Regulated Endocytosis of G-protein-coupled Receptors by a Biochemically and Functionally Distinct Subpopulation of Clathrin-coated Pits

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β-2 Adrenergic receptors (B2ARs) are endocytosed by clathrin-coated pits. This process serves specialized functions in signal transduction and receptor regulation, raising the question of whether B2ARs are associated with biochemically specialized membrane vesicles during their endocytic trafficking. Here we show that B2ARs are endocytosed by a distinct subpopulation of clathrin-coated pits, which represent a limited subset of coated pits present in the plasma membrane, even in cells over-expressing both B2ARs and β-arrestin. Coated pits mediating agonist-induced endocytosis of B2ARs differ from other coated pits mediating constitutive endocytosis of G-protein-coupled receptors in their temperature dependence for fission from the plasma membrane and in the association of their membrane coats with β-arrestin. Endocytosis of these coated pits generates endocytic vesicles selectively enriched in B2ARs, which fuse within ~10 min after their formation with a common population of endosomes containing both B2ARs and transferrin receptors. These observations demonstrate, for the first time, the existence of a functionally and biochemically distinct subpopulation of clathrin-coated pits that mediate the agonist-regulated endocytosis of G-protein-coupled receptors, and they suggest a new model for the formation of compositionally specialized membrane vesicles at the earliest stage of the endocytic pathway.

Many G protein-coupled receptors (GPCRs), such as the β-2 adrenergic receptor (B2AR), are endocytosed by clathrin-coated pits and follow an endocytic pathway similar to constitutively endocytosed nutrient receptors (such as the transferrin receptor (TfnR)) (1–3). Clathrin-mediated endocytosis delivers both classes of receptor to endosomes, where ligand-receptor dissociation and protein sorting occur (4). However, in addition to these conserved functions of the early endocytic pathway, clathrin-mediated endocytosis serves specialized functions in GPCR regulation and signal transduction. Endocytosis of certain GPCRs, such as the B2AR, promotes dephosphorylation and functional resensitization of receptors following agonist-induced desensitization (5, 6). Additionally, clathrin-mediated endocytosis also plays an important role in promoting receptor-dependent activation of mitogen-activated protein kinase (MAPK) cascades (7).

Endocytosis by clathrin-coated pits serves specialized functions for other types of membrane proteins, such as synaptic vesicle membrane proteins and certain membrane transporters. In these cases, the specialized functions of the endocytic pathway are mediated by the formation of distinct endomembrane vesicles (e.g. synaptic vesicles and Glut4-containing vesicles), which diverge from a common early endosomal intermediate and contain a compositionally refined subset of membrane proteins (8–11). Accordingly, these considerations raise the question of whether the endocytic trafficking of GPCRs may also involve specialized membranes. However, in contrast to synaptic vesicle membrane proteins, which are sorted after endocytosis to vesicles distinct from those containing TfnRs, B2ARs and TfnRs colocalize extensively in recycling endosomes (12). Thus, if specialized endocytic membranes containing GPCRs exist, at what point in the endocytic pathway are these vesicles generated, and how is the intracellular trafficking of these vesicles related to the conserved recycling pathway?

We have addressed these questions by performing a detailed comparison of the endocytic membrane trafficking of B2ARs and TfnRs in the same cells. Our studies indicate that whereas internalized B2ARs and TfnRs are extensively colocalized in recycling endosomes, B2ARs differ significantly from TfnRs at earlier stages of endocytic membrane trafficking. These studies establish, for the first time, that functionally distinguishable subpopulations of clathrin-coated pits exist in the plasma membrane of nonpolarized cells.

Experimental Procedures

cDNA Constructs and Mutagenesis

Several epitope-tagged versions of the cloned human β-2 adrenergic receptor (B2AR (13)) were used in these studies: mutant receptors containing an HA or FLAG epitope in the amino-terminal extracellular domain (HAB2AR or SFB2AR, respectively) were described previously and demonstrated to be functional (12, 14). A mutant receptor containing a FLAG epitope in the amino-terminal extracellular domain and an EE epitope in the carboxyl-terminal cytoplasmic domain (SFB2EE) was constructed by appending a sequence encoding the EE epitope (EE-EEYMPME) followed by a stop codon immediately at the 3′-end of the cDNA coding sequence of the SFB2AR. This was accomplished by oligonucleotide-directed mutagenesis using the polymerase chain reaction (Vent polymerase, New England Biolabs). Receptor cDNAs were cloned...
into pcDNA3 (Invitrogen) and verified by dideoxynucleotide sequencing (Sequenase, U. S. Biochemical Corp.). The SF2B2EE receptor, like the HAB2AR and SF2B2AR described previously (12, 14), mediated agonist-dependent activation of adenylyl cyclase when expressed in transfected 293 cells to a similar extent as the wild type B2AR (data not shown), indicating that epitope tagging in this manner did not disturb the functionality of the receptor protein.

Cell Culture and Transfection

Human embryonal kidney 293 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Stably transfected cells expressing epitope tagged B2ARs were generated by transfecting 293 cells in 6-cm dishes with ~5 μg of plasmid DNA by calcium phosphate precipitation, and cells expressing transfectants were selected in 200 μg/ml Genetin (Life Technologies, Inc.). Cells clones expressing comparable amounts of receptor protein (500–1000 fmol/mg) were selected for further study.

