT- $\beta_2\text{AR}$ cells were treated \pm agonist, washed 6 times in ice-cold HE buffer, and scraped into HE buffer containing 10 $\mu\text{g/ml}$ 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 MgCl_2 , 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 - ^{32}P]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 EC_{50} and V_{max} values were determined using Prism software (GraphPad Software, San Diego, CA).

Internalization. The procedure for measuring internalization of the $\beta_2\text{AR}$ 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- $\beta_2\text{AR}$ cells growing on poly-(L-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 $\mu\text{g/ml}$, and a polyclonal anti- $\beta_2\text{AR}$ C terminus antibody at 2 $\mu\text{g/ml}$, 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 \times (1.4 numerical aperture) oil immersion lens, and sections were collected at an optical depth of 150 nm in the z-plane. Images were optimized using DeltaVision deconvolution software and transferred to Adobe Photoshop 6.0 (Adobe Systems, 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- β_2 AR 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 μ g/ml bovine serum albumin, 10 μ g/ml leupeptin, 1.0 mM benzamide, and 1.0 mM phenylmethylsulfonyl fluoride). Cells were homogenized with seven strokes in a Dounce homogenizer, transferred to microcentrifuge tubes, and centrifuged at 600g 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- μ l aliquots were removed for the times indicated in the figure legends and mixed with 2 \times 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- μ l aliquots were removed at the times indicated in the figure legends. The dephosphorylation assay was stopped by addition of 100 μ l of ice-cold 2 \times 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 μ l was centrifuged at 21,000g for 10 min at 0–4°C. The supernatant was removed, and the pellet was suspended in 500 μ l 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 μ M 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 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamide), 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 β_2 AR (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- μ l 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 \times 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 adenylyl 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 \pm 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 β_2 AR PKA consensus site occurred rapidly with an EC₅₀ of approximately 30 pM in HEK 293 cells stably expressing β_2 AR 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 β_2 AR under pretreatment concentrations of agonist that do not promote internalization or GRK site phosphorylation (Tran et al., 2004; Iyer et al., 2006). To determine the rate of dephosphorylation of the PKA site, WT- β_2 AR or β_2 AR-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 μ M propranolol. Addition of propranolol at this concentration causes the removal of agonist stimulation within 10 sec (Stickle 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 $0.080 \pm 0.016/\text{min}$.

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- β_2 AR were treated with 1.0 μ M isoproterenol for 2, 5, and 20 min. In previous studies, we demonstrated that this concentration of agonist results in a rapid ($t_{1/2} \approx 40$ 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 μ M 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 $0.039 \pm 0.006/\text{min}$. 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 μ M isoproterenol stimulates 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 β_2 AR 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 β_2 AR GRK site had not previously been reported, we explored whether dephosphorylation of the GRK sites could be observed in ly-

sates. WT- β_2 AR cells were treated with 1.0 μ M 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 ≈ 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 β_2 AR, we also examined the GRK site dephosphorylation in A431 cells that express endogenous β_2 AR 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 μ M 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 β_2 AR after Agonist Treatment and Addition of Propranolol.

To monitor the intracellular phosphorylation and dephosphorylation of the GRK sites in WT- β_2 AR, 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 β_2 ARs was followed simultaneously with an antibody directed against the receptor's carboxyl terminus. Cells were stimulated with 1.0 μ M 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 β_2 AR 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 β_2 ARs 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 μ M 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

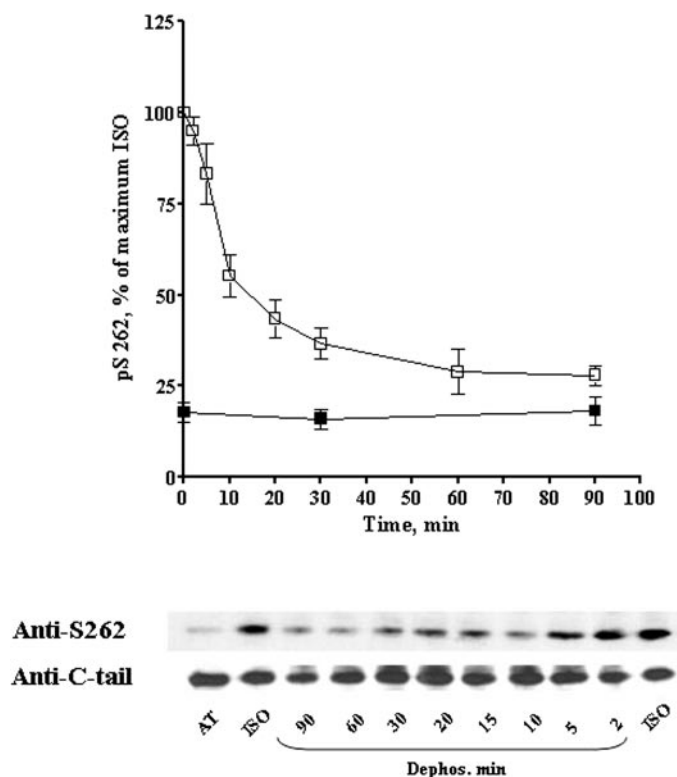


Fig. 1. Dephosphorylation of the β_2 AR PKA site in intact cells. WT- β_2 AR and β_2 AR-SA3 cells growing in 12-well plates were stimulated with 300 pM isoproterenol (ISO) or carrier (AT) for 10 min. The medium was then aspirated (0 time point) and replaced with medium \pm 1.0 μ M propranolol (open symbols). To stop the assay, cells were solubilized at the times indicated and Western blots were performed by sequential blotting with anti-pS262 mAb (2G3) and C-tail antibodies (Santa Cruz) as discussed under *Materials and Methods*. Results were first corrected for β_2 AR levels with C-tail antibody and then normalized to the 10-min isoproterenol treatment (zero time for the dephosphorylation). Data shown are mean \pm S.E. of 10 experiments for the 0 to 30 min values ($n = 4$ and 6 for WT- β_2 AR and β_2 AR-SA3, respectively) and four experiments for the 60- and 90-min values. Each experiment was performed in duplicate. The rate for the dephosphorylation was $k = 0.080 \pm 0.016/\text{min}$. A representative immunoblot is shown below the figure.

