Visualizing of Agonist-induced Sequestration and Down-regulation of a Green Fluorescent Protein-tagged β2-Adrenergic Receptor*

(Received for publication, May 22, 1997, and in revised form, October 22, 1997)

Lorena Kallal, Alison W. Gagnon, Raymond B. Penn, and Jeffrey L. Benovic‡
From the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

To date, the visualization of β2-adrenergic receptor (β2AR) trafficking has been largely limited to immunocytochemical analyses of acute internalization events of epitope-tagged receptors in various transfection systems. The development of a β2AR conjugated with green fluorescent protein (β2AR-GFP) provides the opportunity for a more extensive optical analysis of β2AR sequestration, down-regulation, and recycling in cells. Here we demonstrate that stable expression of β2AR-GFP in HeLa cells enables a detailed temporal and spatial analysis of these events. Time-dependent colocalization of β2AR-GFP with rhodamine-labeled transferrin and rhodamine-labeled dextran following agonist exposure demonstrates receptor distribution to early endosomes (sequestration) and lysosomes (down-regulation), respectively. The observed temporal distribution of β2AR-GFP was consistent with measures of receptor sequestration and down-regulation generated by radioligand-receptor binding assays. Cells stimulated with different β-agonists revealed time courses of β2AR-GFP redistribution reflective of the intrinsic activity of each agonist.

Upon agonist stimulation, β2-adrenergic receptors (β2ARs) are rapidly desensitized by receptor phosphorylation (1). Receptor phosphorylation by G protein-coupled receptor kinases and subsequent binding of non-visual arrestins initiates the internalization of β2ARs via clathrin-coated pits (2, 3). Previous studies have demonstrated that internalized receptors have multiple potential fates. One such fate is the recycling of internalized receptors to the plasma membrane, presumably completing an ill defined “resensitization” process involving β2AR dephosphorylation in an endosomal compartment (4, 5). Depending upon the duration of agonist exposure, internalized β2ARs may ultimately appear “lost” or destroyed (undetectable by radioligand binding), ostensibly trafficking to lysosomes where they are degraded. Previously, the analysis of these events has been heavily dependent on biochemical and pharmacological approaches.

‡ Established investigator of the American Heart Association. To whom correspondence should be addressed: Kimmel Cancer Inst., Thomas Jefferson University, 233 South 10th St., Philadelphia, PA 19107. Tel.: 215-503-4607; Fax: 215-923-1098; E-mail: benovic@lac.jrcjtu.edu.

* This work was supported in part by National Institutes of Health Grants GM44944 and GM47417. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: β2AR, β2-adrenergic receptor; GFP, green fluorescent protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

Agonist-mediated subcellular redistribution of β2ARs was initially inferred from ligand binding studies (6, 7) and the coincident migration of β2ARs with enzyme markers (8, 9) or epidermal growth factor (10, 11) into subcellular fractions resolved through centrifugation. However, direct visualization of β2AR trafficking events remained lacking until von Zastrow and Kobilka (12) provided immunocytochemical evidence of rapid, agonist-induced redistribution of epitope-tagged β2ARs into small, punctate accumulations within the cytoplasm. The time course of β2AR redistribution assessed by confocal microscopy paralleled that of β2AR sequestration measured by radioligand binding. Importantly, internalized β2ARs colocalized with transferrin receptors, suggesting that sequestered β2ARs undergo processing through endosomal compartments in a manner similar to that observed for constitutively internalized receptors.

Advances in the development of proteins conjugated with green fluorescent protein (GFP) have since provided the opportunity for real time optical analysis of protein trafficking events in individual cells (13–15). Green fluorescent protein is a naturally occurring protein isolated from several different species of jellyfish (Aequoria) and sea cucumbers (Renilla). When expressed in cells, proteins conjugated with GFP may be visualized with routine fluorescent microscopy without the need to fix cells. Recently, Barak et al. (16) established the utility of a β2AR-GFP fusion protein in visualizing agonist-mediated β2AR internalization in HEK293 cells transiently expressing the construct. This study also demonstrated that β2AR-GFP is fully functional with regard to ligand binding and adenylyl cyclase stimulation and that it can undergo agonist-dependent phosphorylation and sequestration. Here we demonstrate that stable expression of β2AR-GFP in HeLa cells enables a detailed temporal and spatial analysis of β2AR trafficking events associated with receptor sequestration, down-regulation, and recycling. Colocalization of the β2AR-GFP with rhodamine-labeled transferrin and rhodamine-labeled dextran during agonist treatment revealed sequential localization of receptor in cellular compartments containing these compounds. Moreover, this system appears capable of distinguishing the differing effects of various β-agonists (including the highly hydrophobic ligand salmeterol) on β2AR trafficking, overcoming some of the limitations in previous analyses of these compounds.