HeLa cell lines expressing wild type or dominant negative mutant (K44A) dynamin I under the regulatable tetracycline promoter (kindly provided by Dr. S. Schmid) were grown as described (15). Briefly, cells were cultured expressing tetracycline in Dulbecco’s modified Eagle’s medium supplemented (with 10% fetal bovine serum, 400 μg/ml Geneticin, 1% Pen/Strep, 200 ng/ml puromycin, and 1 μg/ml tetracycline). Receptors were expressed in these cells by transient transfection. Cells grown in 6-cm dishes were transfected with ~5 μg of plasmid DNA by calcium phosphate precipitation. Dynamin expression was induced 24 h after transfection by removing tetracycline from the medium and studies of receptor trafficking in these cells were conducted 48 h later.

Examination of Receptor Endocytosis by Fluorescence Microscopy

Stably transfected 293 cells expressing SF2B2ARs or HeLa cells induced to express wild type or K44A dynamin and transiently transfected with SF2B2ARs were grown on glass coverslips (Corning) and serum-starved at 37 or 16 °C for 30 min in Dulbecco’s modified Eagle’s medium supplemented with 30 mM HEPES, pH 7.4. Cells were incubated with 50 ng/ml of Texas Red-conjugated transferrin (Molecular Probes) using standard methods (16) and Texas Red signal was visualized in frozen sections using a confocal microscope with the use of a rubber stopper, and the top coverslip, now with the plasma membrane attached, was ripped away. The top coverslip was immediately fixed in 3.7% formaldehyde in PBS for 10 min. Following fixation, plasma membranes were ripped away from the membrane and studied by immunofluorescence microscopy.

Biochemical Analysis of Receptor Endocytosis Using Cleaveable Biotin

Stably transfected 293 cells expressing SF2B2EE were grown in 6-cm dishes, washed twice with ice-cold PBS, and biotinylated with 300 μg/ml sulfo-NHS-S-S-biotin (Pierce) in PBS for 30 min at 4 °C. Unreacted biotin was quenched and removed by three washes with ice-cold TBS at 4 °C. Biotinylated cells were then transferred to prewarmed medium (37 or 16 °C) and 10 μM isoproterenol for 30 min to assay endocytosis, and then cells were again chilled on ice to stop membrane trafficking. Biotin attached to proteins still remaining on the cell surface was cleaved by washing cells twice at 4 °C with glutathione strip buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% fetal bovine serum, 5% FBS). Cells were then washed twice for 15 min at 4 °C with iodacetamide buffer (50 mM iodoacetamide, 1% BSA in PBS, 7.4) to quench residual glutathione and cap free sulfhydryl groups present on proteins. Cells were then extracted with Triton X-100 extraction buffer (0.5% (v/v) Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml phenylmethylsulfonyl fluoride, and 1 mg/ml iodoacetamide), and extracts were clarified by centrifugation in a microcentrifuge (12,000 × g) for 10 min prior to immunoprecipitation of receptors.

Immunoprecipitations were washed sequentially with HS1 (0.1% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl), 1 mM NaCl in HSB, and low salt wash buffer (10 mM Tris-HCl, 7.5). Washed beads were extracted with SDS sample buffer, and eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and blocked for 30 min in Blotto (5% dry milk, 0.1% Tween 20 in TBS). Biotinylated proteins were then complexed with horseradish peroxidase by incubating membranes with Vectastain ABC detection system (Vector Laboratories), and biotinylated proteins were detected using ECL (Amersham Pharmacia Biotech). B3/25 immunoprecipitations required the addition of B3/25 antibody (1:500 dilution; Jackson ImmunoResearch) for 30 min to detect FLAG-tagged receptors. Conventional fluorescence microscopy was performed using a Nikon 60X NA1.4 objective and epifluorescence optics; confocal fluorescence microscopy was carried out using a Bio-Rad MRC 1000 and a Zeiss 100X NA1.3 objective.

For time lapse studies, stably transfected cells expressing SF2B2ARs were plated on coverslips and incubated at 4 °C in the presence of M1 anti-FLAG antibody (3 μg/ml) directly conjugated with fluorescein isothiocyanate (Molecular Probes) using standard methods (16) and Texas Red-conjugated transferrin (Molecular Probes) and M1 anti-FLAG antibody (3 μg/ml, Kodak Scientific Imaging Systems) at 4 °C for 30 min to label TfnRs and B2ARs, respectively, and then cells were warmed to 16 or 37 °C for 30 min in the presence of 10 μM isoproterenol (Research Biochemicals). Following this incubation, cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 10 min and then quenched with PBS. Coverslips were permeabilized with 0.1% Triton X-100 (Sigma) in Blotto (3% dry milk in TBS + 1% CaCl2) and then incubated with fluorescence isothiocyanate-conjugated donkey anti-mouse secondary antibody (1:500 dilution; Jackson ImmunoResearch) for 30 min to detect FLAG-tagged receptors. Conventional fluorescence microscopy was performed using a Nikon 60X NA1.4 objective and epifluorescence optics; confocal fluorescence microscopy was carried out using a Bio-Rad MRC 1000 and a Zeiss 100X NA1.3 objective.