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 isoproterenol, washed, suspended, and then incubated with 100 nM calyculin A. This treatment completely blocked dephosphor-

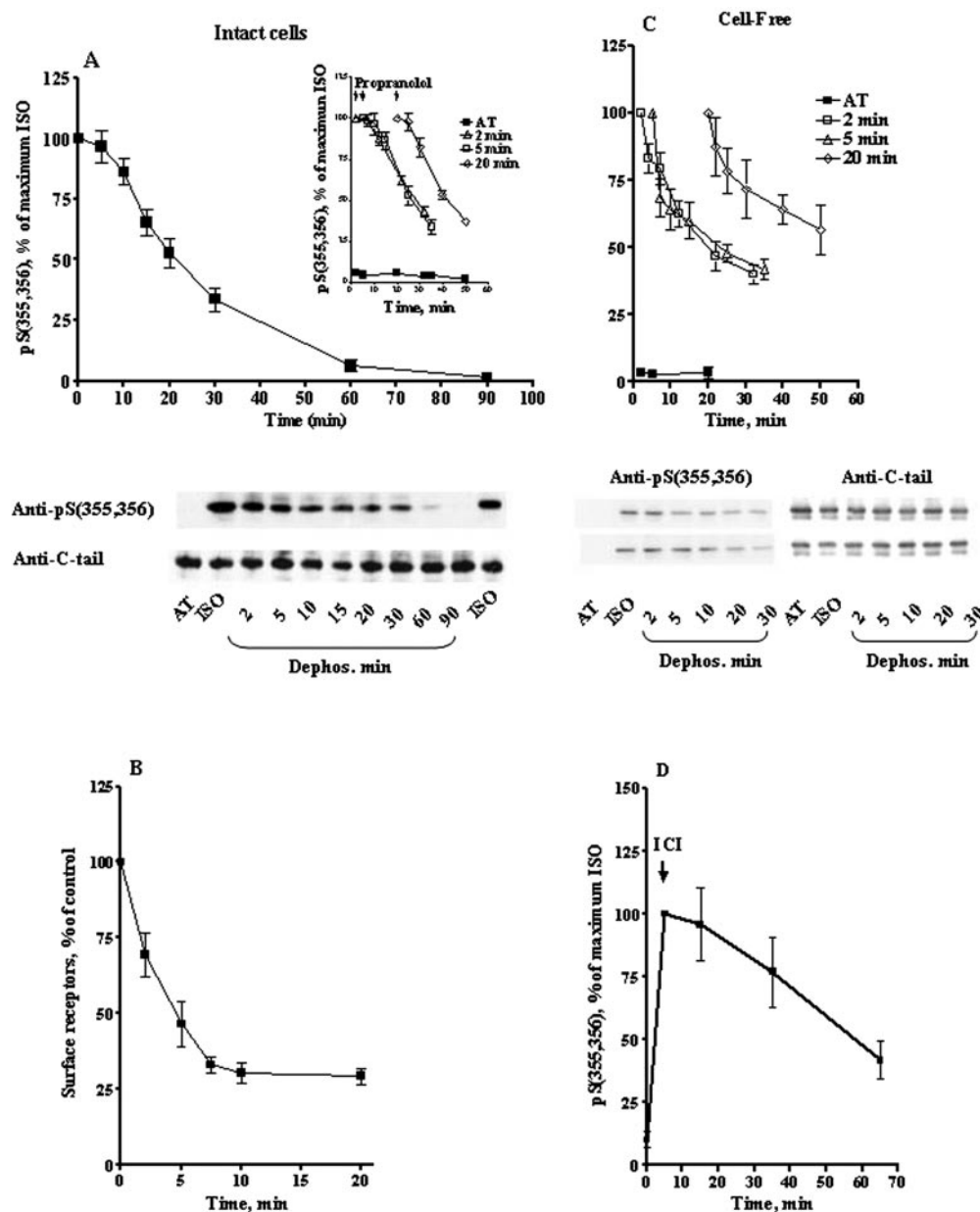


Fig. 2. Dephosphorylation of the β_2 AR GRK site in intact cells and cell-free assays. HEK 293 cells expressing the WT- β_2 AR were grown to confluence in either 12-well plates for intact cell dephosphorylation or 100-mm dishes for cell-free experiments. Cells were pretreated with 1.0 μ M isoproterenol 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 μ M 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 $k = 0.039 \pm 0.006/\text{min}$. 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 μ M isoproterenol for the times indicated, and surface receptors were then measured with [3 H]CGP-12177 as described under *Materials and Methods*. Data presented are the mean \pm 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 are shown as the mean \pm S.E. of three experiments, each performed in duplicate. D, A431 dephosphorylation. The cells were treated with AT or 1 μ M isoproterenol for 5 min, washed, and then incubated with 0.1 μ M 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 \pm 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 μ M) 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 μ M 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 μ M 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 μ M 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

