Differences in endosomal targeting of human β_1- and β_2-adrenergic receptors following clathrin-mediated endocytosis

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Summary

The β_2-adrenergic receptor (β_2AR) undergoes agonist-mediated endocytosis via clathrin-coated pits by a process dependent on both arrestins and dynamin. Internalization of some G protein-coupled receptors, however, is independent of arrestins and/or dynamin and through other membrane microdomains such as caveolae or lipid rafts. The human β_1AR is less susceptible to agonist-mediated internalization than the β_2-subtype, and its endocytic route, which is unknown, may be different. We have found that (i) co-expression of arrestin-2 or -3 enhanced the internalization of both subtypes whereas co-expression of dominant-negative mutants of arrestin-2 or dynamin impaired their internalization, as did inhibitors of clathrin-mediated endocytosis. (ii) Agonist stimulation increased the phosphorylation of β_2AR but not β_1AR. (iii) In response to agonist, each subtype redistributed from the cell surface to a distinct population of cytoplasmic vesicles; those containing β_1AR were smaller and closer to the plasma membrane whereas those containing β_2AR were larger and more perinuclear. (iv) When subcellular fractions from agonist-treated cells were separated by sucrose density gradient centrifugation, all of the internalized β_2AR appeared in the lighter endosomal-containing fractions whereas some of the internalized β_1AR remained in the denser plasma membrane-containing fractions. (v) Both subtypes recycled with similar kinetics back to the cell surface upon removal of agonist; however, recycling of β_2AR but not β_1AR was inhibited by monensin. Based on these results, we propose that the internalization of β_1AR is both arrestin- and dynamin-dependent and follows the same clathrin-mediated endocytic pathway as β_2AR. But during or after endocytosis, β_1AR and β_2AR are sorted into different endosomal compartments.

Key words: β-Adrenergic receptor, Endocytosis, Recycling, Clathrin, Arrestin

Introduction

In response to agonist stimulation, most G protein-coupled receptors (GPCRs) undergo internalization, a process that serves multiple physiological functions and occurs by several distinct mechanisms (Koenig and Edwardson, 1997; Ferguson, 2001; Tsao et al., 2001). The β_2AR has been the prototype GPCR as our understanding of this process has evolved. Initially, agonist-mediated internalization of β_2AR was inferred from subcellular fractionation studies in which some of the receptors redistributed from the plasma membrane to a lighter density membrane fraction (Perkins et al., 1991). The latter receptors remained accessible to hydrophobic but not hydrophilic ligands such as CGP-12177, which paralleled receptor behavior in whole cell binding assays. Because the subcellular localization of the receptors could not be visualized, internalization was often referred to as sequestration. More recently, the internalization of human β_2AR has been observed by confocal fluorescence microscopy using immunodetection of epitope-tagged β_2AR (von Zastrow and Kobilka, 1992; Moore et al., 1995) or direct visualization of β_2AR-green fluorescent protein (GFP) chimeras (Barak et al., 1997; Kallal et al., 1998). The internalized receptors appeared in intracellular vesicles containing transferrin receptors. As the latter receptors are internalized via the clathrin-coated pit endocytic pathway, β_2AR appeared to utilize the same pathway. This was further established by showing that a dominant-negative mutant of dynamin, a GTPase involved in the pinching off of clathrin-coated vesicles, blocks endocytosis and β_2AR internalization (Zhang et al., 1996).

In addition, β_2AR endocytosis is dependent on arrestins (Ferguson et al., 1996; Goodman et al., 1996) that were first identified as mediators of agonist-initiated desensitization (Krupnick and Benovic, 1998; Lefkowitz et al., 1998). Desensitization involves phosphorylation of β_2AR by protein kinase A and GPCR kinases (GRKs). GRK-mediated phosphorylation targets the receptors for binding of arrestins and uncoupling from G_s (Lohse et al., 1990; Gurevich et al., 1995). Arrestins also bind both clathrin and the AP-2 adaptor complex and thereby recruit the receptors into clathrin-coated pits (Goodman et al., 1996; Laporte et al., 2000). Following endocytosis, the receptors traffic through divergent endosomal pathways that lead to different fates. Some are resensitized by dephosphorylation, and recycled back to the cell surface (Yu et al., 1993; Pippig et al., 1995). Others undergo down-regulation by being targeted to lysosomes where they are

degraded (Kallal et al., 1998; Moore et al., 1999). Sorting of β_2AR between the two pathways involves a specific sequence at the C terminus of the receptor (Cao et al., 1999). Mutating this sequence reduces the recycling and increases the down-regulation of β_2AR.

In contrast to our knowledge of β_2AR, less is known about the internalization of β_1AR. As the human β_1AR is less susceptible to agonist-mediated internalization than β_2AR (Suzuki et al., 1992; Green and Liggett, 1994; Zhou et al., 1995; Shiina et al., 2000), it may use a different endocytic pathway. In this regard, not all GPCRs are internalized through the clathrin-mediated, dynamin- and arrestin-dependent pathway including subtypes of the same receptor family (Vickery and von Zastrow, 1999; Claing et al., 2000). Several GPCRs are internalized through caveolae (de Weerd and Leeb-Lundberg, 1997; Feron et al., 1997). Both β_1AR and β_2AR have been reported to be located in caveolae (Schwencke et al., 1999; Ostrom et al., 2001) but other studies have found only β_2AR to be targeted to caveolae (Rybin et al., 2000). If the two subtypes do reside in different plasma membrane microdomains, they may not use the same endocytic pathway. Moreover, even if both subtypes are endocytosed via clathrin-coated pits, they may be sorted into divergent endosomal pathways (Tsao et al., 2001). Whether divergence occurs before or after endocytosis, it may result in differences in the recycling and down-regulation of the two subtypes. It also may regulate subtype interaction with alternative signaling pathways through scaffolding complexes (Pierce et al., 2000; Ferguson, 2001). Thus, β_2AR couples to both G_s and G_i whereas β_1AR only activates G_s, which allows the two subtypes to mediate different physiological responses in the heart, both normal and pathological (Xiao, 2000; Zhu et al., 2001).

The present study was undertaken to identify the endocytic route of β_1AR and compare it with that of β_2AR using a combination of biochemical and morphological approaches. In the course of demonstrating that both subtypes undergo arrestin- and dynamin-dependent, clathrin-mediated endocytosis, we observed that each is sorted to a different endosomal compartment. We believe our findings will have important implications for the function and regulation of both β-subtypes.

Materials and Methods

Materials

Concanavalin A (ConA), (–)-isoproterenol (ISO), (–)-propranolol, isobutylmethylxanthine, poly-L-lysine, methotraxate and Cy3-conjugated mouse anti-Flag M2 antibody were from Sigma (St Louis, MO), G418 and LipofectAMINE Plus from Life Technologies (Gaithersburg, MD), forskolin and monensin from CalBiochem (La Jolle, CA), cholera toxin B-subunit (CT-B) and anti-CT-B from List Biological Laboratories (Campbell, CA), Texas Red®-conjugated transferrin and ProLong® from Molecular Probes (Eugene, OR), and sulfo-biotin-LC-NHS, BCA protein reagent and SuperSignal from Pierce (Rockford, IL). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and streptavidin were from Zymed (South San Francisco, CA), unlabeled and Alexa Fluor 488-conjugated mouse anti-HA.11 from Covance (Princeton, NJ), rabbit anti-Flag and anti-clathrin heavy chain from Affinity Bioreagents (Golden, CO), and fluorescein-, Cy3- and Cy5-conjugated donkey anti-mouse and anti-rabbit from Jackson ImmunoResearch (West Grove, PA). (–)-[^{125}I]iodocyanopindolol (^{125}ICYP; 2200 Ci/mmol) was from New England Nuclear (Boston, MA), ^{125}I-protein A (9 μCi/μg) from ICN (Costa Mesa, CA), and (–)-[^{3}H]CGP-12177 (45 Ci/mmol) from Amersham (Arlington Heights, IL).

Plasmid construction

The expression vectors Zem228c and Zem229 from E. Mulvihill (Zymogenetics, Seattle, WA) have an inducible metallothionein promoter, and neomycin or dihydrofolate reductase selectable markers, respectively. The vector pEGFP-N1, encoding an enhanced, red-shifted GFP variant, was from Clontech (Palo Alto, CA). The following constructs have been described previously: Zem228c-β_1AR and -β_2AR (Zhou et al., 1995), pcDNA3-arrestin-2 and -3, -dynamin-K44A, -arrestin-2-(319-418) (Krupnick et al., 1997; Gagnon et al., 1998) and -Flag-β_2AR and -β_2AR-GFP (Kallal et al., 1998), the latter generously provided by J. Benovic (Thomas Jefferson University, Philadelphia). Zem229-Arr2 was generated by excising the coding region of the arrestin-2 cDNA with *Not*I and *Apa*I and inserting it into the corresponding sites created in the *Bam*HI cloning site of Zem229. pcDNA3.1-HA-β_1AR with an N-terminal hemagglutinin (HA) epitope, Met-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Gly-Ala-Gly-, was generated by PCR. To generate pE-β_1AR-GFP, β_1AR was amplified by PCR using an N-terminal primer, CCCGGAATTCCGC-AGCTCGGCATGGGCGCGG, and a C-terminal primer, CGCCGG-ATCCTCCACCTTGGATTCCGAGGCGAA. This replaced the stop codon with a *Bam*HI site encoding an extra glutamate and aspartate. The PCR product and pEGFP-N1 were cut with *Eco*RI/*Bam*HI, the two purified fragments ligated, and the ligated product cloned. To avoid sequencing the entire PCR-generated β_1AR portion, a *Bgl*I/*Xho*I region was replaced with an *Eco*RI/*Xho*I region of the original β_1AR. The *Bgl*I and *Eco*RI ends were blunted with Klenow before ligation, eliminating both restriction sites in the final product, which was sequenced through the PCR-generated regions.