Examination of Receptor Distribution in Isolated Plasma Membrane Fragments

Stably transfected cells expressing HAB2ARs were grown on poly-L-lysine (Sigma)-coated coverslips and preincubated at 37 or 16 °C for 30 min. Cells were then treated with 10 μM isoproterenol for 30 min and subsequently chilled to 4 °C for 15 min. HAB2ARs and TfnRs present in the plasma membrane were specifically labeled by incubating intact intact cells at 4 °C in the presence of a saturating concentration of rabbit anti-HA monoclonal antibody (~10 μg/ml, Babco) and B3/25 antibody (8 μg/ml) for 1 h at 4 °C. Unbound antibodies were washed away with two washes of ice-cold PBS. Plasma membranes were ripped away from the cells on coverslips using a technique adapted from Sanan and Anderson (18). Briefly, coverslips supporting antibody labeled cells were chilled in an ice water bath, and another poly-L-lysine-coated coverslip was laid on top of the slide. Slight pressure was applied through the use of a rubber stopper, and the top coverslip, now with the plasma membrane attached, was ripped away. The top coverslip was immediately fixed in 3.7% formaldehyde in PBS for 10 min. Following fixation, plasma membrane specimens were blocked with Blotto (3% dry milk, 0.1% Triton X-100 in TBS) and incubated with donkey anti-mouse fluorescein isothiocyanate and donkey anti-rabbit Texas Red secondary antibody.
antibodies (both at 1:500 dilution; Jackson ImmunoResearch) to specifically detect TfnRs and HAB2ARs, respectively, present in the plasma membrane. For colocalization of B2ARs with clathrin, surface-labeled plasma membranes (with rabbit anti-HA antibody) were ripped, fixed, and incubated with a 1:1000 dilution of X.22 (gift from Dr. F. Brodsky) (19), followed by species-specific secondary antibodies conjugated with different fluorochromes (as described above). Control experiments using specimens labeled with single primary antibodies confirmed the immunochromic specificity of the staining procedure and verified the absence of bleedthrough between channels. Specimens were examined by fluorescence microscopy, and images were collected using a cooled charge-coupled device camera. Images were then written to 8-bit data sets using a linear lookup table to allow image registration and quantitation of clusters using National Institutes of Health Image software and to facilitate the display of merged color images using Adobe Photoshop.

**Quantitative Analysis of Receptor Clusters**

**Definition of Receptor Clusters**—B2AR immunoreactivity visualized in plasma membrane sheets prepared from untreated cells was evenly distributed over the entire specimen, without any clusters varying >50% from the mean staining intensity averaged over the plasma membrane fragment. The intensity of this unclustered fluorescence...
staining was ≥9-fold over nonspecific background. The fluorescence intensity of B2AR clusters in isoproterenol-treated cells was typically >5-fold greater than that observed in unclustered regions and was always >20-fold greater than background.

**Colocalization of Receptor Clusters with Clathrin or TfnR—**B2AR clusters (defined above) were identified in dual labeled specimens, and compared with the pattern of clathrin or TfnR immunoreactivity observed in the other fluorescence channel. Clusters of B2AR were scored clathrin or TfnR-positive if a cluster of clathrin immunoreactivity (more than 10-fold brighter than background) or TfnR immunoreactivity (more than 2-fold brighter than background) was centered within 2 pixels of the center of the B2AR cluster, which was judged to be in the resolution limits of our optical system. Clathrin or TfnR clusters (defined above) were scored to be B2AR-positive if a cluster of B2AR immunoreactivity >2-fold greater than the fluorescence staining intensity of the surrounding area was centered within 2 pixels of the center of the clathrin or TfnR cluster.

**Subcellular Fractionation and Immunolocalization of Endocytic Membranes Containing B2ARs**

SFB2EE cells were grown on 15-cm plates and treated for 2, 5, or 15 min with 10 μM isoproterenol at 37 °C. Cell monolayers were then quickly chilled to 4 °C, equilibrated at this temperature for 10 min, and washed twice with isotonic HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose). Cells were harvested with 1.0 ml per plate of HES + 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotonin, 2 μg/ml phenylmethylsulfonyl fluoride, and 100 μg/ml DNase I (Sigma) by scraping with a rubber policeman, and a homogenate was prepared by five strokes in an ice-cold Dounce homogenizer. Unbroken cells, nuclei, and a majority of plasma membrane fragments were removed by differential centrifugation at 19,000 × g for 20 min at 4 °C, and a light membrane fraction was prepared that included the majority of endocytic vesicles. Aliquots of this membrane fraction (3 mg of protein in 1 ml of HES + protease inhibitors) were incubated with 5 μg of EE antibody for 2.5 h at 4 °C. A goat anti-mouse linker antibody (10 μg, Jackson ImmunoResearch) was added to the samples and incubated for an additional 2.5 h at 4 °C. Antibody-bound membranes were isolated by adding 25 μl of fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem), washed twice with HES, and resuspended in 200 μl of HES. Analysis of this fraction indicated that it is highly enriched in B2AR-containing endosomes and contains very low levels of residual plasma membrane (as determined by recovery of only trace amounts of biotinylated plasma membrane proteins in this fraction).

Saturation radioligand binding assays were performed on the immunolabeled CD11b+ cells using [*H]*Hlydiropropranolol (Amersham Pharmacia Biotech) as tracer and alprenolol (1 μM) to define nonspecific binding (which was <10% of total binding in all cases), as described previously (12). Equal amounts of radiolabeled B2AR-containing endosomes, determined by B2AR estimates derived from radioligand binding assay, were solubilized with 25 μl of SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and blotted for B2AR using biotinylated M1 antibodies (prepared by standard methods using Sulfo-NHS biotin; Pierce) B2ARs were detected using horseradish peroxidase-conjugated anti-biotin secondary antibodies (Vector Laboratories) and ECL (Amersham Pharmacia Biotech). In all experiments, equal amounts of B2AR estimated from radioligand binding assay corresponded to equal amounts of receptor protein detected by Western blotting. For detection of TfnRs, blots were probed with H68.4 antibody (a gift from Dr. Ian Trowbridge) (20) followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch) and ECL detection. Relative amounts of TfnR present in B2AR-containing endosomes isolated at various times after endocytosis were quantitated by densitometry of TfnR immunoactivity detected in Western blots of immunolabeled vesicles loaded according to equal B2AR content.