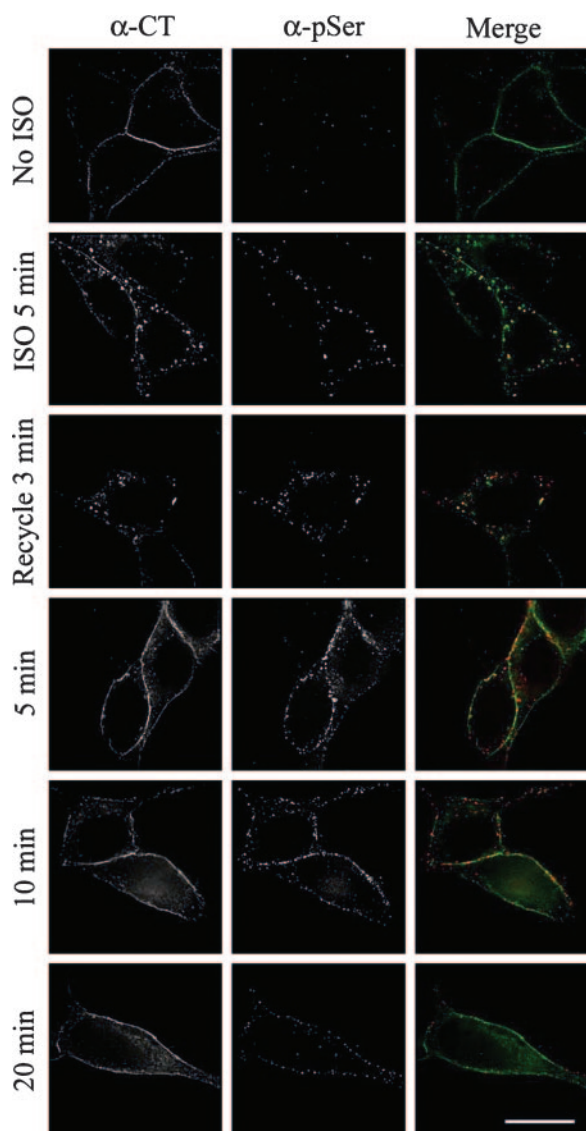


Fig. 3. Cellular localization of GRK site-phosphorylated β_2 ARs. WT- β_2 AR cells were treated with vehicle (AT) or isoproterenol for 5 min and then immediately fixed or extensively washed and incubated with fresh medium for the indicated times before fixation. Cells were labeled as described under *Materials and Methods* using as primary antibodies the polyclonal anti- β_2 AR C-tail antibody (α -CT) to identify all β_2 ARs and monoclonal antibody 5C3 against pS(355,356) (α -pSer) to specifically identify GRK-site phosphorylated receptors. Scale bar, 10 μ m. In the merged panel, green (Alexa 488) shows the C-tail antibody, red (Alexa 594) shows the GRK site antibody, and yellow shows the colocalization.

first stimulated WT- β_2 AR cells with 1.0 μ M 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 μ M 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 gradient fractions with regard to 1) 1.0 μ M isoproterenol-stimulated adenylyl cyclase activity, 2) the levels of β_2 AR determined by using the C-tail antibody, and 3) the extent of GRK site phosphorylation. As shown in Fig. 6B, the isoproterenol-stimulated adenylyl cyclase activity was found in the HV fractions with peak activity in fractions 20 to 25 as previously demonstrated in S49 lymphoma cells (Clark et al., 1985). We found approximately 50% of the receptors were shifted to LV fractions 5 to 11 with isoproterenol pretreatment (Fig. 6C), and there were nearly equivalent levels of

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.

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 β_2 AR (C-tail antibody), and that only the HV fraction showed significant dephosphorylation with intrinsic phosphatase activity ($p = 0.01$). To determine whether the phosphorylated β_2 ARs 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