Cell culture and transfections

Baby hamster kidney (BHK), clone tk$^-$*ts*13, and human embryo kidney (HEK 293) cells were from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. HEK 293 cells stably expressing HA-β_2AR were a generous gift from R. Clark (University of Texas Medical School, Houston). HEK 293 cells were grown in polylysine-coated culture ware. Cells were transfected with a βAR construct or cotransfected with Zem229-Arr2 using calcium phosphate precipitation (Zhou et al., 1995) or LipofectAMINE Plus and selected for resistance to G418, or to both G418 and methotraxate. Clonal lines stably expressing βAR or co-expressing βAR and arrestin-2 were obtained by limiting dilution of the resistant cultures. For transient transfections, cells 80-90% confluent were transfected with equal amounts of plasmid DNA (usually 0.1 μg of each construct per cm^2 of culture area) using LipofectAMINE Plus according to the manufacturer's instructions. After 4 hours, the medium was replaced and the cells were assayed 48 hours post-transfection. For some experiments, cells were subcultured after 24 hours into 48-well plates or on glass coverslips or dishes (see below).

Internalization and recycling assays

βAR internalization was assayed as described previously (Dunigan et al., 2000; Dunigan et al., 2002). Briefly, cells grown in 24-well plates were incubated at 37°C in 1 ml of DMEM/25 mM Hepes and exposed to 1 μM ISO for increasing times up to 30 minutes. The plates were placed on a bed of ice, rapidly washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS), and incubated in 250 μl of Eagle's minimal essential medium (EMEM)/Hepes containing 30 μg/ml of BSA and 5 nM [^{3}H]CGP-12177 with or without 10 μM propranolol at 4°C for 1 hour. The cells were washed as above, dissolved in 1 M

NaOH and assayed for ^{3}H and protein. For cells in 48-well plates, all volumes were reduced by half. We confirmed that binding reached equilibrium for both BHK-hβ_1 and -hβ_2 cells by varying the time from 5 minutes to 4 hours. Maximum binding occurred in <1 hour and half-maximum binding in 9.6 and 6.6 minutes, respectively. For recycling of internalized βAR back to the cell surface, cells exposed to 1 μM ISO for 15-30 minutes were washed twice with warm DMEM/Hepes, incubated in the same medium at 37°C for increasing times up to 1 hour, and assayed for surface receptors as described above.

Internalization of CT-B was determined as described previously (Fishman, 1982; Orlandi and Fishman, 1998). Briefly, cells in 24-well plates were incubated with or without inhibitors for 30 minutes at 37°C and then with 10 nM CT-B for 30 minutes at 4°C. After the cells were washed with DPBS, some were warmed to 37°C with or without fresh inhibitors for 1 hour. The cells were incubated with anti-CT-B and then with ^{125}I-protein A, each for 1 hour at 4°C followed by three washes with DPBS. Finally, the cells were dissolved in 1 M NaOH and assayed for ^{125}I and protein.

Confocal fluorescence microscopy

A Zeiss LSM 410 or 510 laser scanning confocal microscope with a 63×1.40 NA oil immersion objective was used to examine the cells. Viable cells were grown in 35 mm glass-bottomed dishes (MatTek), and on the microscope stage, were maintained at 37°C in Phenol Red-free medium and exposed to ISO for increasing times. Otherwise, HEK 293 cells were grown on polylysine-coated coverslips, exposed to Texas Red®-conjugated transferrin and ISO as indicated in the figure legends, washed with ice-cold DPBS, fixed with 4% paraformaldehyde in DPBS without calcium and magnesium at room temperature for 10 minutes, and washed three times with DPBS. For antibody staining, the cells were permeabilized with 0.2% Triton X-100 in DPBS for 10 minutes, blocked with Blotto (3% dry milk and 0.05% Triton X-100 in DPBS) for 20 minutes and incubated with primary and then secondary antibodies in Blotto each for 60 minutes with 3×5-minute washes with DPBS after each antibody and a 20 minute block in Blotto between antibodies. Concentrations of antibodies (μg/ml) were: anti-HA, 4; anti-Flag, 1; anti-clathrin, 1; anti-β_1AR C-tail, 7, and secondary, 3. The coverslips were mounted on slides with ProLong. In some experiments, HEK 293 cells co-expressing HA-β_1AR and Flag-β_2AR were stained at 4°C with Cy3-conjugated anti-Flag (10 μg/ml) and Alexa Fluor 488-conjugated anti-HA (2 μg/ml), washed, warmed up to 37°C for 5 minutes and incubated for 15 minutes in the absence and presence of ISO and then fixed. The images were processed using Adobe Photoshop 5.5 or 6.0.

Phosphorylation experiments

Phosphorylation of receptors in BHK-hβ_1 and -hβ_2 cells was done using a modification of published methods (Seibold et al., 2000; Dunigan et al., 2002). Briefly, cells were incubated for 3 hours in phosphate-free medium containing 100 μCi/ml of [^{32}P]orthophosphate, stimulated with 1 μM ISO for up to 15 minutes, washed and lysed in 1 mM Tris-HCl/2 mM EDTA, pH 7.4, containing 1 mM EGTA, 100 nM okadaic acid and protease inhibitors. The lysates were centrifuged at 500 ***g*** for 5 minutes and the postnuclear supernatants containing 90% of the receptors were mixed with $\frac{1}{4}$ volume of 5×RIPA buffer containing phosphatase inhibitors. The samples were rotated for 45 minutes, centrifuged at 14,000 ***g*** for 20 minutes, and the supernatants containing the soluble receptors were immunoprecipitated with anti-βAR antibodies preabsorbed to protein A-agarose (Dunigan et al., 2002). The immunoprecipitates were washed four times with RIPA buffer and eluted with SDS sample buffer. The elutes were resolved by SDS-PAGE and the gels were dried and exposed to a Bio-Rad storage phosphor screen-BI. The screen was scanned and the labeled bands were detected and quantified using a Bio-Rad GS525 Molecular Imager System and Multi-Analysis/PC software.

Subcellular fractionation

Subcellular fractionation by sucrose density gradient centrifugation was done as described previously (Kassis and Sullivan, 1986) with modifications. Cells grown in 75 cm^2 flasks were treated as described above for internalization, washed and incubated at 4°C in EMEM/Hepes containing 0.25 mg/ml of ConA for 30 minutes to increase the resolution of the plasma membranes on the gradients (Waldo et al., 1983). The cells were washed, allowed to swell for 10 minutes in 1 mM Tris-HCl/2 mM EDTA, pH 7.4, then scraped and lysed in 1 ml of the same solution. Portions (400 μl) were layered on top of discontinuous sucrose gradients formed in 0.5×2-inch ultracentrifuge tubes from 1.1, 2.5 and 1.5 ml portions of 15, 30 and 45% sucrose in 10 mM Tris-HCl, pH 7.4. The gradients were centrifuged in a Beckman SW55Ti rotor at 35,000 rpm for 1 hour at 4°C, and fractions were collected from the top of the gradient and assayed for binding.

Other methods

Protein was measured using BCA reagent and a microtiter plate assay. Cells were assayed for cAMP accumulation by radioimmune assay (Fishman, 1982). Membranes were prepared and assayed for adenylyl cyclase activity (Zhou et al., 1995). Cell lysates were assayed for saturation and competition binding with ^{125}ICYP (Dunigan et al., 2000). βARs were detected by western blotting with rabbit antibodies against synthetic peptides corresponding to the C-tails of β_1AR (Ala^{456}-Ser^{475}) and β_2AR (Val^{394}-Leu^{413}) (Dunigan et al., 2002). Cells were biotinylated and biotin-labeled βARs detected by immunoprecipitation and blotting with HRP-streptavidin as described previously (Dunigan et al., 2002). Unless otherwise indicated, all experiments were done with at least two clonal lines, each experiment was repeated at least three times and within an experiment, each data point was done in triplicate. Data were fitted to curves by nonlinear regression analysis and analyzed for statistical significance by a two-tailed *t*-test using Prism 3 (GraphPad Software, San Diego, CA).

Results

Effect of arrestins on internalization of βAR subtypes

When BHK-hβ_1 and -hβ_2 cells stably expressing similar levels of each βAR subtype were exposed to the agonist ISO, the time-dependent internalization of cell surface β_2AR was 2.3-fold greater than that of β_1AR (Fig. 1A). This difference was observed over a wide range of receptor densities (Fig. 1B). The amount of receptor that was internalized was directly proportional to the initial receptor density up to ~1.5 pmol/mg protein of both subtypes, after which saturation appeared to be reached. Similar differences were observed in HEK 293-hβ_1 and -hβ_2 cells although both subtypes were internalized more than in BHK cells (Fig. 1C). It has been reported that β_1AR expressed in HEK 293 cells does not internalize upon agonist stimulation (Shiina et al., 2000) whereas others workers found that 40% is internalized (McLean and Milligan, 2000). The differing observations may reflect variations in cell strains.

To determine the effects of arrestins on agonist-mediated internalization, we used cells stably cotransfected with arrestin-2 and either β_1AR or β_2AR. Arr2-BHK-hβ_1 and -hβ_2 cells exhibited 3.2- and 1.6-fold more internalization (Fig. 1D) and expressed ~50-fold more arrestins (data not shown) than BHK-hβ_1 and -hβ_2 cells, respectively. To compare arrestin-2 and -3, we used transiently cotransfected BHK and HEK 293 cells. In both cell lines, β_1AR was internalized less than β_2AR, and again internalization was greater in HEK 293 than in BHK cells (Fig. 2). Co-expression of either arrestin-2 or -3 in BHK