**RESULTS**

**Dynamin-dependent Endocytosis of Adrenergic Receptors Is Selectively and Completely Blocked at 16 °C, whereas Transferrin Receptors Continue to Endocytose Rapidly at This Temperature—**Agonist-induced endocytosis of B2ARs in 293 cells has been reported previously to be mediated by clathrin-coated pits and a similar vesicular pathway as that of constitutive endocytosis and recycling of TfnRs (1, 2, 12, 21). Consistent with this, in the presence of the adrenergic agonist isoproterenol, epitope-tagged B2ARs were translocated within minutes from the plasma membrane to endocytic vesicles (Fig. 1, compare panels a and b), many of which contained endocytosed transferrin (Fig. 1, b and c). Endocytosis of both receptors was undetectable in cells incubated at 4 °C (not shown). However, significant differences were observed in the temperature dependence of B2AR and TfnR endocytosis at intermediate temperatures. In particular, endocytosis of B2ARs was undetectable in cells incubated at 16 °C, even in the presence of 10 μm isoproterenol (Fig. 1c). This is a saturating concentration of agonist that strongly activates receptors at 16 °C, as determined by assay of receptor-mediated activation of adenyl cyclase and agonist-induced phosphorylation of receptors at this temperature (not shown). In contrast to the failure of B2ARs to endocytose at 16 °C, endocytosis of TfnRs was readily observed at this temperature in the same cells. This was indicated by the bright punctate staining of endocytic vesicles containing internalized Texas Red-conjugated transferrin (Fig. 1f). These puncta were confirmed to represent endocytosed vesicles, rather than clusters in the plasma membrane, both by optical sectioning using confocal microscopy and because they were resistant to stripping under conditions that remove labeled transferrin from the cell surface (not shown). This difference in the temperature dependence for endocytosis of B2ARs and TfnRs was observed in multiple clones of stably transfected cells, as well as in transiently transfected cells expressing different levels of receptor protein.

A similar assay was used to compare the endocytosis of B2ARs and TfnRs in HeLa cells, in order to determine whether the selective blockade of B2AR endocytosis observed at 16 °C is unique to 293 cells or whether it reflects a general property of receptor endocytosis. As in 293 cells, endocytosed B2ARs and TfnRs visualized in agonist-stimulated HeLa cells were observed in a similar population of endocytic membranes, many of which could be colocalized by confocal fluorescence microscopy (Fig. 2, a and d). Furthermore, endocytosis of both receptors was specifically inhibited by K44A mutant dynamin I (Fig. 2, b and e), supporting the hypothesis that both receptors are
endocytosed by clathrin-coated pits (15, 22) in HeLa cells as in 293 cells. However, as observed in 293 cells, endocytosis of B2ARs was undetectable by this assay in cells incubated at 16 °C, whereas endocytosis of TfnRs was readily observed at this temperature in the same cells (Fig. 2, c and f).

The selective blockade of B2AR endocytosis at 16 °C was confirmed using a biochemical assay to measure endocytosis of B2ARs and TfnRs in the same stably transfected 293 cells. Both B2ARs and TfnRs, analyzed by specific immunoprecipitation from the same cell lysates, were readily detected in the plasma membrane of surface-biotinylated 293 cells. B2ARs resolved as a glycosylated band resolving with an apparent molecular mass between 60 and 80 kDa, whereas TfnRs resolved at ~190 kDa by SDS-polyacrylamide gel electrophoresis under nonreducing conditions (Fig. 3, lane 1, top and bottom panels, respectively). Biotin was cleaved quantitatively in the presence of a membrane-impermeant reducing agent (Fig. 3, lane 2), allowing endocytosis of both receptors to be detected in the same cells by their resistance to cleavage under these conditions. Using this highly sensitive biochemical assay, a small amount of B2AR endocytosis was observed in the absence of agonist (Fig. 3, lane 3, top panel), which was strongly stimulated in the presence of isoproterenol (lane 4, top panel). Constitutive endocytosis of TfnRs was readily detectable in the same cell lysates, both in cells incubated in the absence and presence of isoproterenol (lanes 3 and 4, bottom panel).

In cells incubated at 16 °C, agonist-induced endocytosis of B2ARs was completely blocked (Fig. 3, lane 6, top panel). In contrast, endocytosis of TfnRs was readily detectable and was only partially inhibited relative to that observed at 37 °C (compare lanes 3 and 4 to lanes 5 and 6, bottom panel). These results were quantitated in multiple experiments using densitometric scanning of films exposed in the linear range. This analysis confirmed a complete (~95%) inhibition of agonist-induced endocytosis of B2ARs at 16 °C. No agonist-induced endocytosis was detected when assays were conducted for different time periods ranging from 15 min to 1 h (not shown), further confirming that the inhibition of agonist-induced endocytosis of B2ARs observed at 16 °C represents an essentially complete blockade rather than a quantitative effect on endocytic rate. In contrast, this analysis indicated that the partial inhibition of TfnR endocytosis observed at 16 °C represents a moderate (~2.3-fold) reduction in the rate of TfnR endocytosis. Thus incubation of cells at 16 °C selectively and nearly completely blocked the agonist-induced endocytosis of B2ARs, whereas it only partially inhibited the rate of constitutive endocytosis of TfnRs measured in the same cells.