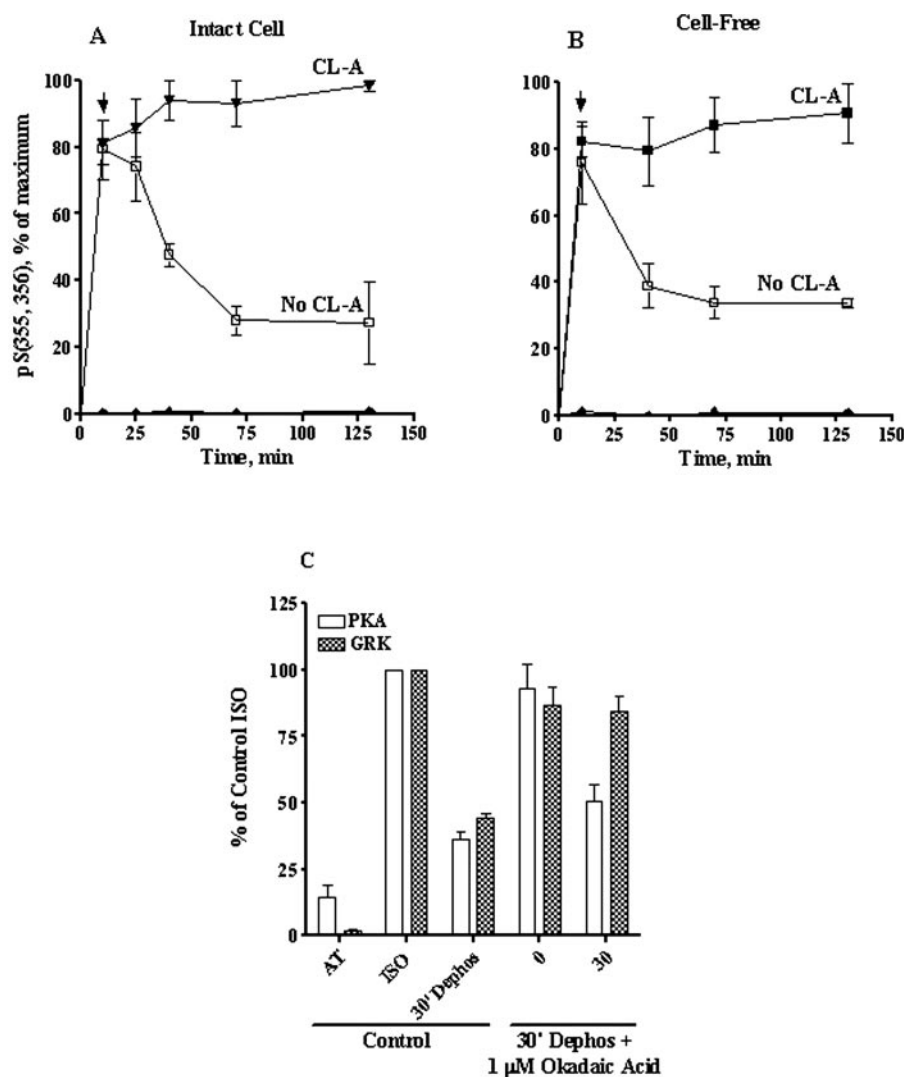


Fig. 4. Inhibition of GRK and PKA site dephosphorylation of the β_2 AR with calyculin A and okadaic acid in intact cells and cell-free assay. **A**, for the intact cell experiment, WT- β_2 AR cells were grown to confluence in 100-mm dishes and stimulated with either 100 nM isoproterenol or carrier (AT) 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 \pm 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 \pm S.E. of three experiments. **C**, for the effect of okadaic acid on intact cell dephosphorylation, WT- β_2 AR cells in 12-well dishes were preincubated \pm 1.0 μ M okadaic acid for 60 min. Cells were then stimulated with either 300 pM isoproterenol for either 10 min for the PKA site dephosphorylation, or 2 min with 1.0 μ M isoproterenol for the GRK site dephosphorylation. The medium was removed and replaced with medium plus 1 μ M 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 \pm 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 β_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 μ M metoprolol, a low affinity (240 nM K_d) pan-blocker of agonist stimulation of β_1 ARs and β_2 ARs (Contreras et al., 1986; January et al., 1997, 1998). Cells were stimulated for 15 min with or without 1.0 μ M isoproterenol, rapidly washed three times with medium at 37°C, and subsequently incubated for 2 to 30 min with medium containing 100 μ M metoprolol. To stop resensitization, cells were washed and lysed, and mem-

branes 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_{max} to emphasize the receptor level desensitization/resensitization, because we have demonstrated previously that the receptor-level desensitization in β_2 AR-overexpressing cells is almost exclusively reflected in the EC_{50} shift, and the decrease in V_{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 $\approx 55\%$ 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 ($p \leq 0.001$ 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