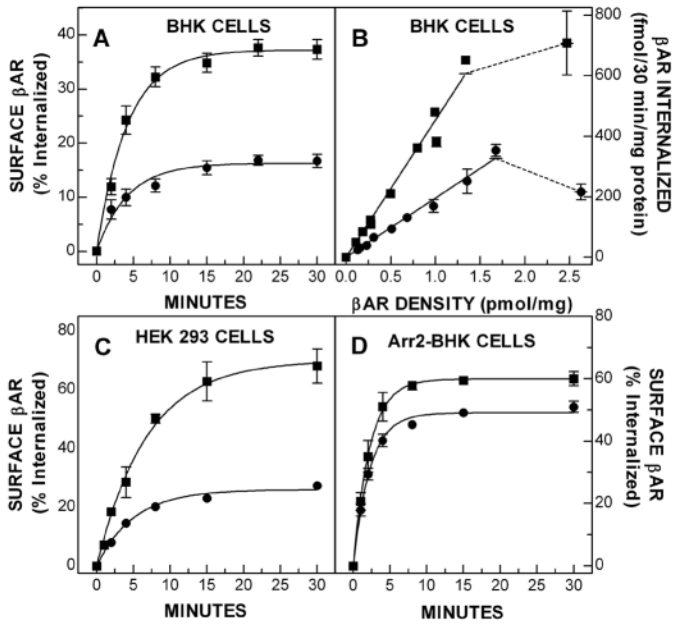

Fig. 1. Comparison of agonist-mediated internalization of receptor subtypes. Cells stably expressing β_1AR (●) or β_2AR (■) were exposed to 1 μM ISO for the indicated times or 30 minutes, washed and assayed for [^{3}H]CGP-12177 binding to surface receptors as described in Materials and Methods. (A) Time course of internalization in BHK-hβ_1 and -hβ_2 cells expressing 978±47 (*n*=6) and 1010±84 (*n*=5) fmol of βAR/mg protein. (B) Effect of βAR density on internalization. Different clonal lines of BHK-hβ_1 and -hβ_2 cells were assayed for the amount of internalization after 30 minutes of agonist treatment. Some were induced with zinc sulfate for 24 hours. Internalization is plotted as a function of initial surface receptor density. The slopes of the linear regression lines are 196 and 454 fmol/30 minutes/pmol, a difference of 2.3-fold. (C) Same as A except for HEK 293-hβ_1 and -hβ_2 cells expressing 1790±42 (*n*=3) and 1580±31 (*n*=4) fmol of βAR/mg protein. (D) Same as A except for Arr2-BHK-hβ_1 and -hβ_2 expressing 1680±204 (*n*=6) and 1310±169 (*n*=5) fmol of βAR/mg protein.

cells increased internalization of β_1AR ~6- to 8-fold and β_2AR ~2-fold. Significant but smaller increases occurred in HEK 293 cells, as internalization of both subtypes was high without arrestin co-expression. These cell-specific differences may be due to BHK cells having no endogenous arrestin-2 and only one-third of the arrestin-3 found in HEK 293 cells, the latter having both in a 2:3 ratio (Santini et al., 2000).

β_1AR internalization is dependent on arrestins, dynamin and clathrin

To further establish a role for arrestins in β_1AR internalization, we transiently cotransfected the cells with each subtype and arrestin-2-(319-418), a dominant-negative mutant containing the C-terminal clathrin-binding domain. Expression of the mutant totally blocked agonist-mediated internalization of β_1AR in BHK cells and significantly reduced it in HEK 293 cells as well as blocking internalization of β_2AR in both cell lines (Fig. 2). Similar results were obtained when dynamin-K44R, a dominant-negative mutant defective in GTP binding, was co-expressed. Thus, the internalization of β_1AR appeared to be dependent on arrestins and dynamin as has been found

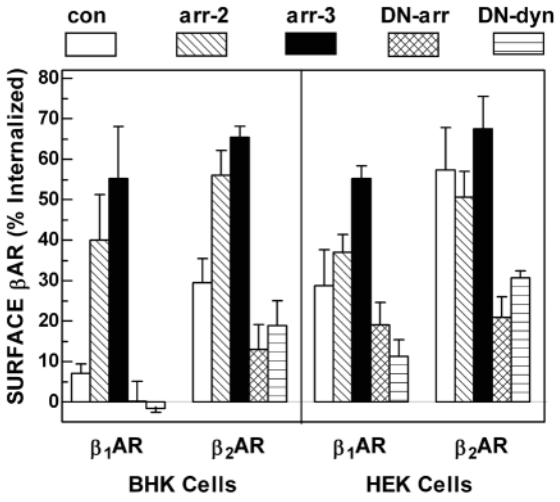

Fig. 2. Effect of arrestins and dominant-negative mutants of arrestin-2 and dynamin on agonist-mediated internalization of β-subtypes. Cells were transiently cotransfected with equal amounts of total plasmid DNA containing Zem228c-β_1AR or -β_2AR, and either pcDNA3 (con), pcDNA3-arrestin-2 (arr-2), -arrestin-3 (arr-3), -arrestin-2-(319-418) (DN-arr), or -dynamin-K44A (DN-dyn). After 48 hours, the cells were incubated for 30 minutes with or without 1 μM ISO and assayed for surface receptors. Results represent the means±s.e.m. of 3-6 experiments for each condition. Although βAR expression levels varied, they were similar for both subtypes at each condition.

for β_2AR. To determine whether it was also dependent on clathrin-coated pits, we treated stably transfected cells with two known blockers of clathrin-mediated endocytosis, ConA and hypertonic sucrose (Yu et al., 1993; Pippig et al., 1995). As ConA may inhibit clathrin-independent endocytosis (Hansen et al., 1993), we also measured the internalization of cholera toxin B-subunit, which enters cells through caveolae (Orlandi and Fishman, 1998). Both treatments inhibited the internalization of both βAR subtypes, but did not block that of cholera toxin B-subunit (Table 1).

Differences in phosphorylation of βAR subtypes

As arrestin binding to β_2AR is facilitated by GRK-catalyzed phosphorylation of the receptor C-tail (Gurevich et al., 1995), we investigated whether β_1AR was less susceptible than β_2AR to phosphorylation. BHK-hβ_1 and -hβ_2 cells, as well as untransfected BHK cells, were labeled with [^{32}P]orthophosphate, stimulated with agonist for 15 minutes, and the receptors were solubilized, immunoprecipitated and analyzed for incorporation of label. No labeled proteins were detected in the immunoprecipitates from untransfected cells (Fig. 3A). Labeled proteins were detected in the transfected cells (Fig. 3A) and identified as receptors by western blotting (not shown). Whereas agonist stimulation increased the phosphorylation of β_2AR 3-fold, it had no effect on the phosphorylation of β_1AR (Fig. 3B). The phosphorylation of β_2AR was rapid, reaching a maximum level in 5 minutes whereas that of β_1AR showed little change (Fig. 3C).

Characterization of β_1AR-GFP

For visualizing the endocytic route used by β_1AR, we

Table 1. Effect of ConA and hypertonic sucrose on internalization of β_1AR and β_2AR and cholera toxin B-subunit in BHK cells

Treatment	% Internalized		
	β_1AR	β_2AR	CT-B
None	24.9±3.5	60.6±2.1	34.6±6.2
0.25 mg/ml Con A	4.85±1.9	14.8±4.75	39.1±5.4*
0.6 M Sucrose	1.92±3.6	31.1±1.6	35.8±5.1*

Cells expressing β_1AR or β_2AR were treated with the indicated inhibitor for 30 minutes, incubated in the absence and presence of 1 μM ISO for an additional 30 minutes and assayed for surface receptors as described in Materials and Methods. In addition, some of the cells were incubated with 10 nM CT-B for 30 minutes at 4°C, washed and either kept at 4°C or warmed in medium containing fresh inhibitor for 1 hour, and assayed for surface CT-B. Results shown represent the mean±s.e.m. of three to five independent experiments. *Not significant compared with no inhibitor.

constructed a β_1AR-GFP chimera in which GFP was fused to the C terminus of the receptor, transfected BHK cells with the construct and isolated clonal cell lines stably expressing β_1AR-GFP. We analyzed several different clonal lines and found the pharmacological and functional properties of β_1AR-GFP were very similar to those of wild-type β_1AR (Fig. 4). Both had a similar affinity for the radioligand ^{125}ICYP and the β_1-selective antagonist CGP-20712A, whereas β_1AR-GFP had a slightly lower affinity for the agonist ISO (Fig. 4C). β_1AR-GFP was functional as it mediated agonist-stimulated cAMP formation in intact cells (Fig. 4D) with a similar EC_{50} value but a one-third lower V_{max} value compared with wild-type β_1AR. When cell membranes were assayed for adenylyl cyclase activity, the respective EC_{50} and V_{max} values were not significantly different (data not shown). Furthermore, β_1AR-GFP underwent agonist-mediated internalization but not as effectively as β_2AR-GFP (30% versus 78%). Thus both GFP-tagged subtypes were internalized ~2-fold more than their wild-type counterparts (compare with Fig. 1A) without any change in the rate ($t_{1/2}$≈3 minutes for all). Possibly, the increased size of the C terminus makes the receptors more susceptible to agonist-mediated internalization. Our results differ from another study using stably transfected HEK 293 cells in which each βAR-GFP internalizes more slowly than its wild-type counterpart but to the same extent (McLean and Milligan, 2000).

Western blotting was used to determine whether all the receptors in cells expressing β_1AR-GFP contained the GFP tag and retained it during agonist-mediated internalization. Wild-type β_1AR appeared as a doublet of 57 and 74 kDa proteins that increased to 86 and 97 kDa for β_1AR-GFP (not shown). The increases are consistent with the 28 kDa mass of GFP. The receptors from control and agonist-treated cells appeared the same and there was no evidence of wild-type receptors being expressed or generated in cells transfected with GFP-tagged receptors.