Adrenergic Receptors Associate with Clathrin-coated Pits That Fail to Endocytose at 16 °C—A relatively simple explanation for the selective blockade of B2AR endocytosis observed at 16 °C could be that B2ARs are immobile in the plasma membrane at this temperature and are therefore unable to enter
coated pits. Although B2ARs are rapidly mobile in the plasma membrane at 37 °C (23) and have been shown previously to associate with coated pits at both 37 and 16 °C (1, 2), the relative extent to which receptor clustering in the plasma membrane may be inhibited at reduced temperature is not known. To examine this question, we utilized a previously described technique that allows receptor distribution to be examined relative to clathrin-coated pits in large regions of the plasma membrane viewed en face (18).

Using this technique, B2ARs were observed in a diffuse distribution in the plasma membrane of cells incubated in the absence of agonist (Fig. 4A, panel a). In the presence of isoproterenol at 37 °C, marked clustering of B2ARs in the plasma membrane was observed (Fig. 4A, panel b). This agonist-induced clustering of receptors was completely blocked at 4 °C (not shown). However, at 16 °C, pronounced agonist-induced clustering of B2ARs occurred in the plasma membrane that was qualitatively indistinguishable from that observed at 37 °C (Fig. 4A, panel c). Quantitative analysis of fluorescence staining intensity in these specimens indicated that the relative density of B2AR clusters observed in the plasma membrane was not significantly different in cells incubated with isoproterenol at 37 °C compared with 16 °C (Fig. 4B). Furthermore, the concentration of B2AR immunoactivity present in individual clusters formed at 16 °C was similar to, or even slightly greater than, that observed in clusters formed at 37 °C (Fig. 4C).

To determine whether clusters of B2ARs formed at 16 °C are associated with clathrin-coated regions of the plasma membrane, the localization patterns of B2ARs and clathrin were compared in the same plasma membrane fragments. A representative region of the plasma membrane is shown at high magnification in Fig. 5A. Most B2AR clusters present in the plasma membrane (Fig. 5A, panel a) colocalized with puncta of intense clathrin immunoactivity (panel b), causing them to appear yellow in the merged image (panel c, arrowhead). B2AR clusters on the plasma membrane also colocalized extensively with AP-2 (data not shown). These clathrin-positive puncta have been demonstrated previously to represent clathrin-coated pits and flat lattices that represent intermediates in coated pit formation (18). Quantitation of these observations over a large number of plasma membrane fragments confirmed that the vast majority (~90%) of B2AR clusters present in the plasma membrane were associated with clathrin coats (Fig. 5C). Many of these structures had the morphology of typical coated pits when observed by electron microscopy of ultrathin sections (Fig. 5B), consistent with a previous study in which agonist-induced redistribution of B2ARs into coated pits was observed at 16 °C (1). Thus, clathrin-coated pits containing concentrated B2ARs form efficiently in the plasma membrane at 16 °C but fail to endocytose at this temperature, indicating that the rate-limiting step for B2AR endocytosis under these conditions is downstream of the agonist-induced clustering and association of receptors with clathrin-coated regions of the plasma membrane.

**Transferrin Receptors Are Observed in a Compositionally Distinct Subpopulation of Clathrin-coated Pits**—The ability of B2ARs to associate efficiently with clathrin-coated regions of the plasma membrane that fail to endocytose at 16 °C raises the question of how TfnRs are able to continue to undergo
date, the plasma membrane specimens. Error bars
represent the standard deviation of these data from the mean.

dynamin-dependent endocytosis at 16 °C in the same cells. One
possibility is that B2ARs may concentrate in a limited subset of
coated pits in the plasma membrane, which differ in their
temperature dependence for endocytosis. Supporting this hy-
pothesis, numerous clathrin-coated pits were observed in the
plasma membrane that did not contain a detectable concentra-
tion of B2ARs, causing them to appear red in the merged image
(Fig. 5A, panel c; a representative B2AR-negative coated pit is
indicated by an arrow in panels a–c). Quantitation of these
observations indicated that even in cells overexpressing B2ARs
at high levels (−1 pmol/mg), >20% of clathrin-coated pits were
devoid of detectable concentration of B2AR immunoreactivity
(Fig. 5D). An alternative interpretation of this observation is
that these B2AR-negative puncta may represent deeply invag-
nated coated pits, which are inaccessible to macromolecules such as antibodies (24). Thus, B2ARs, even if present in these
structures, could be undetectable by immunocytochemical
staining. To address this possibility directly, we compared the
distribution of B2ARs and TfnRs receptors in the same plasma
membrane fragments using the same technique. Both B2ARs
and TfnRs were labeled in parallel using monoclonal antibodies
that specifically recognize ectodomains of each receptor, to
ensure that differences in the localization pattern of B2AR and
TfnR immunoreactivity were not caused by differences in the
accessibility of individual coated pits to antibodies. These ex-
periments confirmed that, indeed, significant compositional
heterogeneity exists among individual clathrin-coated pits in
the plasma membrane. Significantly, many coated pits were
observed in the plasma membrane containing readily detecta-
ble concentrations of either TfnRs or B2ARs, but not both (red
and green puncta, respectively, in Fig. 6A, panel c). In addition,
consistent with the ability of some coated pits to endocytose a
mixed membrane cargo, coated pits containing both types of
receptor were also observed (yellow structures in Fig. 6A,
panel c).

Quantitation of these differences in receptor immunoreactiv-
ity by digital fluorescence imaging confirmed that individual
coated pits differed substantially in relative concentration of
B2AR and TfnR. All coated pits scored as B2AR-negative con-
tained no detectable concentration of B2AR immunoreactivity
above background levels (measured in unclustered regions of
the plasma membrane) and were determined to be depleted
>5-fold of B2ARs compared with coated pits containing detect-
able B2ARs. Similarly, TfnR-negative coated pits contained no
TfnR immunoreactivity above background and were deter-
mined to be depleted of TfnRs by >5-fold compared with TfnR-
positive coated pits (see the legend to Fig. 6A). Quantitation of
the relative numbers of these structures observed in multiple
experiments indicated that ~50% of the coated pits observed in
the plasma membrane contained readily detectable amounts of
either B2ARs or TfnRs, but not both (Fig. 6B, middle and right
columns). These data confirm the existence of a substantial
number of compositionally distinct coated pits in the plasma
membrane, even when using relatively stringent experimental
criteria (>25-fold difference in relative concentration of B2AR
versus TfnR immunoreactivity) that may underestimate the
absolute number of these structures.