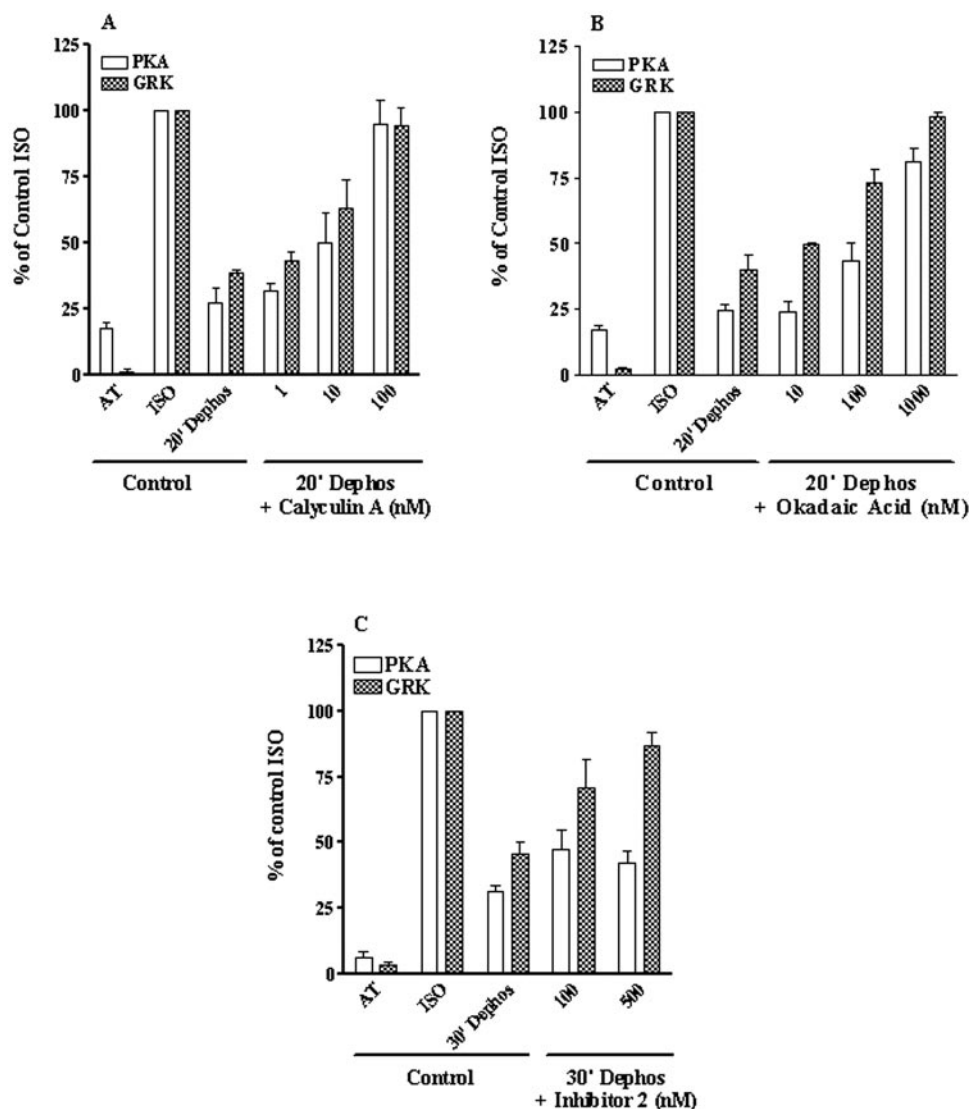


Fig. 5. Inhibition of GRK and PKA site dephosphorylation of the β_2 AR with calyculin A, okadaic acid, and PP1-inhibitor 2 in cell-free assays. To determine the effective concentrations of calyculin A (A), okadaic acid (B), and PP1-inhibitor 2 (C), cells in 100-mm dishes were stimulated for either 10 min with 300 pM (PKA site) or 2 min with 1.0 μ M (GRK site) isoproterenol, and the post-600g supernatant was incubated for 20 or 30 min with or without the various concentrations of inhibitors. The dephosphorylation was then followed in the post-600g 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 \pm 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.

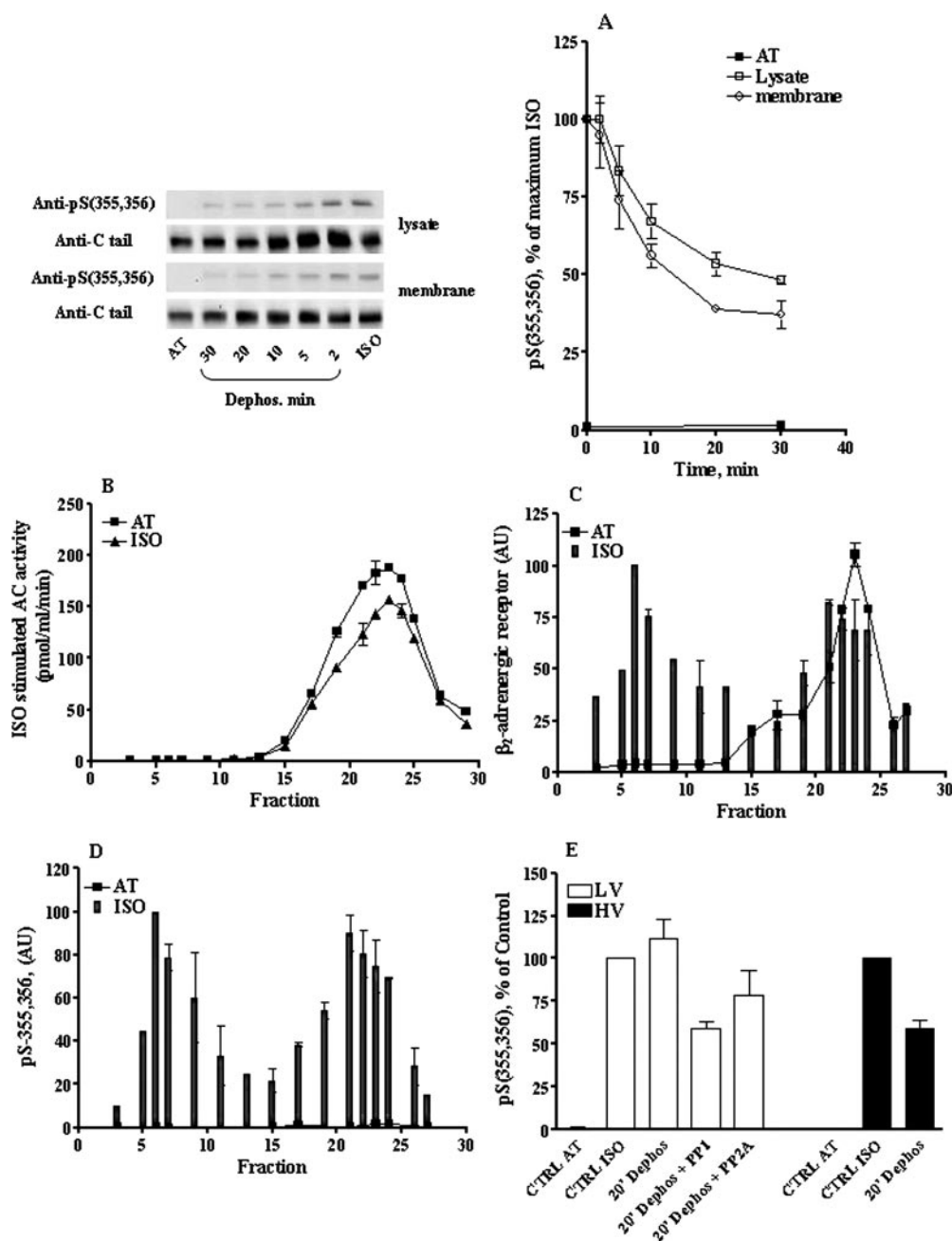


Fig. 6. Dephosphorylation of GRK site of WT- β_2 ARs in the crude particulate fraction and in heavy and light vesicle fractions. A, WT- β_2 AR were grown to confluence in 100-mm dishes and stimulated with 1.0 μ M isoproterenol or carrier AT for 2 min. Cells were then washed, lysed, and homogenized in 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 μ l 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 μ M 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- μ l 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. To measure the dephosphorylation, aliquots were incubated for 20 min and then mixed with 2 \times solubilization buffer (20' Dephos \pm 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 \pm 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 μ M 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 ($\approx 0.039/\text{min}$ and $0.08/\text{min}$, respectively) after the lag occurred far more slowly than the rate of resensitization ($\approx 0.43/\text{min}$).