EXPERIMENTAL PROCEDURES

Construction of a β2AR-GFP Fusion Expression Construct—To create a Flag-tagged β2AR-GFP fusion protein, PCR was used to amplify both the β2AR and Aequoria victoria GFP-S65T. An amino-terminal β2AR primer, TGCCGCATGCGGCAAC, was combined with a primer designed against the carboxyl terminus, GCCTTAGACAGCAGTGGAT-CATTGT, in which the stop codon is replaced by an XhoI site that encodes two extra amino acids, serine and arginine. Similarly, primers

To create a Flag-tagged β2AR-GFP fusion protein, PCR was used to amplify both the β2AR and Aequoria victoria GFP-S65T. An amino-terminal β2AR primer, TGCCGCATGCGGCAAC, was combined with a primer designed against the carboxyl terminus, GCCTTAGACAGCAGTGGAT-CATTGT, in which the stop codon is replaced by an XhoI site that encodes two extra amino acids, serine and arginine. Similarly, primers

Printed in U.S.A.
were designed to amplify GFP from the pGFP-S67 T template (CLON-TECH) with an XbaI site at the NH2 terminus, GCTTCAGAGGTTGAGCAGAACGGCGAC and a SalI site at the COOH terminus, AACAGTGTGCGAAGCTTCTGAGCAGCGTGC. The two PCR products were cut with SalI/XbaI and inserted for the β2AR or XbaI site into pBCflag, the third clone into NcoI/SalI digested pBCflag2AR (provided by B. Kobilka). An NcoI/NcoI fragment of the pBC backbone was recloned in, and to circumvent the entire open reading frame of the β2AR, an NcoI/EcoRV region was replaced with that portion of the original pBCflag2AR construct. The final ligated product was sequenced through the regions that were generated by PCR. This construct was further modified by exchanging a small NcoI/EcoRV fragment containing the region encoding the flag epitope with an identical fragment generated by PCR that contains a Kozak consensus sequence for translation initiation (5′CCATG). For the present studies, the region encoding the entire Flag-tagged β2AR-GFP fusion was removed with HindIII/SalI and cloned into the BamHI site of pcDNA3 (Invitrogen) using BamHI linkers. This final construct, pcDNA3-β2AR-GFP, was used in transient transfections and for making the stable HeLa cell line. A Flag-tagged β2AR construct in pcDNA3 was created by digesting pBCflag2AR with SalI followed by Klenow to blunt the 3′ end. The insert was excised with HindIII and the –2 kilobase fragment was ligated into HindIII/EcoRV digested pcDNA3.

**Transient and Stable Transfection of HeLa Cells**—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10–15% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 95% air/5% CO2. Cells grown to 80–90% confluence were trypsinized, diluted, and replated in media supplemented with 1 mg/ml Geneticin (Life Technologies, Inc.). Media were subsequently replaced every 3 days with complete media containing 0.5 mg/ml Geneticin. Stable transformants were isolated approximately 2 weeks after transfection, and clonal expression was confirmed by examining cells grown on coverslips by fluorescent microscopy.