Visualization of β_1AR and β_2AR internalization

Having confirmed that β_1AR-GFP was similar to wild-type β_1AR, we used confocal fluorescence microscopy to visually follow the internalization of the receptor upon agonist stimulation. In unstimulated BHK cells, β_1AR-GFP predominantly appeared on the cell surface with some intracellular fluorescence localized mostly in small vesicles around the perinuclear region (Fig. 5, top row). When the cells were exposed to ISO, there was a modest decrease in surface fluorescence and small punctate structures appeared in the cytoplasm. Even after 20 minutes, however, β_1AR-GFP mostly remained at the cell surface. In unstimulated cells expressing β_2AR-GFP, there was more intracellular fluorescence than in cells expressing β_1AR-GFP (Fig. 5, second row). Upon agonist stimulation, there was a rapid and extensive redistribution of cell surface β_2AR-GFP to large perinuclear structures, and by 20 minutes, only a faint surface fluorescence remained. When we co-expressed arrestin-2 (Fig. 5, third row) or arrestin-3 (Fig. 5, bottom row) in the cells, the agonist-mediated redistribution of β_1AR-GFP was greatly enhanced and by 20 minutes very little surface fluorescence was visible. The internalized β_1AR-GFP, however, appeared in smaller, more peripheral structures than β_2AR-GFP. Co-expression of arrestins had little effect on β_2AR-GFP

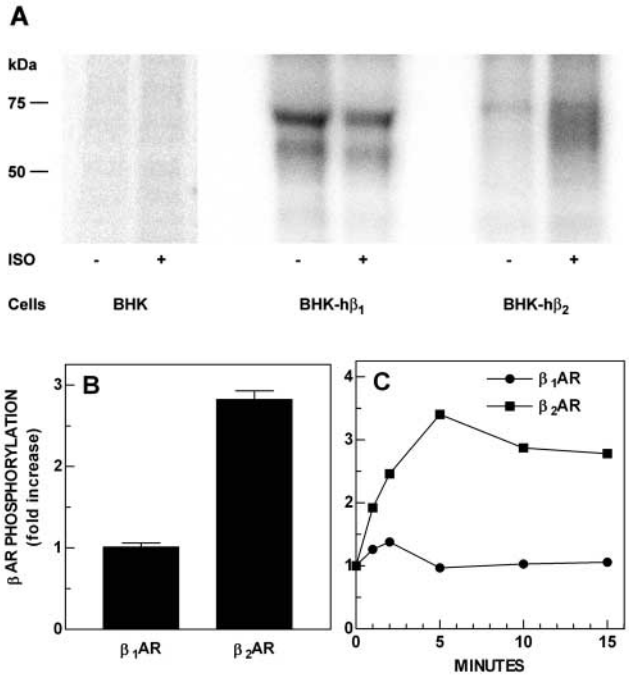

Fig. 3. Differences in agonist-stimulated phosphorylation of β_1AR and β_2AR in BHK cells. Untransfected cells and cells stably expressing β_1AR or β_2AR were incubated for 3 hours with [^{32}P]orthophosphate, incubated with or without 1 μM ISO for 15 minutes or the times indicated, washed and lysed. After solubilization, receptors were immunoprecipitated with antibodies to β_1AR (BHK and BHK-hβ_1 cells) or β_2AR (BHK-hβ_2 cells) and separated by SDS-PAGE. Receptor phosphorylation was detected on the dried gels and quantified by phosphor imaging as described in Materials and Methods. (A) A representative grayscale image of ^{32}P-labeled receptors from control and ISO-treated cells. Immunoprecipitates of equal amounts of protein (0.5 mg), β_1AR (0.6 pmol) or β_2AR (0.45 pmol) were loaded on the gel. (B) Summary of the quantification of receptor phosphorylation. Results are expressed as fold stimulation by agonist and are the means±s.e.m. of three separate experiments. (C) Time course of agonist-stimulated phosphorylation of β_1AR (●) and β_2AR (■) in BHK cells. Results shown are from a single experiment.

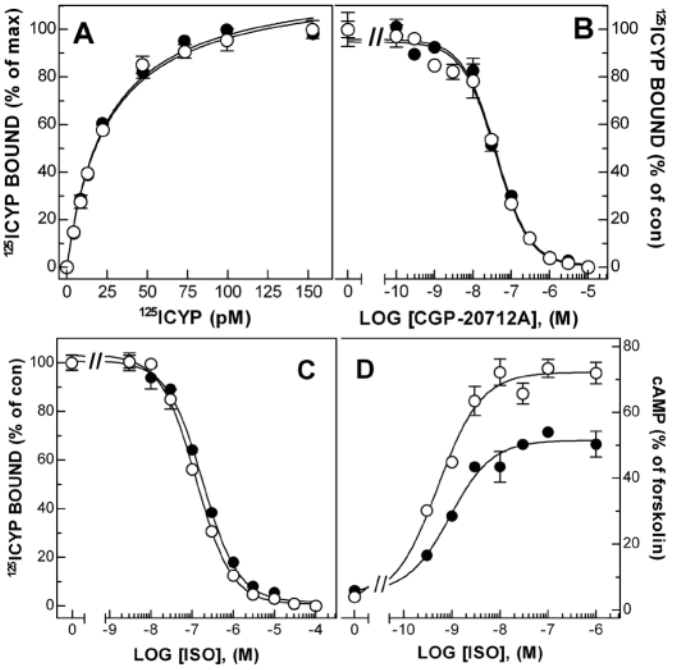

Fig. 4. Pharmacological and functional properties of wild-type β_1AR and β_1AR-GFP. BHK cells stably expressing β_1AR (○) and β_1AR-GFP (●) at ~0.7-1 pmol/mg protein were assayed for binding or cAMP accumulation as described in Materials and Methods. (A) Saturation binding of radioligand. Cell lysates were incubated for 3 hours at 30°C with increasing concentrations of ^{125}ICYP with or without 10 μM propranolol. K_d values for ICYP were 18.2±3.0 and 20.4±1.9 pM (P>0.05). (B) Antagonist competition binding. Same as A except ~30 pM ^{125}ICYP and increasing concentrations of CGP-20712A were used. K_i values for CGP-2012A were 9.7±3.9 and 14.1±3.0 nM (P>0.05). (C) Agonist competition binding. Same as B except ISO plus 100 μM GTP were used. K_d values for ISO were 57.0±1.4 and 87.6±3.4 nM (P<0.002). (D) Cells were incubated with increasing concentrations of ISO or 100 μM forskolin for 10 minutes and assayed for cAMP. EC_{50} values for ISO were 0.80±0.1 and 1.26±0.3 nM (P>0.05). V_{max} values for ISO as a percentage of forskolin stimulation were 74.7±1.3 and 50.0±3.6% (P<0.002). A representative experiment is shown in each panel. Values are the means±s.e.m. of 3 or 4 experiments.

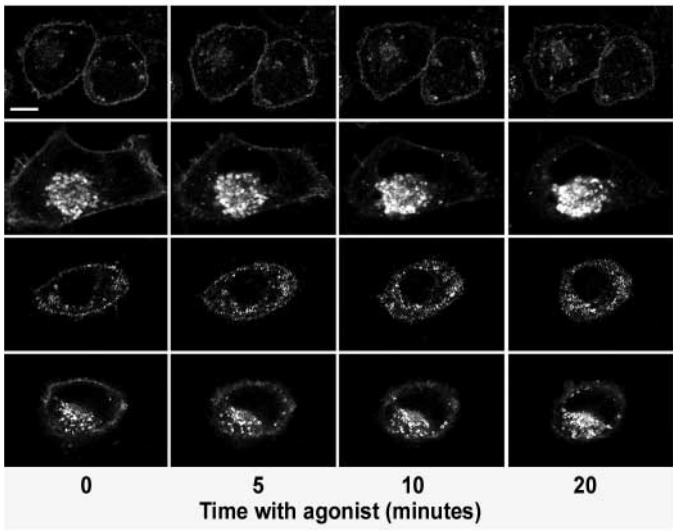

Fig. 5. Visualization of agonist-mediated internalization of β_1AR- and β_2AR-GFP. BHK cells stably expressing β_1AR-GFP (top row) or β_2AR-GFP (second row) or β_1AR-GFP and transiently co-expressing arrestin-2 (third row) or arrestin-3 (bottom row) were observed by confocal fluorescence microscopy during stimulation with 1 μM ISO for 0, 5, 10 and 20 minutes at 37°C as described in Materials and Methods. Whereas agonist treatment resulted in the redistribution of most of β_2AR-GFP from plasma membrane to large perinuclear vesicles, there was little redistribution of β_1AR-GFP until arrestin-2 or -3 was co-expressed. Under these conditions, most of β_1AR-GFP appeared in small, peripheral vesicles that were distinct from those containing β_2AR-GFP. Bar, 10 μm.

redistribution (data not shown). Thus, the qualitative fluorescence results were consistent with the quantitative binding data shown in Figs 1 and 2.

To further identify the β_1AR endocytic pathway, HEK 293 cells were transiently transfected with β_1AR-GFP alone or with arrestin-2. The cells were exposed to Texas-Red®-labeled transferrin, an established marker for clathrin-mediated endocytosis, incubated with or without agonist, fixed and processed for immunofluorescence (Fig. 6A-I). In unstimulated cells, β_1AR-GFP (green in Fig. 6) was mainly on the surface, transferrin (red) was mostly in endosomes and colocalization of the two (yellow) was minimal (Fig. 6A-C). In agonist-treated cells, localization of transferrin with β_1AR-GFP in endosomes was enhanced (Fig. 6D-F). Co-expression of arrestin-2 increased both the internalization of β_1AR-GFP (Fig. 6G-I) and the extent of its localization with transferrin (Fig. 6F compared with 6I). As was observed in BHK cells, β_1AR-GFP appeared in small vesicles and when we stained similar cells with an anti-clathrin antibody (Fig. 6J-R), we were able to observe considerable colocalization (yellow) of β_1AR-GFP (green) and clathrin (red) in control cells (Fig. 6J-L) and ISO-treated cells without (Fig. 6M-O) and with (Fig. 6P-Q) arrestin-2.

In order to exclude the possibility that β_1AR-GFP may traffic differently than wild-type β_1AR, HEK 293 cells were cotransfected with HA-β_1AR, Flag-β_2AR and arrestin-2 (to enhance β_1AR internalization), incubated for 15 minutes with and without agonist, fixed, permeabilized and stained with anti-HA and anti-Flag antibodies and fluorescent second antibodies (Fig. 7A-F). We also incubated the cells at 4°C with fluorescent anti-HA and anti-Flag antibodies, warmed them up for 15 minutes with and without agonist, and then fixed them (Fig. 7G-L). In unstimulated cells, both HA-β_1AR and Flag-β_2AR were localized to the plasma membrane with only modest colocalization (Fig. 7C,I). In agonist-stimulated cells, HA-β_1AR was redistributed to small endosomes as had been observed for β_1AR-GFP (Fig. 7D,J). In contrast, Flag-β_2AR in the same cells was redistributed to larger endosomes (Fig. 7E,H) and had little localization with HA-β_1AR (Fig. 7F,L). Similar results were obtained with HEK 293 cells stably expressing HA-β_2AR and co-transfected with β_1AR and arrestin-2, stained with anti-HA and anti-β_1AR C-tail antibodies and fluorescent second antibodies (Fig. 7M-Q). Even though both β_1AR (green) and HA-β_2AR (red) were cleared from the plasma membrane by agonist stimulation, colocalization (yellow) was not extensive (Fig. 7P-R).