Discussion

Previous studies of the phosphorylation and dephosphorylation of the β_2 AR 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 β_2 AR 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 EC_{50} of 30 pM in cells overexpressing the WT receptor. In contrast, the EC_{50} 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 pM 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 $t_{1/2}$ of ≈ 9 min, and that the GRK site dephosphorylation proceeded with a relatively slower $t_{1/2}$ 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 ($t_{1/2} = 1.6$ 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 EC_{50} for isoproterenol stimulation and, after 5 min, a 4-fold reduction in the EC_{50} ; these are times at which little dephosphorylation was observed, equivalent to restoration of $\approx 50\%$ 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 dephosphorylated β_2 AR is not required for this phase of resensitization. Whereas previous studies of resensitization suggested that internalization and dephosphorylation were

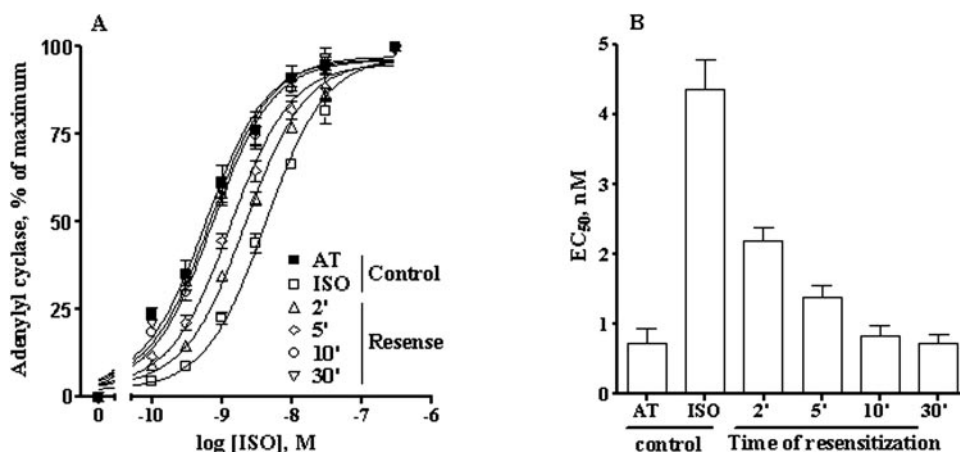


Fig. 7. Resensitization of β_2 AR stimulation of adenylyl cyclase activity after a 15-min treatment with 1.0 μ M isoproterenol. WT- β_2 AR cells in 100-mm dishes were treated for 15 min with 1.0 μ M isoproterenol or carrier AT. To measure resensitization, cells were washed rapidly three times with warm medium and incubated in medium containing 100 μ M metoprolol to block residual isoproterenol stimulation. At times from 2 to 30 min after washout, the medium was removed, cells were washed, and membranes were prepared on sucrose step gradients for adenylyl cyclase assays as given under *Materials and Methods*. A, the resensitization was measured by the decrease in the EC_{50} values for isoproterenol stimulation of adenylyl cyclase (means \pm S.E.M.; $n = 5$). Basal activity was first subtracted and the data normalized to the V_{max} for each experiment. B, a plot of the EC_{50} values from the data of Fig. 7A. All values of the EC_{50} s for the resensitization relative to control were significant ($p \leq 0.001$). The rate constant for the resensitization was $k = 0.43/\text{min}$.

required for resensitization (Sibley et al., 1986; Yu et al., 1993; Pippig et al., 1995; Zhang et al., 1997), the rapid kinetics of adenylyl 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 β_2 ARs documented that in response to high agonist concentrations, arrestin binding to GRK-phosphorylated receptor was required for the major desensitization in subsequent assays with reconstituted G_s (Benovic et al., 1987; Lohse et al., 1992). Phosphorylation of the GRK sites, β -arrestin translocation and binding to the β_2 AR, 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 β_2 AR even if the receptor is phosphorylated. It was previously demonstrated that the $t_{1/2}$ for the dissociation of GFP-arrestin from the β_2 AR in HEK 293 cells was ≈ 6 –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 β_2 AR 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.

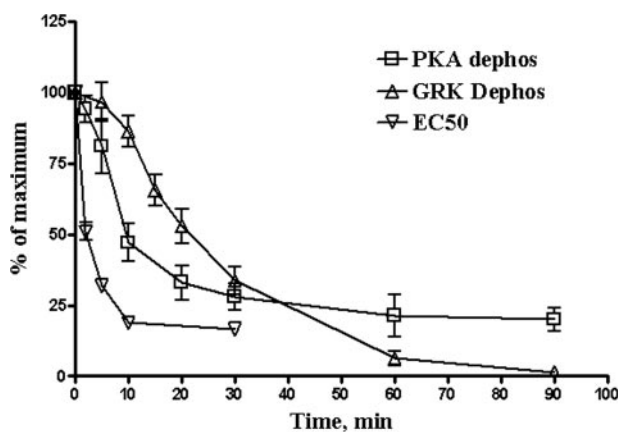


Fig. 8. Comparison of the time course of PKA and GRK site dephosphorylation with that of resensitization of adenylyl cyclase activity: To directly compare the kinetics of resensitization of agonist stimulation of adenylyl cyclase with PKA and GRK site dephosphorylation, the data from Figs. 1, 2A (data for the 5-min treatment only), and 7 were plotted normalized to the maximum value in each experiment. Rate constants for the resensitization and GRK and PKA site dephosphorylations were $k = 0.42$, 0.039 , and $0.080/\text{min}$, respectively, and $t_{1/2}$ values were 1.6, 18, and 8.7 min, respectively.