cAMP Assays—cAMP assays were performed using CHW cells (provided by R. Lefkowitz), a hamster fibroblast line that does not express endogenous β2ARs. CHW cells were transfected using a modification of an adenovirus-assisted transfection procedure (17). Briefly, harvested cells in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum were mixed with 40 μg/ml DEAE-dextran, 100 μl of replication-defective adenovirus (GPT-Ads; a gift from P. Garcia), and 2 μg of plasmid DNA. Transfected cells were passaged the next day onto 12-well plates, and experiments examining agonist-mediated cAMP production were performed 5 days after transfection. Cells were washed with cold phosphate-buffered saline (PBS) and stimulated at 37 °C for 1–5 min with 500 μl of PBS containing 300 μM ascorbic acid, 1 mM isobutylmethylxanthine, and no addition (basal), 10−10−3 M (−)-isoproterenol, or 100 μM forskolin. Reactions were stopped by placing the plates on ice, aspirating the media, and adding 500 μl of ice-cold ethanol. The contents of each well were collected, lyophilized, resuspended, and assayed for cAMP content by radioimmunoassay using 125I-labeled cAMP (NEP Life Science Products) and anti-cAMP antibody (a gift from M. Ascoli) as described previously (18). β2AR Binding and Ligand Competition Assays—For determination of β2AR density in control and transfected cells, whole cells were harvested with trypsin/EDTA, or membranes were prepared (for competition binding assays). Whole cells were incubated in PBS containing 200 pM [125I]-iodopindolol (NEP Life Science Products, 2200 Ci/mol) or 10 μM (−)-alprenolol at 37 °C for 1 h as described previously (18). Competition binding studies on cell membranes were performed as described previously (18). Briefly, cells were collected into cold homogenization buffer (25 mM Tris, pH 7.5, 5 mM EDTA, 1 mM EGTA, 0.02 mg/ml leupeptin, 0.2 mg/ml benzamidine, 0.5 mM phenylmethylsulfonyl fluoride) and homogenized by Polytron disruption. Homogenates were then centrifuged at 10,000 g at 4 °C to remove debris, and supernatants were collected. Approximately 5 μg (transfected cells) or 50 μg (untransfected cells) of membrane protein were incubated at 37 °C for 1 h with 30 pM [125I]-iodopindolol in the presence of 10−11–10−4 M (−)-isoproterenol. For down-regulation studies, cells were treated with isoproterenol for various times, washed, and homogenized in lysis buffer (20 mM Tris, pH 8, 5 mM EDTA, 2 mM EGTA, 5 μg/ml leupeptin, 0.2 mg/ml benzamidine) using a Polytron (2 × 30 s at 25,000 rpm), and approximately 50 μg of lysate were incubated at 37 °C for 1 h with 1 nM [125I]-iodopindolol. All binding reactions were terminated by the addition of 5 × 4 ml of ice-cold 25 mM Tris, pH 7.5, 2 mM MgCl2 followed by filtration through Whatman GF/C filters using a Brandwein cell harvester.

β2AR Sequestration Assays—HeLa cells stably expressing β2AR-GFP were grown to 80–90% confluence, and internalization of receptor was assayed as described previously using the hydrophilic ligand [3H]CGP-12177 (3). Briefly, cells were harvested by trypsinization, resuspended in PBS, and incubated at 37 °C for 0–60 min with 10 μM (−)-isoproterenol. Incubations were stopped by the addition of ice-cold PBS, and cells were resuspended thoroughly with cold PBS. Cells were resuspended in cold PBS, and cell surface β2AR density was assessed in binding assays using 10–15 nM [3H]CGP-12177 ± 10 μM (−)-alprenolol at 14 °C for 3 h. For sequestration studies in transiently transfected HeLa cells, cells were harvested 48 h after transfection, incubated at 37 °C for 30 min with 1 μM (−)-isoproterenol, and then assayed for cell surface receptors using [3H]CGP-12177 following a 0-, 20-, or 60-min agonist washout.

Fluorescence Microscopy and Single Cell Time Courses—Fluorescence microscopy was performed on a Bio-Rad MRC-Zeiss Axiosvert 100 confocal microscope (Hemmelholstede, UK), using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective. Cells were grown on glass coverslips and mounted on an imaging chamber (Warner Instrument Corp) with an inlet port through which media and drugs could be perfused. The media used for microscopy did not contain phenol red or antibiotics. For time course studies, the temperature was maintained at 37 ± 1 °C using an adjustable warm air flow and digital temperature probe (Yellow Springs Instrument Co., Inc.). For labeling of the lysosomal compartments, cells were incubated overnight with 1 mg/ml rhodamine-labeled dextran (Molecular Probes, Eugene, OR) on glass coverslips. The dextran was washed out of cells by rinsing once with media and then placing fresh media on cells 1.5 h before imaging. Agonists were added to the media and/or rhodamine-dextran mixture depending on the treatment. For the transferrin experiments, the cells were incubated in media lacking serum for 30 min, followed by incubation with 20 or 200 μg/ml rhodamine-labeled transferrin for 30–60 min at 37 °C before imaging. The rhodamine-transferrin was only briefly rinsed off of cells three times with PBS before imaging. Incubations were timed so that agonist exposure end points coincided with the rhodamine-dextran or-transferrin loading and washing procedure end points. Cells for the rhodamine colocalization studies were rinsed quickly three times with PBS, incubated for 10 min at room temperature in 3.7% formaldehyde to fix, rinsed again in PBS, and mounted on a microscope slide with Slowfade® mounting medium (Molecular Probes) before imaging by confocal microscopy.