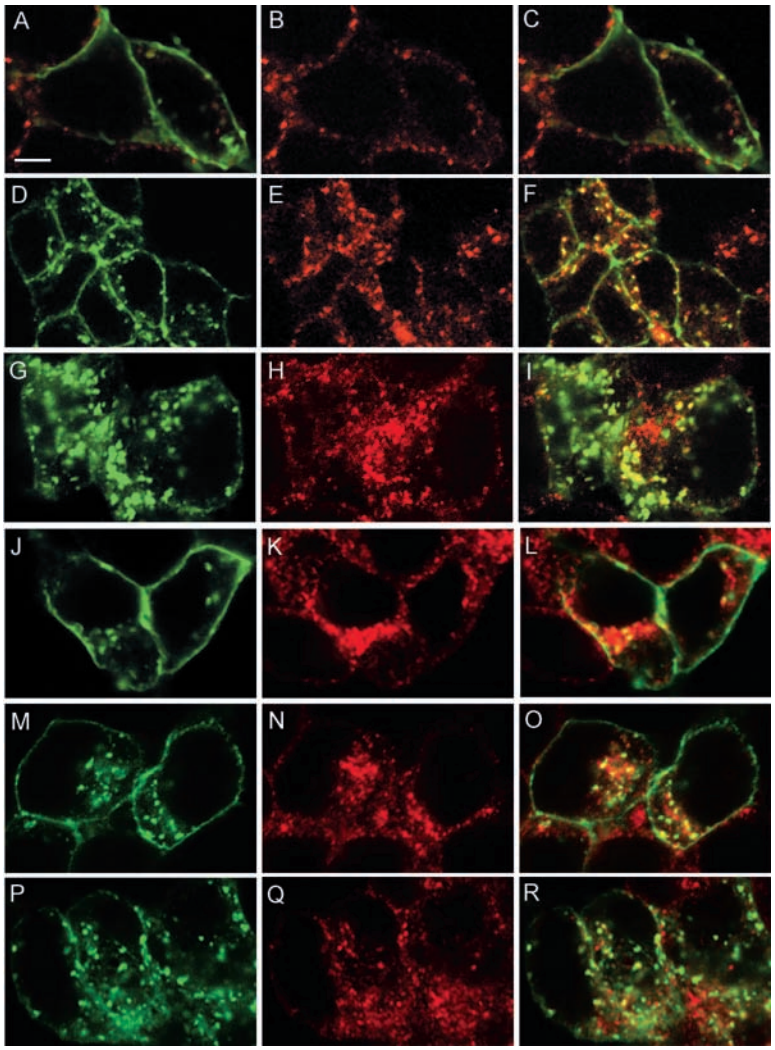

Fig. 6. Colocalization of β_1AR-GFP with transferrin and with clathrin. (A-I) HEK cells transiently transfected with β_1AR-GFP without (D-F) or with (A-C,G-I) arrestin-2 were incubated at 4°C with Texas Red®-labeled transferrin (20 μg/ml) for 30 minutes and with 1 μM ISO at 37°C for another 20 minutes (D-I). The cells were washed and fixed as described in Materials and Methods. The distribution of β_1AR-GFP (green) and transferrin (red) was examined using a confocal microscope. Colocalization of transferrin with β_1AR-GFP (yellow) can be observed in the merged images. (J-R) Same as A-I except transferrin was omitted and the cells were permeabilized and stained for clathrin (red) with rabbit anti-clathrin followed by Cy5-conjugated anti-rabbit (color changed from blue). Colocalization of clathrin with β_1AR-GFP (yellow) can be observed in the merged images. Scale bar: 5 μm.

β-Subtypes differ in agonist-mediated redistribution to subcellular fractions

The redistribution of receptors from plasma membranes to endosomes in response to agonist can also be determined by using sucrose density gradient centrifugation to separate subcellular fractions (Waldo et al., 1983; Kassis and Sullivan, 1986; Marullo et al., 1999). Initially, we used linear 15-40% sucrose gradients but were unable to resolve plasma membranes from endosomes. We then tried discontinuous gradients (Kassis and Sullivan, 1986) and readily observed that β_2AR from agonist-treated BHK cells appeared in both dense and light gradient fractions, but were unable to detect a similar distribution of β_1AR (data not shown). This may be because of the limited internalization of β_1AR in BHK cells (Fig. 1A). Therefore, we turned to Arr2-BHK-hβ_1 and -hβ_2 cells, in which agonist-mediated internalization is >50% for both subtypes (Fig. 1D). In fractionated control cells assayed for ^{125}ICYP binding, the bulk of each subtype appeared in the dense gradient fractions with small amounts in the lighter fractions near the top of the gradients (Fig. 8A,B). When the fractions were assayed for [^{3}H]CGP-12177 binding, the hydrophilic radioligand bound to the receptors in the dense but not in the light fractions. When fractions from agonist-treated cells were similarly assayed, differences were observed in the distribution of β_1AR and β_2AR (Fig. 8C,D). As expected, β_2AR was about equally distributed between the plasma membrane and endosomal fractions; in contrast, more β_1AR remained in the plasma membrane fractions and the distribution was ~2:1. In addition, most of the β_2AR (88.3±3.3%, n=3) remaining in the plasma membrane fractions was detected by [^{3}H]CGP-12177 binding, whereas only 63.7±4.2% (n=4) of the β_1AR was detected.

As the procedure separates subcellular organelles mainly by their density, it appeared that some of the internalized β_1AR was in structures that sediment with the plasma membranes. To rule out the possibility that some β_1AR had not undergone endocytosis, but was still on the plasma membrane and only inaccessible to the hydrophilic radioligand, we used a biotinylation procedure that differentiates between cell surface and internal proteins. Briefly, control and agonist-treated cells were labeled at 4°C with a non-permeable biotin derivative that reacts with amino groups, then lysed and extracted with detergent. βARs in the soluble extracts were immunoprecipitated with anti-βAR antibodies, separated by SDS-PAGE and detected by blotting with HRP-streptavidin (Fig. 9A). The biotinylation of both β_1AR and β_2AR was reduced in the agonist-treated cells and the decreases were essentially the same as the decreases in [^{3}H]CGP-12177 binding (Fig. 9B). By contrast in cells first labeled with biotin and then exposed to agonist, the levels of biotin-labeled receptors were similar to those of control cells. This is consistent with the loss of surface receptors by internalization and not by down-regulation. As we assayed the cells for ^{125}ICYP binding in the same experiments, we confirmed there was little if any down-regulation during the initial 15-30 minutes of agonist treatment (mean ± s.e.m. for β_1AR, 96.4±1.8 and for β_2AR, 97.1±2.1% of control; n=6-8).

Recycling of β_1AR and β_2AR differ in sensitivity to monensin

As the two subtypes appeared to be sorted to different endosomal compartments, we determined whether they also differed in their ability to be recycled. We exposed BHK-hβ_1 and -hβ_2 cells to ISO for 30 minutes to induce maximum

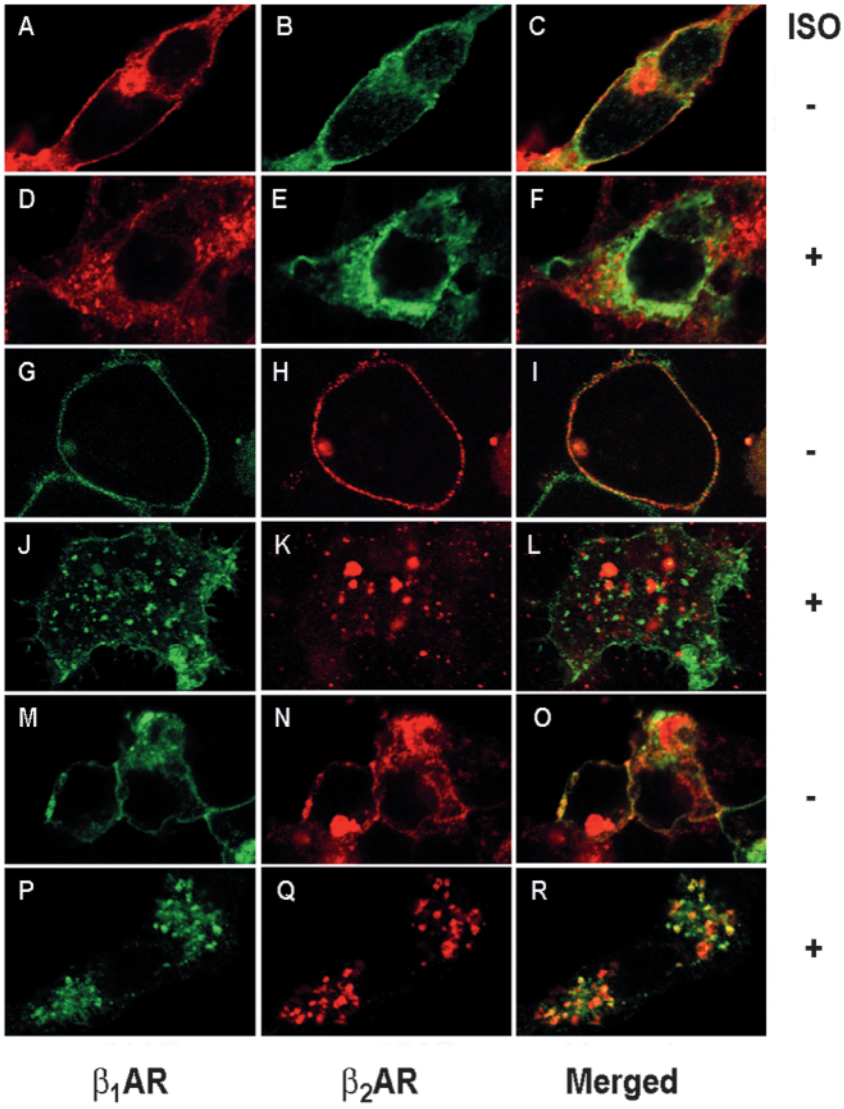

Fig. 7. Agonist-mediated endocytosis of β_1AR and β_2AR co-expressed in HEK 293 cells. Three different approaches were used to show the distribution of β_1AR (left panels) and β_2AR (middle panels) and any colocalization (right panels) in control cells (–ISO) and cells stimulated with 1 μM ISO for 15 minutes (+ISO). (A-F) Cells stably expressing HA-β_1AR were transiently transfected with Flag-β_2AR and arrestin-2, incubated with and without agonist, washed and fixed. The cells were then processed for immunofluorescence as described in Materials and Methods. HA-β_1AR (red) and Flag-β_2AR (green) are shown separately and as merged images to show colocalization (yellow). (G-L) The same as described above except the cells were incubated at 4°C with fluorescent anti-HA and anti-Flag, washed, warmed up for 5 minutes, incubated with or without agonist and fixed. HA-β_1AR (green) and Flag-β_2AR (red) are shown separately and as merged images to show colocalization (yellow). (M-R) Cells stably expressing HA-β_2AR were transiently transfected with β_1AR and arrestin-2, incubated with or without agonist, washed, fixed and processed for immunofluorescence. β_1AR (green) and HA-β_2AR (red) are shown separately and as merged images to show colocalization (yellow).