The mechanisms for β_2 AR 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 β_2 AR 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 β_2 AR 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 phosphorylation when dephosphorylation was inhibited by calyculin A or okadaic acid. Second, the rate of GRK phosphorylation ($k = 1$ –2/min) exceeded that of dephosphorylation ($k = 0.04/\text{min}$) by 30-fold, predicting that steady-state GRK site phosphorylation should be near saturation, as was demonstrated by mass spectrometry of the β_2 AR (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 β_2 AR in the heavy vesicle plasma membrane fraction, but not the endosomal receptor, was dephosphorylated by intrinsic phosphatase activity. Fifth, immunofluorescence of the phosphorylated β_2 AR 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 (≈ 31 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 β_2 AR, as was shown for the CXCR2 receptor (Fan et al., 2001), that could include second-messenger regulation of phosphatase and β_2 AR 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 IC_{50} 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 β_2 AR PKA and GRK sites (20 and 10% of the resensitization rate, respectively), compared with the rapid kinetics of resensitization of agonist stimulation of adenylyl 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 adenylyl cyclase activity after removal of agonist is the fast dissociation of β -arrestin from membrane-bound β_2 AR 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 are not achieved by perfusion or the addition of antagonist. 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 reinsertion of the β_2 AR 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

- Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG, and Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc Natl Acad Sci USA* **84**:8879–8882.
- Clark RB, Friedman J, Prashad N, and Ruoho AE (1985) Epinephrine-induced sequestration of the beta-adrenergic receptor in cultured S49 WT and cyc- lymphoma cells. *J Cyclic Nucleotide Protein Phosphor Res* **10**:97–119.

- Clark RB, Knoll BJ, and Barber R (1999) Partial agonists and G protein-coupled receptor desensitization. *Trends Pharmacol Sci* **20**:279–286.
- Contreras ML, Wolfe BB, and Molinoff PB (1986) Kinetic analysis of the interactions of agonists and antagonists with beta adrenergic receptors. *J Pharmacol Exp Ther* **239**:136–143.
- Fan GH, Yang W, Sai J, and Richmond A (2001) Phosphorylation-independent association of CXCR2 with the protein phosphatase 2A core enzyme. *J Biol Chem* **276**:16960–16968.
- Gupta RC, Neumann J, Watanabe AM, and Sabbah HN (2002) Inhibition of type 1 protein phosphatase activity by activation of beta-adrenoceptors in ventricular myocardium. *Biochem Pharmacol* **63**:1069–1076.
- Gurevich VV and Gurevich EV (2004) The molecular acrobatics of arrestin activation. *Trends Pharmacol Sci* **25**:105–111.
- Huang HB, Horiuchi A, Watanabe T, Shih SR, Tsay HJ, Li HC, Greengard P, and Nairn AC (1999) Characterization of the inhibition of protein phosphatase-1 by DARPP-32 and inhibitor-2. *J Biol Chem* **274**:7870–7878.
- Iyer V, Tran TM, Foster E, Dai W, Clark RB, and Knoll BJ (2006) Differential phosphorylation and dephosphorylation of beta(2)-adrenoceptor sites Ser262 and Ser355,356. *Br J Pharmacol* **147**:249–259.
- January B, Seibold A, Allal C, Whaley BS, Knoll BJ, Moore RH, Dickey BF, Barber R, and Clark RB (1998) Salmeterol-induced desensitization, internalization and phosphorylation of the human beta2-adrenoceptor. *Br J Pharmacol* **123**:701–711.
- January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrunn A, Barber R, and Clark RB (1997) β_2 -Adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J Biol Chem* **272**:23871–23879.
- Kelly E (2006) G-protein-coupled receptor dephosphorylation at the cell surface. *Br J Pharmacology* **147**:235–236.
- Krasel C, Bunemann M, Lorenz K, and Lohse MJ (2005) Beta-arrestin binding to the β_2 -adrenergic receptor requires both receptor phosphorylation and receptor activation. *J Biol Chem* **280**:9528–9535.
- Krueger KM, Daaka Y, Pitcher JA, and Lefkowitz RJ (1997) The role of sequestration in G protein-coupled receptor resensitization. Regulation of β_2 -adrenergic receptor dephosphorylation by vesicular acidification. *J Biol Chem* **272**:5–8.
- Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38**:289–319.
- Liang W, Curran PK, Hoang Q, Moreland RT, and Fishman PH (2004) Differences in endosomal targeting of human (beta)1- and (beta)2-adrenergic receptors after clathrin-mediated endocytosis. *J Cell Sci* **117**:723–734.
- Liang W and Fishman PH (2004) Resistance of the human beta1-adrenergic receptor to agonist-induced ubiquitination: a mechanism for impaired receptor degradation. *J Biol Chem* **279**:46882–46889.
- Lohse MJ, Andexinger S, Pitcher J, Trukawinski S, Codina J, Faure JP, Caron MG, and Lefkowitz RJ (1992) Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of β -arrestin and arrestin in the β_2 -adrenergic receptor and rhodopsin systems. *J Biol Chem* **267**:8558–8564.
- Lohse MJ, Benovic JL, Caron MG, and Lefkowitz RJ (1990a) Multiple pathways of rapid β_2 -adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* **265**:3202–3211.
- Lohse MJ, Benovic JL, Codina J, Caron MG, and Lefkowitz RJ (1990b) β -Arrestin: a protein that regulates beta-adrenergic receptor function. *Science (Wash DC)* **248**:1547–1550.
- Lohse MJ, Lefkowitz RJ, Caron MG, and Benovic JL (1989) Inhibition of beta-adrenergic receptor kinase prevents rapid homologous desensitization of beta 2-adrenergic receptors. *Proc Natl Acad Sci USA* **86**:3011–3015.
- Malbon CC, Tao J, and Wang HY (2004) AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. *Biochem J* **379**:1–9.
- McLean AJ and Milligan G (2000) Ligand regulation of green fluorescent protein-tagged forms of the human beta(1)- and beta(2)-adrenoceptors; comparisons with the unmodified receptors. *Br J Pharmacol* **130**:1825–1832.
- Morrison KJ, Moore RH, Carsrud ND, Trial J, Millman EE, Tuvim M, Clark RB, Barber R, Dickey BF, and Knoll BJ (1996) Repetitive endocytosis and recycling of the β_2 -adrenergic receptor during agonist-induced steady state redistribution. *Mol Pharmacol* **50**:692–699.
- Pippig S, Andexinger S, and Lohse MJ (1995) Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666–676.
- Pitcher JA, Payne ES, Csontos C, DePaoli-Roach AA, and Lefkowitz RJ (1995) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc Natl Acad Sci USA* **92**:8343–8347.
- Seibold A, January BG, Friedman J, Hipkin RW, and Clark RB (1998) Desensitization of β_2 -adrenergic receptors with mutations of the proposed G protein-coupled receptor kinase phosphorylation sites. *J Biol Chem* **273**:7637–7642.
- Seibold A, Williams B, Huang ZF, Friedman J, Moore RH, Knoll BJ, and Clark RB (2000) Localization of the sites mediating desensitization of the β_2 -adrenergic receptor by the GRK pathway. *Mol Pharmacol* **58**:1162–1173.
- Shenoy SK and Lefkowitz RJ (2005) Receptor-specific ubiquitination of β -arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *J Biol Chem* **280**:15315–15324.
- Shih M, Lin F, Scott JD, Wang HY, and Malbon CC (1999) Dynamic complexes of β_2 -adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J Biol Chem* **274**:1588–1595.
- Sibley DR, Strasser RH, Benovic JL, Daniel K, and Lefkowitz RJ (1986) Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylyl cyclase and subcellular distribution. *Proc Natl Acad Sci USA* **83**:9408–9412.
- Sike S and Shenolikar S (2005) Protein phosphatase inhibitor-1, an amplifier of cAMP signals. *Cellscience Rev* **2**.
- Sohlemann P, Hekman M, Puzicha M, Buchen C, and Lohse MJ (1995) Binding of