Clathrin-coated Pits Containing B2ARs or TfnRs Differ in
the Protein Composition of Their Membrane Coats—The het-
erogeneity observed among clathrin-coated pits at 16 °C was also
seen at 37 °C (data not shown), indicating that compositionally
distinct subpopulations of clathrin-coated pits exist at physi-
ological temperatures. Interestingly, despite their ability to seg-
regate among distinct coated pits, quantitative analysis of re-
ceptor concentration in individual coated pits suggested that
B2ARs and TfnRs do not compete for the same binding sites in
the coated pits (Fig. 6, legend). These observations suggest that
individual coated pits may differ in the composition of the
clathrin-associated proteins that interact selectively with
B2ARs or TfnRs. Endocytosis of B2ARs is promoted by receptor
interaction with β-arrestins, which have the ability to bind to
clathrin and to associate with clathrin coated pits (2). To ex-
amine whether β-arrestin specifically marks the subpopulation
of coated pits that contain B2ARs, we used HAB2AR cells
transiently transfected with arrestin3-GFP (17). Cells were
treated with 10 μM of isoproterenol at 37 °C and quickly chilled
to 4 °C to inhibit any further endocytosis. Surface B2ARs and
TfnRs were labeled with antibodies recognizing the ectodomain
of the receptors under nonpermeabilized conditions, thereby
labeling only those receptors present in the plasma membrane.
When cells were fixed and stained with secondary antibodies,
B2ARs were seen as discrete clusters present in the plasma
membrane (Fig. 7A). B2AR-containing coated pits colocalized
extensively with arrestin3-GFP (Fig. 7B), consistent with pre-
previous studies demonstrating colocalization between B2ARs and β-arrestin in coated pits (2). This extensive colocalization is indicated in Fig. 7c, in which B2ARs (red) and arrestin3-GFP (green) give rise to yellow clusters when the images are merged. In contrast, arrestin3-GFP was associated only with a minority of coated pits containing TfnRs (Fig. 7, d and e), consistent with the existence of numerous coated pits containing TfnRs but no detectable B2ARs. Although some coated pits that contained TfnRs were associated with arrestin3-GFP (yellow puncta), many coated pits containing TfnRs were not associated with arrestin3-GFP (red puncta). Moreover, numerous arrestin-positive coated pits were observed that excluded TfnRs (green puncta). These results confirm the existence of compositionally distinct subpopulations of clathrin-coated pits in the plasma membrane, even in intact cells examined under steady state conditions at physiological temperature, and they identify a specific difference in the coat protein composition of these coated pits. Furthermore, because coated pits containing B2ARs colocalized extensively with β-arrestin at 16 °C (data not shown), these results suggest that B2AR-containing coated pits present in the plasma membrane at 16 °C contain a full complement of coat components yet still fail to endocytose at this temperature.

Endocytosis of Adrenergic and Transferrin Receptors by Different Clathrin-coated Pits Mediates the Formation of Compositionally Specialized Primary Endocytic Vesicles, Which Fuse with a Common Population of Early Endosomes—The observation of compositionally distinct clathrin-coated pits in the plasma membrane at 37 °C suggests that these coated pits may mediate the selective endocytosis of B2ARs and TfnRs into different endocytic vesicles. To test this hypothesis, the endocytosis of B2ARs and TfnRs was compared in the same cells at 37 °C using time-lapse fluorescence microscopy. Cells were incubated with isoproterenol, and B2ARs and TfnRs present in the plasma membrane were specifically labeled with different fluorophores at 4 °C. Then, the movement of receptors from the plasma membrane was determined at different times after warming cells rapidly to 37 °C in the continued presence of isoproterenol. Within 2 min after warming cells from 4 to 37 °C, both B2ARs and TfnRs were translocated from the plasma membrane to small endocytic vesicles (as indicated by resistance to antibody stripping in nonpermeabilized cells), which were located close to the plasma membrane (Fig. 8A, panel a, green puncta and red puncta, respectively). Some of these vesicles contained readily detectable amounts of both B2ARs and TfnRs, yielding yellow staining of these structures in the merged image (Fig. 8A, panel a, large arrow), consistent with the observation of coated pits in the plasma membrane containing both receptors. In addition, numerous small endocytic vesicles were observed in the cell periphery, which were highly enriched for B2ARs but contained no detectable TfnRs (green vesicles in Fig. 8A, panel a, small arrow) or were enriched for TfnRs without detectable B2AR immunoreactivity (red vesicles in Fig. 8A, panel a, arrowhead). These observations support the hypothesis that compositionally specialized coated pits func-

![Fig. 7. B2AR- and TfnR-coated pits differ in their membrane coat composition.](image-url)