internalization, washed out the agonist and allowed recycling to occur for increasing times (Fig. 10A). While both subtypes recycled back to the cell surface, the extent of β_1AR recycling was very small because of its limited ability to be internalized. To increase internalization, recycling was measured in Arr2-BHK-hβ_1 and -hβ_2 cells and both subtypes were found to recycle with similar kinetics (Fig. 10B). The respective $t_{\frac{1}{2}}$ values (means ± s.e.m., n=3-5) were 13.8±1.4 and 13.7±1.3 minutes. Similar results were obtained with HEK 293-hβ_1 and -hβ_2 cells (Fig. 10C); the $t_{\frac{1}{2}}$ values were 10.8±1.2 and 10.7±1.25 minutes. For both subtypes, a greater proportion of the internalized receptors was recycled in HEK 293 than in Arr2-BHK cells. In the presence of monensin, an inhibitor of recycling (Pippig et al., 1995), β_2AR recycling was substantially reduced in both cell lines whereas monensin had little if any effect on β_1AR recycling (Fig. 10C,D). Although we initially used 100 μM monensin (Pippig et al., 1995), we found that 10 μM was as effective at inhibiting β_2AR recycling.

Discussion

We present evidence for two major conclusions. The first is that the internalization of the human β_1AR is dynamin- and arrestin-dependent and via the clathrin-mediated endocytic pathway. We showed that in both BHK and HEK 293 cells, co-expression of arrestin-2 or -3 enhanced β_1AR internalization to equal that of β_2AR whereas co-expression of dominant-negative mutants of arrestin-2 or dynamin inhibited β_1AR internalization. Arrestins act as adaptor proteins between β_2AR and the clathrin AP-2 adaptor complex and thus promote receptor endocytosis via clathrin-coated pits (Goodman et al., 1996; Laporte et al., 2000). Our results indicate that arrestins function in the same way for β_1AR. Dynamin-dependent endocytosis was initially associated with clathrin-coated pits (Zhang et al., 1996) but dynamin also is involved in caveolae-mediated uptake (Henley et al., 1998; Oh et al., 1998). We found, however, that two known blockers of clathrin-mediated endocytosis inhibited β_1AR internalization. β_2AR, which undergoes clathrin-mediated endocytosis, and CT-B, which is internalized through caveolae, served as positive and negative controls, further reinforcing our conclusion. Caveolae were of particular interest as both subtypes are localized to these membrane microdomains when expressed in COS cells (Schwencke et al., 1999) and BHK and HEK 293 cells (Shor et al., 2001). Although the latter cells are reported to lack caveolin, they contain caveolae-like structures to which heterologously expressed β_1AR and β_2AR are targeted (Rybin et al., 2000). Others have reported the presence of caveolins in HEK 293 cells (Roseberry and Hosey, 2001; Shor et al., 2001).

To directly visualize endocytosis, we constructed a β_1AR-GFP that had properties similar to wild-type β_1AR. We showed colocalization of the chimera with clathrin and with transferrin. Although we now have established that β_1AR uses the same

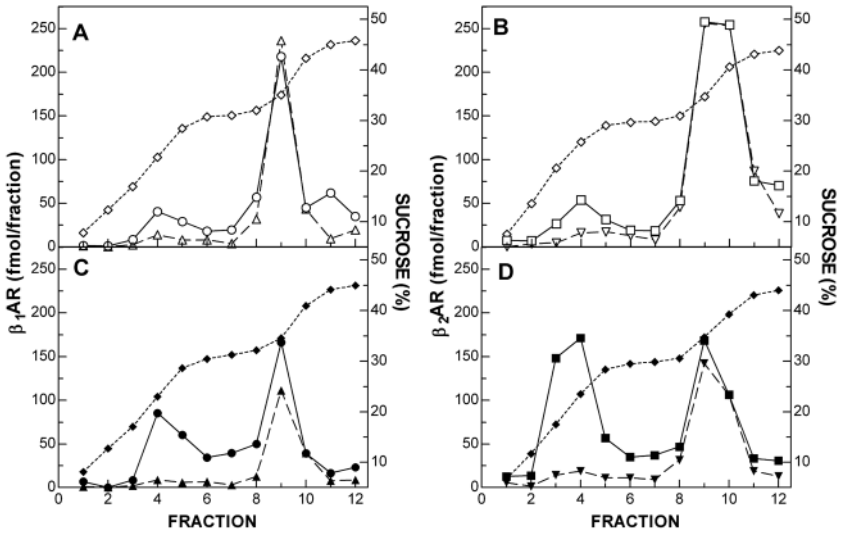

Fig. 8. Subcellular fractionation of control and agonist-treated cells by sucrose density gradient centrifugation. Arr2-BHK-hβ_1 (A,C) and -hβ_2 (B,D) cells were induced with zinc sulfate for 24 hours. Lysates prepared from control cells (open symbols) and cell treated with ISO for 15 minutes (closed symbols) were fractionated by sucrose density gradient centrifugation and the fractions assayed for binding with ^{125}ICYP (○,□,●,■) and [^{3}H]CGP-12177 (△,▽,▲,▼) along with the sucrose concentration (◇,◆) as described in Materials and Methods. Distributions of total receptors between plasma membrane and endosomal fractions were for β_1AR: control, 82.7±0.6 and 17.3±0.6%; ISO-treated, 63.1±3.6 and 36.9±3.6% (n=4); and for β_2AR: control, 87.8±2.4 and 12.2±2.4%; ISO-treated, 49.0±1.1 and 51±1.1% (n=3).

endocytic pathway as β_2AR, this could not be deduced a priori. In fact, given the well-documented quantitative differences in their internalization, it was equally possible that each subtype used a different pathway. Also, considering the many studies comparing the internalization of the subtypes, it is somewhat unexpected that the endocytic route of β_1AR had not been previously identified.

Although quantitative differences in agonist-mediated internalization of β_1AR and β_2AR are well documented both here (Fig.1) and elsewhere (Suzuki et al., 1992; Green and Liggett, 1994; Zhou et al., 1995; Shiina et al., 2000), the underlying basis has not been completely identified. We showed that the endocytosis of β_1AR was increased to that of β_2AR by co-expressing arrestins. Our results differ from those of other workers who found that arrestin-2 or -3 co-expression in HEK 293 cells has little effect on β_1AR internalization (Shiina et al., 2000). They also found that arrestins have a lower affinity for β_1AR than β_2AR, that arrestins recognize the receptor C-tails and third intracellular loops, and that using chimeras, these two regions have the most influence on internalization. We obtained similar results when we expressed the same chimeras in BHK cells (unpublished observations). It had been shown that deletion of a proline-rich region in the third intracellular loop of β_1AR increases its internalization (Green and Liggett, 1994), and that GRK-catalyzed phosphorylation of the β_2AR C-tail promotes binding of arrestins (Gurevich et al., 1995). Arrestins also bind to non-phosphorylated β_2AR with lower affinity (Gurevich et al., 1995) and rescue the internalization of β_2AR mutants in which the potential GRK phosphorylation sites are removed by substitution or truncation (Ferguson et al., 1996). Thus differences between β_1AR and β_2AR as substrates for GRK-mediated phosphorylation may explain their dissimilar capacities for arrestin-dependent endocytosis. In support of this concept, agonist-stimulated phosphorylation of β_1AR is much less than that of β_2AR in HEK 293 cells (Shiina et al., 2001) and we found that in BHK cells, agonist stimulation increased the phosphorylation of β_2AR by 3-fold, but had little effect on the phosphorylation of β_1AR.

Our other major finding is that although both subtypes use the same pathway of endocytosis, they are sorted to different

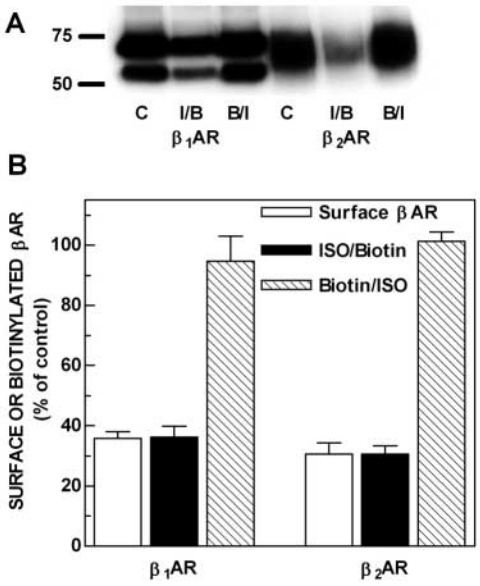

Fig. 9. Detection of agonist-mediated internalization of β-subtypes by cell surface biotinylation. Arr2-BHK-hβ_1 and -hβ_2 cells were induced with zinc sulfate for 24 hours, incubated with or without 1 μM ISO for 15 minutes, and subjected to cell surface biotinylation for 30 minutes at 4°C, or biotinylated first and then exposed to agonist. The cells were lysed, extracted with detergent and clarified by centrifugation. The soluble receptors were immunoprecipitated with antibodies to β_1AR or β_2AR C-tail preabsorbed to protein A-agarose. The bound receptors were subjected to SDS-PAGE and blotting with HRP-streptavidin followed by chemiluminescence detection and densitometry. In addition, portions of the lysates were assayed for ^{125}ICYP binding, and separately, intact cells were assayed for [^{3}H]CGP-12177 binding, to quantify the amounts of total and internalized receptors. (A) Blots of biotin-labeled β_1AR (left) and β_2AR (right) from control cells **(**C**)**, cells exposed to ISO and biotinylated **(**I/B**)** and cells biotinylated and treated with ISO **(**B/I**)**. Equal amounts of receptor (10 fmol of total βAR) were loaded on the gel. (B) Summary of the quantification of cell surface receptor internalization by [^{3}H]CGP-12177 binding and biotinylation by densitometry). Values are expressed as percentage of control and represent the means±s.e.m. of three separate experiments, each assayed in triplicate.