- purified recombinant beta-arrestin to guanine-nucleotide-binding-protein-coupled receptors. *Eur J Biochem* **232**:464–472.
- Stickle D and Barber R (1989) Evidence for the role of epinephrine binding frequency in activation of adenylate cyclase. *Mol Pharmacol* **36**:437–445.
- Stickle D and Barber R (1991) Estimation of the kinetic constants for binding of epinephrine to beta-adrenergic receptors of the S49 cell. *Biochem Pharmacol* **42**:1069–1077.
- Tran TM, Friedman J, Qunaibi E, Baameur F, Moore RH, and Clark RB (2004) Characterization of agonist stimulation of cAMP-dependent protein kinase and G protein-coupled receptor kinase phosphorylation of the β_2 -adrenergic receptor using phosphoserine-specific antibodies. *Mol Pharmacol* **65**:196–206.
- Trester-Zedlitz M, Burlingame A, Kobilka B, and von Zastrow M (2005) Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. *Biochemistry* **44**:6133–6143.
- Vaughan DJ, Millman EE, Godines V, Friedman J, Tran TM, Dai W, Knoll BJ, Clark RB, and Moore RH (2006) Role of the G protein-coupled receptor kinase site serine cluster in β_2 -adrenergic receptor internalization, desensitization, and β -arrestin translocation. *J Biol Chem* **281**:7684–7692.
- Whaley BS, Yuan N, Birnbaumer L, Clark RB, and Barber R (1994) Differential expression of the β -adrenergic receptor modifies agonist stimulation of adenylyl-cyclase: a quantitative evaluation. *Mol Pharmacol* **45**:481–489.
- Yang SD, Fong YL, Benovic JL, Sibley DR, Caron MG, and Lefkowitz RJ (1988) Dephosphorylation of the β_2 -adrenergic receptor and rhodopsin by latent phosphatase 2. *J Biol Chem* **263**:8856–8858.
- Yu SS, Lefkowitz RJ, and Hausdorff WP (1993) β -Adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J Biol Chem* **268**:337–341.
- Zhang J, Barak LS, Anborgh PH, Laporte SA, Caron MG, and Ferguson SS (1999) Cellular trafficking of G protein-coupled receptor/ β -arrestin endocytic complexes. *J Biol Chem* **274**:10999–11006.
- Zhang J, Barak LS, Winkler KE, Caron MG, and Ferguson SS (1997) A central role for β -arrestins and clathrin-coated vesicle-mediated endocytosis in β_2 -adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. *J Biol Chem* **272**:27005–27014.

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