**RESULTS AND DISCUSSION**

**Pharmacological and Functional Properties of β2AR-GFP**—The complete GFP-S67 T open reading frame was directly fused to the carboxyl terminus of a flag-tagged β2AR. Preliminary experiments were then performed to establish the suitability of β2AR-GFP as a model. Radioligand binding experiments using HeLa cells transiently expressing the receptor constructs demonstrated pharmacological properties of β2AR-GFP that were similar to those observed for the wild type β2AR. Competition of [125I]-iodopindolol binding with isoproterenol revealed comparable IC50 values (50 nM) measured using cells expressing either β2AR-GFP or wild type β2AR (Fig. 1A). Similar results were also obtained by Barak et al. (16), although calculated Kd values for both β2AR-GFP and wild type β2AR expressed in HEK293 cells were considerably higher, perhaps reflecting differences in receptor-G protein coupling between the respective model cells.

The capacity of β2AR-GFP to promote agonist-mediated cAMP production was also examined (Fig. 1B). CHW cells, which lack endogenous β2ARs, were transiently transfected with either wild type β2AR or β2AR-GFP, and the dose-dependent response to isoproterenol was examined. Isoproterenol was both efficacious (maximal cAMP reached approximately 2.5-fold basal) and potent (EC50 = 1–2 nM) in stimulating cAMP production in cells expressing β2AR-GFP. Moreover, β2AR-GFP responsiveness was similar to that observed for the wild type β2AR. Collectively, these data suggest that fusion of the
238-amino acid GFP protein to the carboxyl terminus of the β2AR does not alter the salient pharmacological or functional properties of the receptor.

Assessment of agonist-mediated internalization of β2AR-GFP was performed in HeLa cells stably expressing β2AR-GFP at ~200 fmol/mg protein (Fig. 1C). Cells were pretreated with 10 μM isoproterenol for 0–60 min, and cell surface β2AR density was subsequently assessed using the hydrophilic β-agonist [3H]CGP-12177. The results demonstrate a classical time-dependent sequestration of β2ARs. Within 15 min of agonist treatment an ~30% loss of cell surface receptors was observed, reaching ~50% by 60 min. These results are also comparable with those observed by Barak et al., in which flow cytometry was used to measure agonist-mediated sequestration (16). In addition, these results imply that mechanisms involving the acute trafficking events of the wild type β2AR (3) appear applicable to β2AR-GFP. Indeed when transiently expressed in COS-1 cells, agonist-mediated β2AR-GFP internalization was enhanced by co-expression of β-arrestin (data not shown). These results suggest that β-arrestin is capable of binding to the β2AR-GFP and mediating its internalization, as has been observed with the wild type β2AR (2, 3).

Alterations in cellular β2AR content following chronic exposure to β-agonist were subsequently determined in HeLa cells (Fig. 1D). Cells were treated with 10 μM isoproterenol for 0–24 h, and total β2AR density was measured in crude cell lysates by radioligand binding using the hydrophobic β-antagonist [125I]iodopindolol. As with the sequestration data, the temporal profile of β2AR-GFP down-regulation was typical of that observed for wild type β2AR expressed in various cell systems (19, 20).

Visualization of Time-dependent Trafficking of β2AR-GFP—Having established β2AR-GFP as an appropriate model of β2AR function and trafficking, we subsequently performed experiments designed to visualize the subcellular distribution of β2AR-GFP following acute and chronic exposure to β-agonist. These experiments were performed using HeLa cells in which stable transfection of β2AR-GFP resulted in expression levels of 200–700 fmol/mg protein. Examination of numerous cells suggests that the β2AR-GFP is diffusely distributed on the cell surface before agonist treatment (seen in Fig. 2, left panels in top and bottom rows). However, significant perinuclear staining was also visible in some cells (e.g., Fig. 2, bottom row). Cyclohexamide treatment reveals that although some of the perinuclear staining is likely due to Golgi localization of receptors, some receptors are also in a presently unknown cellular compartment.