Cells transfected with arrestin3-GFP were treated for 30 min at 37 °C with 10 μM isoproterenol to drive endocytosis and recycling of B2ARs to steady state (12). Surface HAB2ARs, labeled under nonpermeabilized conditions (a), extensively colocalized with arrestin3-GFP (b), yielding yellow puncta in the merged image (c) (red, B2AR; green, arrestin3-GFP). Surface TfnRs (d) only partially colocalized with arrestin3-GFP (e), as demonstrated by the merged color image (f). Although some coated pits containing TfnRs were associated with arrestin3-GFP (yellow puncta), many coated pits containing TfnRs were not associated with arrestin3-GFP (red). In addition, numerous arrestin3-GFP-positive puncta were observed that did not contain TfnRs (green).
tion at 37 °C and can mediate the endocytosis of B2ARs and TfnRs into different primary endocytic vesicles. Interestingly, upon continued incubation of cells at 37 °C, endocytosed B2ARs and TfnRs, present initially in different endocytic vesicles, merged into the same endocytic membranes, which were located deeper in the cytoplasm and typically had a tubulovesicular morphology (Fig. 8A, panel b, arrows). Examination of the same cells after an additional 8 min of incubation (10 min total) revealed that B2ARs and TfnRs became extensively colocalized (b, yellow structures) in vesicles that had a tubulovesicular morphology (b, arrows). B, endocytic vesicles containing epitope tagged B2ARs were immunoisolated from a light membrane fraction (LM) to yield a highly purified population of B2AR-containing endocytic vesicles (ILN), as indicated by anti-B2AR immunoblotting of the membrane fractions. No membranes were recovered in control isolates prepared from the LM fractions of cells expressing B2ARs without the epitope tag (CTL), confirming the biochemical specificity of the immunoisolation procedure. C, B2AR-containing vesicles were immunoisolated after 2, 5, and 15 min of isoproterenol treatment. Aliquots of endocytic membranes containing equal amounts of B2AR were analyzed for content of TfnR by immunoblotting using H68.4 monoclonal antibody. B2AR-containing endocytic membranes isolated within 2 min after endocytosis (left lane) were relatively depleted of TfnRs, whereas the relative amount of immunoreactive TfnR increased in a time-dependent manner (middle and right lanes indicate endocytic membranes isolated 5 and 15 min after endocytosis, respectively).

![Fig. 8](image-url)

**Fig. 8. Endocytosis of B2ARs and TfnRs by distinct primary endocytic vesicles.** A, internalization of surface-labeled SFB2ARs and TfnRs was examined in the same cells by time-lapse fluorescence microscopy, as described under “Experimental Procedures.” Receptors internalized after 2 min were observed in small endocytic vesicles located at the cell periphery close to the plasma membrane. Some of these vesicles contained both B2ARs and TfnRs, appearing yellow in the merged color image (a, large arrow). Another population of primary endocytic vesicles were brightly stained with endocytosed B2ARs but did not contain detectable amounts of endocytosed TfnR, appearing green in the merged color image (a, small arrow). Conversely, endocytic vesicles highly enriched in TfnRs but containing no detectable B2ARs were also observed in the same cells, appearing as red structures (a, arrowhead). Examination of the same cells after an additional 8 min of incubation (10 min total) revealed that B2ARs and TfnRs became extensively colocalized (b, yellow structures) in vesicles that had a tubulovesicular morphology (b, arrows). B, endocytic vesicles containing epitope tagged B2ARs were immunoisolated from a light membrane fraction (LM) to yield a highly purified population of B2AR-containing endocytic vesicles (ILN), as indicated by anti-B2AR immunoblotting of the membrane fractions. No membranes were recovered in control isolates prepared from the LM fractions of cells expressing B2ARs without the epitope tag (CTL), confirming the biochemical specificity of the immunoisolation procedure. C, B2AR-containing vesicles were immunoisolated after 2, 5, and 15 min of isoproterenol treatment. Aliquots of endocytic membranes containing equal amounts of B2AR were analyzed for content of TfnR by immunoblotting using H68.4 monoclonal antibody. B2AR-containing endocytic membranes isolated within 2 min after endocytosis (left lane) were relatively depleted of TfnRs, whereas the relative amount of immunoreactive TfnR increased in a time-dependent manner (middle and right lanes indicate endocytic membranes isolated 5 and 15 min after endocytosis, respectively).
selectively into different primary endocytic vesicles by clathrin-mediated endocytosis, and they indicate that these vesicles fuse within 10 min after their formation with a common population of early and/or recycling endosomes. From the early endosome, synaptic vesicle proteins diverge from the common endocytic pathway to form synaptic vesicles. In contrast, a different and novel mechanism for membrane specialization, a divergent model, can be used to describe the endocytic trafficking of the B2AR. B2ARs and TfnRs are endocytosed by functionally distinct subpopulations of clathrin-coated pits that co-exist in the plasma membrane. These subpopulations of coated pits differ functionally in their temperature dependence for endocytosis because fission from the plasma membrane of the B2AR-containing coated pits (whether or not they also contain TfnRs) is selectively blocked at 16 °C. Following endocytosis, compositionally distinct primary endocytic vesicles are formed from these subpopulations of coated pits and remain in the cytoplasm for up to 10 min before converging with a common population of tubulovesicular endosomes. The convergence of B2ARs and TfnRs into a common endosomal compartment accounts for the extensive colocalization observed between these receptors at later stages after endocytosis (Fig. 8) and at steady state (12). We propose that primary endocytic vesicles containing B2AR function in receptor-mediated activation of MAPK. Ligand dissociation and dephosphorylation of receptors may occur after receptor delivery to a common population of endosomes.