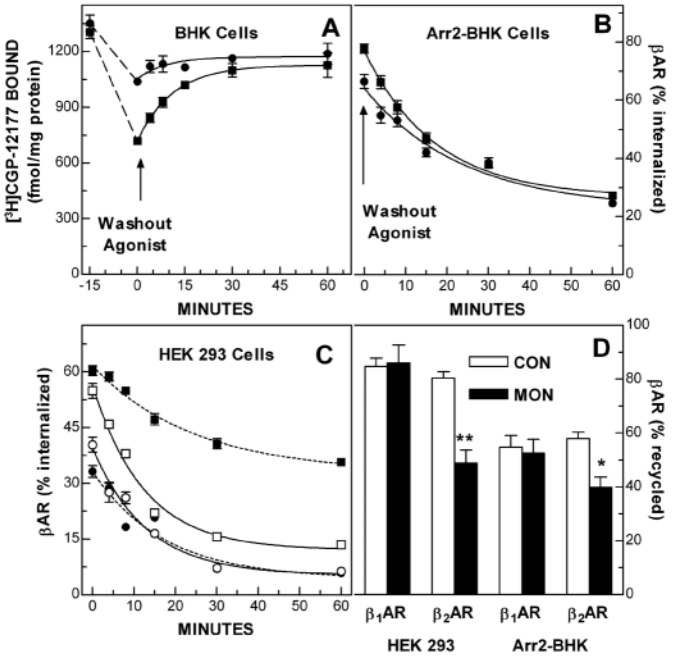

Fig. 10. β_1AR and β_2AR recycle with similar kinetics but differ in sensitivity to monensin. Cells stably expressing β_1AR and β_2AR were treated with 1 μM ISO for 15-30 minutes to induce maximum internalization, washed to remove the agonist and allowed to recycle for the indicated times. The cells were then assayed for surface receptors as described in Materials and Methods. Recycling of β_1AR (●) and β_2AR (■) in (A) BHK cells and (B) Arr2-BHK cells. (C) Recycling of β_1AR (○,●) and β_2AR (□,■) in control and monensin-treated HEK 293 cells. Cells were treated with 100 μM monensin (closed symbols) for 30 minutes before adding agonist, and monensin was present during the recycling period. (D) Summary of βAR recycling in control and monensin-treated cells. Values are expressed as the percentage recycling of internalized receptors, and represent the means±s.e.m. of 3-5 independent experiments. Differences between control and monensin-treated β_1AR-expressing cells were not significant. *P<0.01; **P<0.001.

endosomal compartments. The following supports this conclusion. First, using confocal fluorescence microscopy, we observed that in response to agonist stimulation, β_1AR appeared in small cytoplasmic vesicles close to the cell membrane whereas β_2AR accumulated in large perinuclear vesicles. It is unlikely that the quantitative differences in subtype internalization account for the qualitative difference in trafficking as co-expression of arrestin-2 increased β_1AR endocytosis without affecting its trafficking. When both subtypes were co-expressed in HEK 293 cells, the differences in subcellular distribution were very obvious as was the limited colocalization. The latter indicates that there is some sharing of endosomal compartments by the two subtypes. Probably sharing occurs early after endocytosis as colocalization of each subtype and transferrin is observed. Second, the cells were subjected to subcellular fractionation by sucrose density gradient centrifugation, which separated more dense plasma membranes from less dense endosomes. Whereas β_2AR redistributed from the plasma membrane fractions to the endosomal fractions in proportion to the amount of agonist-mediated internalization that it underwent, β_1AR redistribution was not proportional. Some of the internalized β_1AR remained in the plasma membrane-containing fractions, but inaccessible to the hydrophilic [^{3}H]CGP-12177. As this pool of β_1AR was not labeled by surface biotinylation of intact cells, it most probably represents internalized receptors in vesicles that co-sediment with the plasma membranes. In some ways, these vesicles resemble the peripheral endosomes in which β_2AR accumulates when internalization is done at low temperatures (Marullo et al., 1999). The latter are smaller and denser than the perinuclear endosomes to which β_2AR is targeted at 37°C.

Finally, when we compared the recycling of the two subtypes, we found that recycling of β_2AR was monensin-sensitive whereas recycling of β_1AR was not. Although monensin acts by inhibiting endosomal acidification, it blocks recycling but not dephosphorylation of β_2AR (Pippig et al., 1995). This is somewhat unexpected as during the recycling process, β_2AR is dephosphorylated by a specific endosomal-associated phosphatase and neutralization of the endosomes with NH_4Cl blocks the dephosphorylation (Krueger et al., 1997). Thus, monensin must be acting at a later step in the recycling pathway. As to the effect of endosomal pH on the dephosphorylation of cytoplasmic domains of the receptor, it has been proposed that endosomal acidification induces a conformational change in the receptor that facilitates the interaction of the phosphatase with the cytoplasmic domains (Krueger et al., 1997).

One possible explanation for the dissimilar effects of monensin on recycling by the subtypes is that they traffic to different populations of recycling endosomes that vary in sensitivity to monensin. Although there is evidence for more than one recycling pathway, they have different kinetics. Many GPCRs including β_2AR are recycled with rapid kinetics whereas others, typified by the vasopressin V2 receptor, are recycled very slowly via a distinct 'long' pathway (Innamorati et al., 2001). Given the almost identical $t_{\frac{1}{2}}$ values for recycling of β_1AR and β_2AR in two different cell lines, it is unlikely that β_1AR is taking the 'long' pathway. A second possibility that is consistent with our other findings is that after endocytosis, β_1AR, not requiring dephosphorylation, is targeted by default to recycling endosomes whereas β_2AR has to traffic through the dephosphorylation compartment before entering the recycling compartment. It is this latter step that may be inhibited by monesin. As is discussed below, recycling of β_2AR requires binding of a specific sorting protein to its C-terminus after the latter has been dephosphorylated. Binding may depend on a pH-induced conformation of the receptor that is blocked by monensin, and results in dissociation of the sorting protein and interruption of receptor recycling.

There is substantial evidence that the endocytosis and subsequent trafficking of GPCRs are determined and controlled by the interactions of cellular 'sorting' proteins with specific cytoplasmic sequences in the receptors (Tsao et al., 2001). The C-terminal four residues (DSLL) of β_2AR represent a binding motif for PDZ domain-containing proteins of the Na^+/H^+-exchanger regulatory factor (NHERF) family, and when the motif is mutated or phosphorylated by GRK-5, receptor trafficking is shifted from recycling to degradation (Cao et al., 1999). β_1AR also terminates in a PDZ-interacting sequence (ESKV) that is recognized by PSD-95 and MAGI-2, which respectively inhibits and enhances receptor

internalization (Hu et al., 2000; Xu et al., 2001). The interaction between PSD-95 and β_1AR is inhibited when the sequence is phosphorylated by GRK-5 (Hu et al., 2002). Both PSD-95 and MAGI-2 are only expressed in highly differentiated cells such as neurons where they function as post-synaptic scaffolding proteins. Finally, mutation of the PDZ motif of mouse β_1AR enhances agonist-mediated endocytosis in mouse cardiac myocytes, and the mutated β_1AR is able to interact with G_i (Xiang et al., 2002).

In summary, we have demonstrated that the agonist-mediated endocytosis of the human β_1AR was through clathrin-coated pits and was both arrestin- and dynamin-dependent. Although the same mechanism is used for endocytosis of the human β_2AR, the two subtypes are sorted to different endosomal compartments. In turn, this compartmentalization may have physiological consequences by providing each subtype with a different milieu for alternate signaling and further trafficking. In cardiac myocytes, each subtype has a different physiological role: β_1AR only couples to G_s, does not activate ERK and is pro-apoptotic, whereas β_2AR couples to both G_s and G_i, activates ERK and is anti-apoptotic (Xiao, 2000; Zhu et al., 2001; Shizukuda and Buttrick, 2002). Finally, β_1AR is more resistant than β_2AR to agonist-mediated down-regulation (Suzuki et al., 1992; Zhou et al., 1995; Dunigan et al., 2002). Thus, each subtype may be in endosomes that differ in their trafficking to lysosomes.

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References

Barak, L. S., Ferguson, S. S. G., Zhang, J., Martenson, C., Meyer, T. and Caron, M. G. (1997). Internal trafficking and surface mobility of a functionally intact β_2-adrenergic receptor-green fluorescent protein conjugate. *Mol. Pharmacol.* **51**, 177-184.

Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999). A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. *Nature* **401**, 286-290.

Claing, A., Perry, S. J., Achiriloaie, M., Walker, J. K., Albanesi, J. P., Lefkowitz, R. J. and Premont, R. T. (2000). Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity. *Proc. Natl. Acad. Sci. USA* **97**, 1119-1124.

de Weerd, W. F. C. and Leeb-Lundberg, L. M. F. (1997). Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Gα subunits Gα_q and Gα_i in caveolae in DDT1 MF-2 smooth muscle cells. *J. Biol. Chem.* **272**, 17858-17866.

Dunigan, C. D., Curran, P. K. and Fishman, P. H. (2000). Detection of β-adrenergic receptors by radioligand binding. *Methods Mol. Biol.* **126**, 329-343.