Initial studies examined the rapid internalization of β2AR-

Fig. 1. A, competition binding in transiently transfected HeLa cells. Wild type β2AR and β2AR-GFP were expressed transiently in HeLa cells using LipofectAMINE to levels of ~2 and 3.5 pmol/mg membrane protein, respectively (25–40-fold above endogenous β2AR levels in HeLa cells). Binding experiments contained 30 pM [125I]iodopindolol, which was competed with the indicated concentrations of isoproterenol. Results represent duplicate binding assays. B, cyclic AMP generation in CHW cells. Receptors were expressed transiently in CHW cells as described under “Experimental Procedures,” and the cells were then treated with various concentrations of isoproterenol for 10 min at 37 °C. CAMP was assayed by radioimmunoassay and was compared with receptor-independent cAMP levels generated by incubation with 10 μM forskolin. Results represent the means ± S.E. of two experiments performed in triplicate. C, agonist-promoted sequestration of β2AR-GFP. HeLa cells stably expressing β2AR-GFP (~200 fmol/mg protein) were treated for the indicated times with 10 μM isoproterenol, and binding to the hydrophilic radioligand [3H]CGP-12177 was assessed. Binding at various time points was compared with the unstimulated cells. Results represent the means ± S.E. of four experiments performed in triplicate. D, down-regulation in HeLa cells stably expressing β2AR-GFP. HeLa cells stably expressing β2AR-GFP (~200 fmol/mg protein) were treated for the indicated times with 10 μM isoproterenol, the cells were washed and lysed in a hypotonic buffer, and binding to the hydrophobic antagonist [125I]iodopindolol was assessed. Results represent the means ± S.E. of three to six experiments performed in triplicate.
GFP following exposure of HeLa cells to 10 μM isoproterenol (Fig. 2). Fluorescent images obtained from single cells using confocal microscopy demonstrate the rapid appearance of a punctate staining pattern with accumulations that become progressively larger and more numerous over a 20-min course of agonist exposure (Fig. 2, top row). These images are consistent with the time course of sequestration suggested by our radioligand binding data (Fig. 1C) as well as with those studies utilizing immunocytochemistry of fixed and permeabilized cells to visualize internalization of epitope-tagged β2ARs (3, 21).

We then observed cells in which a 10-min agonist treatment was followed by media washout and exposure to the β2AR antagonist alprenolol (Fig. 2, lower panels). Antagonist was included in the wash to prevent agonist rebinding during the washout. The punctate localization pattern of the receptor was shown to revert to a more diffuse pattern within 20 min of agonist removal, suggesting that receptors are recycling back to the plasma membrane. Radioligand binding experiments in transiently transfected HeLa cells indicate that the return of the β2AR-GFP to the cell surface after agonist removal is temporally and quantitatively similar to that of the wild type β2AR (Fig. 3). However, complete recycling of internalized receptor was not observed until 60 min after agonist washout. Our findings agree with other pharmacological studies, suggesting that the receptor relocates to the plasma membrane upon removal of agonist (7), and with results from immunocytochemistry experiments using fixed cells (21). The ability to observe this process in live cells should provide unique insight into receptor recycling, and what signals, if any, affect this dynamic process.