**DISCUSSION**

The specialized role of clathrin-mediated endocytosis in GPCR function raises the question of whether GPCRs may be targeted to a distinct subpopulation of membrane vesicles at any time during their endocytic trafficking. As one approach to address this question, we performed a detailed comparison of the endocytic membrane trafficking of B2ARs and TfnRs in the same cells. Our studies demonstrate that compositionally distinct endocytic vesicles containing concentrated B2ARs do exist. Surprisingly, in contrast to other compositionally refined endocytic membranes that are formed after the delivery of membrane cargo to early endosomes, endocytic membranes selectively enriched in B2ARs are generated at the earliest stages of the endocytic pathway. This was shown initially by immunocytochemical and biochemical assays demonstrating that the agonist-induced endocytosis of B2ARs is selectively and almost completely blocked at 16 °C. In marked contrast, constitutive endocytosis of TfnRs by coated pits was readily observed and only partially inhibited at this temperature. This selective temperature block did not result from reduced lateral mobility of B2ARs in the plasma membrane because both B2ARs and TfnRs concentrated in clathrin-coated pits at 16 °C and did so to a similar extent as observed at 37 °C. Further investigation of this phenomenon revealed that individual subpopulations of clathrin-coated pits differ also in whether or not their membrane coats contain β-arrestin. Thus two distinct subpopulations of clathrin-coated pits can be resolved in the plasma membrane, which differ both functionally (in their temperature dependence for endocytosis) and biochemically (in the composition of their clathrin coat-associated proteins).

The selective association of B2AR-containing coated pits with β-arrestin is consistent with the ability of this protein to promote the association of B2ARs with clathrin-coated pits (2). Interestingly, B2ARs and TfnRs were observed in a limited subpopulation of coated pits even in cells overexpressing both B2ARs and arrestins, suggesting that the formation of a distinct subpopulation of B2AR-containing coated pits cannot be explained by the presence of receptors or β-arrestin in limiting amounts. Conversely, it also appears unlikely that coated pit subpopulations are generated by the random assortment of receptors among individual coated pits, because a large number of coated pits are observed in the plasma membrane that differ greatly in receptor concentration, even in cells overexpressing B2ARs. Further studies will be necessary to understand the mechanistic basis for the selectivity of receptor association with individual subpopulations of coated pits and to elucidate why individ-
ual subpopulations of coated pits differ in their temperature dependence for fission from the plasma membrane.

Although distinct subpopulations of coated pits were first observed in cells incubated at reduced temperature, they were also observed in cells incubated at 37 °C and mediated the internalization of B2ARs and TfnRs into distinguishable subpopulations of endocytic vesicles at this temperature. These vesicles represent compositionally specialized early intermediates in the endocytic pathway, which exist as separate structures in the cytoplasm for up to ~10 min after their formation and then fuse with a common population of tubulovesicular endosomes containing both B2ARs and TfnRs. These observations, which were established both by time-lapse fluorescence microscopy of live cells and by biochemical analysis of B2AR-containing endocytic vesicles immunosolated at various times after receptor internalization, reconcile the present results with previous studies demonstrating colocalization of B2ARs and TfnRs in the same recycling endosomes (12).

To our knowledge, the present observation that B2ARs are endocytosed by a biochemically and functionally distinct subpopulation of clathrin-coated pits is completely novel. The present studies have focused exclusively on the B2AR as a model GPCR that undergoes agonist-induced endocytosis by clathrin-coated pits. However, studies in progress indicate that agonist-induced endocytosis of several other GPCRs is also blocked at 16 °C (data not shown), suggesting that the present observations may be relevant to the regulated endocytosis of other receptors. Interestingly, morphological studies of ligand-induced endocytosis of receptor tyrosine kinases suggest that these signaling receptors may also associate with a subset of coated pits (25, 26). Although these earlier studies did not identify biochemical or functional distinctions among coated pits containing receptor tyrosine kinases, they suggest the possibility that the endocytosis of ligand-regulated receptors by distinct clathrin-coated pits may be a rather general phenomenon.

The present observations establish a new model for the generation of membrane specialization in the early endocytic pathway. In contrast to other specialized membrane proteins, which are first delivered to a common population of early endosomes and are subsequently packaged into distinct vesicles that diverge from this common pathway (Fig. 9A), compositionally refined membranes containing B2ARs are generated at the earliest stage of the endocytic pathway and subsequently merge with a common population of endosomes (Fig. 9B). This model for “convergent” membrane trafficking of specialized endocytic membranes is unprecedented and may be of particular importance for the specialized functions of the endocytic pathway in signal transduction. In the case of B2AR, for example, endocytosis by clathrin-coated pits is specifically required for receptor-mediated signaling via MAPK. Significantly, the ability of these receptors to signal to MAPK requires both agonist-activation of the receptor and receptor phosphorylation (27). However, early endosomes mediate dephosphorylation of internalized receptors and also promote ligand dissociation, suggesting that internalized receptors may be unable to signal to MAPK following delivery to early endosomes (28). Thus, it is possible that the packaging of GPCRs into refined endocytic membranes at this early stage in the endocytic pathway provides a membrane environment that supports the selective signaling of internalized receptors to MAPK, before ligand dissociation and receptor dephosphorylation occur in early endosomes (Fig. 9B). Further studies will be required to test this hypothesis and to identify putative signaling proteins that associate with activated receptors in these endocytic membranes.

In conclusion, the present studies establish that agonist-induced endocytosis of GPCRs is mediated by a subpopulation of clathrin-coated pits, which form compositionally refined primary endocytic vesicles that are capable of fusing with a common population of early endosomes. The existence of biochemical and functional specificity at the earliest stage of the endocytic pathway is a novel observation that may be important to understanding the specialized functions of endocytosis in signal transduction and receptor regulation. These studies also suggest a new model for convergent membrane trafficking of compositionally specialized endocytic vesicles, which may be of general relevance to understanding differences between constitutive and regulated endocytosis of other membrane proteins.

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