Dunigan, C. D., Hoang, Q., Curran, P. K. and Fishman, P. H. (2002). Complexity of agonist- and cyclic AMP-mediated downregulation of the human β_1-adrenergic receptor: Role of internalization, degradation, and mRNA destabilization. *Biochemistry* **41**, 8019-8030.

Ferguson, S. S. G. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1-24.

Ferguson, S. S. G., Downey, W. E., III, Colapietro, A.-M., Barak, L. S., Ménard, L. and Caron, M. G. (1996). Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science* **271**, 363-366.

Feron, O., Smith, T. W., Michel, T. and Kelly, R. A. (1997). Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J. Biol. Chem.* **272**, 17744-17748.

Fishman, P. H. (1982). Internalization and degradation of cholera toxin by cultured cells: relationship to toxin action. *J. Cell Biol.* **93**, 860-865.

Gagnon, A. W., Kallal, L. and Benovic, J. L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the β_2-adrenergic receptor. *J. Biol. Chem.* **273**, 6976-6981.

Goodman, O. B., Jr, Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. and Benovic, J. L. (1996). β-Arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. *Nature* **383**, 447-450.

Green, S. A. and Liggett, S. B. (1994). A proline-rich region of the third intracellular loop imparts phenotypic β_1-versus β_2-adrenergic receptor coupling and sequestration. *J. Biol. Chem.* **269**, 26215-26219.

Gurevich, V. V., Dion, S. B., Onorato, J. J., Ptasienski, J., Kim, C. M., Sterne-Marr, R., Hosey, M. M. and Benovic, J. L. (1995). Arrestin interactions with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, β_2-adrenergic, and m2 muscarinic cholinergic receptors. *J. Biol. Chem.* **270**, 720-731.

Hansen, S. H., Sandvig, K. and van Deurs, B. (1993). Molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. *J. Cell Biol.* **123**, 89-97.

Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998). Dynamin-mediated internalization of caveolae. *J. Cell Biol.* **141**, 85-99.

Hu, L. A., Chen, W., Premont, R. T., Cong, M. and Lefkowitz, R. J. (2002). G protein-coupled receptor kinase 5 regulates β_1-adrenergic receptor association with PSD-95. *J. Biol. Chem.* **277**, 1607-1613.

Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J. and Hall, R. A. (2000). β_1-Adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of β_1-adrenergic receptor interaction with N-methyl-D-aspartate receptors. *J. Biol. Chem.* **275**, 38659-38666.

Innamorati, G., Le Gouill, C., Balamotis, M. and Birnbaumer, M. (2001). The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. *J. Biol. Chem.* **276**, 13096-13103.

Kallal, L., Gagnon, A. W., Penn, R. B. and Benovic, J. L. (1998). Visualization of agonist-induced sequestration and down-regulation of a green fluorescent protein-tagged β_2-adrenergic receptor. *J. Biol. Chem.* **273**, 322-328.

Kassis, S. and Sullivan, M. (1986). Desensitization of the mammalian β-adrenergic receptor: analysis of receptor redistribution on nonlinear sucrose gradients. *J. Cyclic Nucleotide Protein Phosphor. Res.* **11**, 35-46.

Koenig, J. A. and Edwardson, J. M. (1997). Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol. Sci.* **18**, 276-287.

Krueger, K. M., Daaka, Y., Pitcher, J. A. and Lefkowitz, R. J. (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, J. G. and Benovic, J. L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu. Rev. Pharmacol. Toxicol.* **38**, 289-319.

Krupnick, J. G., Goodman, O. B., Keen, J. H. and Benovic, J. L. (1997). Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. *J. Biol. Chem.* **272**, 15011-15016.

Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S. and Caron, M. G. (2000). The inter-action of β-arrestin with the AP-2 adaptor, rather than clathrin, is required for the clustering of β_2-adrenergic receptor into clathrin-coated pits. *J. Biol. Chem.* **275**, 23120-23126.

Lefkowitz, R. J., Pitcher, J., Krueger, K. and Daaka, Y. (1998). Mechanisms of β-adrenergic receptor desensitization and resensitization. *Adv. Pharmacol.* **42**, 416-420.

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1990). β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science* **248,** 1547-1550.

Marullo, S., Faundez, V. and Kelly, R. B. (1999). β_2-Adrenergic receptor endocytic pathway is controlled by a saturable mechanism distinct from that of transferrin receptor. *Recept. Channels* **6**, 225-269.

McLean, A. J. and Milligan, G. (2000). Ligand regulation of green fluorescent protein-tagged forms of the human β_1- and β_2-adrenoceptors: comparisons with the unmodified receptors. *Br. J. Pharmacol.* **130**, 1825-1832.

Moore, R. H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J. A., Carsrud, N. D., Dickey, B. F. and Knoll, B. J. (1995). Ligand-stimulated β_2-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. *J. Cell Sci.* **108**, 2983-2991.

Moore, R. H., Tuffaha, A., Millman, E. E., Dai, W., Hall, H. S., Dickey, B.

F. and Knoll, B. J. (1999). Agonist-induced sorting of human β_2-adrenergic receptors to lysosomes during downregulation. *J. Cell Sci.* **112**, 329-338.

Oh, P., McIntosh, D. P. and Schnitzer, J. E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* **141**, 101-114.

Orlandi, P. A. and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905-915.

Ostrom, R. S., Gregorian, C., Drenan, R. M., Xiang, Y., Regan, J. W. and Insel, P. A. (2001). Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. *J. Biol. Chem.* **276**, 42063-42069.

Perkins, J. P., Hausdorf, W. P. and Lefkowitz, R. J. (1991). Mechanism of ligand-induced desensitization of beta-adrenergic receptors. In *The Beta-Adrenergic Receptors* (ed. J. P. Perkins), pp. 73-124. Clifton, NJ: Humana Press.

Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, D. K. and Lefkowitz, R. J. (2000). Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc. Natl. Acad. Sci. USA* **97**, 1489-1494.

Pippig, S., Andexinger, S. and Lohse, M. J. (1995). Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47**, 666-676.

Roseberry, A. G. and Hosey, M. M. (2001). Internalization of the M_2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae. *J. Cell Sci.* **114,** 739-746.

Rybin, V. O., Xu, X., Lisanti, M. P. and Steinberg, S. F. (2000). Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J. Biol. Chem.* **275**, 41447-41457.

Santini, F., Penn, R. B., Gagnon, A. W., Benovic, J. L. and Keen, J. H. (2000). Selective recruitment of arrestin-3 to clathrin coated pits upon stimulation of G protein-coupled receptors. *J. Cell Sci.* **113**, 2463-2470.

Schwencke, C., Okumura, S., Yamamoto, M., Geng, Y. J. and Ishikawa, Y. (1999). Colocalization of β-adrenergic receptors and caveolin within the plasma membrane. *J. Cell. Biochem.* **75**, 64-72.

Seibold, A., Williams, B., Huang, Z. F., Friedman, J., Moore, R. H., Knoll, B. J. and Clark, R. B. (2000). Localization of the sites mediating desensitization of the β_2-adrenergic receptor by the GRK pathway. *Mol. Pharmacol.* **58**, 1162-1173.

Shiina, T., Arai, K., Tanabe, S., Yoshida, N., Haga, T., Nagao, T. and Kurose, H. (2001). Clathrin box in G protein-coupled receptor kinase 2. *J. Biol. Chem.* **276**, 33019-33026.

Shiina, T., Kawasakii, A., Nagao, T. and Kurose, H. (2000). Interaction with β-arrestin determines the difference in internalization behavior between β_1- and β_2-adrenergic receptors. *J. Biol. Chem.* **275**, 29082-29090.

Shizukuda, Y. and Buttrick, P. M. (2002). Subtype specific roles of β-adrenergic receptors in apoptosis of adult rat ventricular myocytes. *J. Mol. Cell. Cardiol.* **34**, 823-831.

Shor, J., Curran, P. K. and Fishman, P. H. (2001). Targeting β-adrenergic receptor subtypes to caveolae: Differences between endogenous and heterologously expressed β_1- but not β_2-adrenergic receptors. *Mol. Biol. Cell* **12**, 443s.

Suzuki, T., Nguyen, C. T., Nantel, F., Bonin, H., Valiquette, M., Frielle, T. and Bouvier, M. (1992). Distinct regulation of β_1- and β_2-adrenergic receptors in Chinese hamster fibroblasts. *Mol. Pharmacol.* **41**, 542-548.

Tsao, P., Cao, T. and von Zastrow, M. (2001). Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **22**, 91-96.

Vickery, R. G. and von Zastrow, M. (1999). Distinct dynamin-dependent and -independent mechanisms target structurally homologous dopamine receptors to different endocytic membranes. *J. Cell Biol.* **144**, 31-43.

von Zastrow, M. and Kobilka, B. K. (1992). Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267**, 3530-3538.

Waldo, G. L., Northup, J. K., Perkins, J. P. and Harden, T. K. (1983). Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. *J. Biol. Chem.* **258**, 13900-13908.

Xiang, Y., Devic, E. and Kobilka, B. K. (2002). The PDZ binding motif of the β_1-adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. *J. Biol. Chem.* **277**, 33783-33790.

Xiao, R. P. (2000). Cell logic for dual coupling of a single class of receptors to G(s) and G(i) proteins. *Circ. Res.* **87**, 635-637.

Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A. and Hall, R. A. (2001). β_1-Adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. *J. Biol. Chem.* **276**, 41310-41317.

Yu, S. S., Lefkowitz, R. J. and Hausdorff, W. P. (1993). β-Adrenergic receptor sequestration. A potential mechanism of receptor resensitization. *J. Biol. Chem.* **268**, 337-341.

Zhang, J., Ferguson, S. S. G., Barak, L. S., Ménard, L. and Caron, M. G. (1996). Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J. Biol. Chem.* **271**, 18302-18305.

Zhou, X. M., Pak, M., Wang, Z. and Fishman, P. H. (1995). Differences in desensitization between human β_1- and β_2-adrenergic receptors stably expressed in transfected hamster cells. *Cell. Signal.* **7**, 207-217.

Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K. and Xiao, R. P. (2001). Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes. *Proc. Natl. Acad. Sci. USA* **98**, 1607-1612.