We next examined the localization patterns of β2AR-GFP induced by exposure to β-agonists of differing pharmacological properties (Fig. 4). We hypothesized that the rate of observable punctate pattern formation would correlate with the intrinsic activity and/or onset of action of the various β-agonists tested. The effect of formoterol, a long acting β-agonist of high intrinsic activity and rapid onset of action, on β2AR-GFP redistribution is depicted in Fig. 4A. The rate and pattern of vesicular formation were comparable with that induced by isoproterenol. Conversely, albuterol, a short acting agonist with moderate intrinsic activity, exhibited a slightly slower rate of receptor relocation than that observed with either isoproterenol or
formoterol (Fig. 4B). Multiple experiments suggest that cells exposed to albuterol required approximately 25–30 min to reach a pattern of $\beta_2$AR-GFP distribution caused by a 20-min exposure to isoproterenol (Figs. 2 and 4 and data not shown). Most striking, however, were the data obtained using salmeterol, a long acting $\beta$-agonist with low intrinsic activity and a very slow rate of onset of action, characteristics determined in part by the very hydrophobic nature of the compound (22, 23). Cells exposed to salmeterol (Fig. 4C) required 70 min of treatment to exhibit a level of receptor internalization comparable with that observed at the 20-min isoproterenol time point. Yet it was possible to show that salmeterol clearly caused relocalization of receptors to intracellular vesicles, providing evidence of salmeterol-induced sequestration not obtainable in previous studies (23, 24). Because treatment of cells with salmeterol causes stable activation of adenylyl cyclase that survives extensive wash procedures and sucrose-gradient purification of plasma membrane fractions (23), radioligand binding is rendered an unreliable tool for the analysis of salmeterol effects on $\beta_2$AR trafficking. Here we demonstrate that direct visualization of $\beta_2$AR-GFP following acute (sequestration) versus chronic (associated with down-regulation) exposure to $\beta$-agonists, we examined the time-dependent colocalization of $\beta_2$AR-GFP with additional fluorescent compounds (rhodamine-labeled transferrin and dextran) known to accumulate in distinct subcellular compartments. Transferrin primarily internalizes with transferrin receptors and constitutively recycles with the receptors through early endosomes to a recycling compartment and then back to the cell surface (25). Conversely, dextran has recently been used to demonstrate lysosomal localization of the thyrotropin-releasing hormone receptor (27). Fig. 5A demonstrates that in unstimulated cells, the $\beta_2$AR-GFP distribution displays a relatively diffuse membrane localization pattern, whereas 20-min incubation of these cells with transferrin results in accumulation of transferrin in small vesicles. 30 min after treatment with isoproterenol, a significant portion of $\beta_2$AR-GFP is seen to distribute into early endosomal compartments coincident with the presence of transferrin (Fig. 5B, colocalization shown in yellow). In contrast, when cells are exposed to isoproterenol for 3.5 h followed by a 1-h treatment with transferrin in the absence of $\beta$-agonist, minimal co-local-
ization of βAR-GFP and transferrin is evident (Fig. 5C). These results demonstrate that under such conditions a large fraction of the βAR-GFPs remain in internal vesicles that lack transferrin receptors, possibly in late endosomes and/or lysosomes.

The agonist- and time-dependent localization of βAR-GFP with lysosomes is revealed in cells loaded with rhodamine-labeled dextran. Cells were incubated with 1 mg/ml rhodamine-labeled dextran for 24 h and then washed for 1.5 h in the absence of dextran to remove any accumulation in early endosomes. During the latter portion of these incubations the cells were also incubated with 10 μM isoproterenol for 30 min, 1 h, 3.5 h, or 24 h prior to fixing the cells. Unstimulated cells display dextran localized to large vesicles typical of lysosomes, many of which are centrally located in the cells (Fig. 6, upper left). Following 30 min of isoproterenol exposure, the βAR-GFP has moved into early endosomes and shows minimal co-localization with dextran. After 1 h of isoproterenol treatment, the βAR-GFP exhibits significant co-localization with dextran, with this colocalization increasing somewhat at the 3.5- and 24-h time points. Cells treated in a time-dependent manner with salmeterol displayed a similar pattern, although the progression of colocalization of βAR-GFP with dextran was slower. Although salmeterol treatment for 24 h results in images similar to those obtained with isoproterenol treatment, colocalization of dextran with βAR-GFP was not evident until after 3 h of treatment with salmeterol (data not shown), suggesting that the relatively slow kinetics of salmeterol binding/activation of the βAR translate into attenuated rates of βAR sequestration and down-regulation.

In conclusion, we have utilized βAR-GFP to visualize real time cellular redistribution of βARs in live cells responding to agonists. We have demonstrated the rapid colocalization of the βAR in transferrin-containing endosomes following acute β-agonist exposure. Following prolonged (but not acute) exposure to β-agonists, βAR-GFP is shown to colocalize with dextran in lysosomes. Experiments examining the effects of various β-agonists suggest that βAR distribution, sequestration, and down-regulation are regulated by the intrinsic activity and onset of action of a ligand. In this regard, the βAR-GFP was especially advantageous for the examination of the lipophilic compound salmeterol. Our results suggest that GFP conjugated G protein-coupled receptors are powerful tools for visualizing the dynamics of receptor trafficking in living cells.

Acknowledgments—We thank Drs. J. Keen and C. Schmutte and members of the Benovic and Keen labs for helpful discussions, J. Dispoto and P. Hingorani for confocal microscopy, and H. Alder and the Kimmel Nucleic Acids facility for DNA synthesis and sequencing. We also thank Dr. B. Kobilka for the Flag tagged βAR construct, Dr. R. Lefkowitz for CHW cells, Dr. M. Ascoli for the cAMP antibody, Dr. P. Garcia for the GPT-Ad5, and Seprocare Inc. for providing formoterol, albuterol, and salmeterol.

REFERENCES
1. Sterne Marr, R., and Benovic, J. L. (1995) in Vitamins and Hormones (Litwack, G., ed) pp. 193–234, Academic Press, New York.
2. Ferguson, S. S. G., Downey, W. E. III, Colapietro, A. M., Barak, L. S., Menard, L., and Caron, M. G. (1996) Science 271, 363–365.
3. Goodman, O. B. J., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 385, 447–450.
4. Pippig, S., Andexinger, S., and Lohse, M. (1995) Mol. Pharmacol. 47, 666–676.
5. Kruger, K. M., Daaka, Y., Pitcher, J. A., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 5–8.
6. Toews, M. L., Liang, M., and Perkins, J. P. (1987) Mol. Pharmacol. 32, 737–742.
7. Kurz, J. B., and Perkins, J. P. (1992) Mol. Pharmacol. 41, 375–381.
8. Chuang, D. M. (1982) Biochem. Biophys. Res. Commun. 105, 1466–1472.
9. Wald, G. L., Northup, J. K., Perkins, J. P., and Harden, T. K. (1983) J. Biol. Chem. 258, 19209–19216.
10. Waksman, E., Hertel, C., O’Keefe, E. J., and Perkins, J. P. (1985) J. Cell. Biochem. 29, 127–141.
11. Liao, J.-F., and Perkins, J. P. (1993) Mol. Pharmacol. 44, 364–370.
12. von Zastrow, M., and Kobilka, B. K. (1992) J. Biol. Chem. 267, 3530–3538.

Fig. 6. Colocalization of the βAR-GFP with dextran during β-agonist treatment. HeLa cells stably expressing βAR-GFP were grown onto coverslips, incubated with 1 mg/ml rhodamine-labeled dextran for 24 h, and then washed in media without dextran for 1.5 h. During the latter portion of this incubation period, the cells were incubated with 10 μM isoproterenol or salmeterol for the times indicated above each photograph. Cells were fixed and imaged as described in the legend to Fig. 4.
13. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992) *Gene (Amst.)* **111**, 229–233

14. Kain, S. R., Adams, M., Kondepudi, A., Yang, T.-T., Ward, W. W., and Kitts, P. (1995) *BioTechniques* **19**, 650–654

15. Inouye, S., and Tsuji, F. I. (1994) *FEBS Lett.* **341**, 277–280

16. Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T., and Caron, M. G. (1997) *Mol. Pharmacol.* **51**, 177–184

17. Forsayeth, J. R., and Garcia, P. D. (1994) *BioTechniques* **17**, 354–358

18. Penn, R. B., Kelsen, S. G., and Benovic, J. L. (1994) *Am. J. Respir. Cell Mol. Biol.* **11**, 496–505

19. Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2979–2983

20. Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., and Lefkowitz, R. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5089–5093

21. von Zastrow, M., and Kohlka, B. K. (1994) *J. Biol. Chem.* **269**, 18448–18452

22. Linden, A., Rabe, K. G., and Lofdahl, C.-G. (1996) *Lung* **174**, 1–22

23. Clark, R. B., Allal, C., Friedman, J., Johnson, M., and Barber, R. (1996) *Mol. Pharmacol.* **48**, 182–189

24. Green, S. A., Spadea, A. P., Coleman, R. A., Johnson, M., and Liggett, S. B. (1996) *J. Biol. Chem.* **271**, 24029–24035

25. Schmid, S. L., Fuchs, R., Male, P., and Mellman, I. (1988) *Cell* **52**, 73–83

26. Ohkuma, S., and Poole, B. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3327–3331

27. Petrou, C., Chen, L., and Tashjian, A. H., Jr. (1997) *J. Biol. Chem.* **272**, 2326–2333