Recruitment of Activated G Protein-coupled Receptors to Pre-existing Clathrin-coated Pits in Living Cells*

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The process of clathrin-mediated endocytosis tightly regulates signaling of the superfamily of seven-transmembrane G protein-coupled receptors (GPCRs). A fundamental question in the cell biology of membrane receptor endocytosis is whether activated receptors can initiate the formation of clathrin-coated pits (CPs) or whether they are simply mobilized to pre-existing CPs. Here, using various approaches, including a dynamic assay to monitor the distribution of CPs and GPCR-β-arrestin complexes in live HeLa cells, we demonstrate for the first time that activated GPCRs do not initiate the de novo formation of CPs but instead are targeted to pre-existing CPs.

Many cell surface receptors and membrane proteins are internalized through specialized structures of the plasma membrane, called clathrin-coated pits (CPs), which gradually invaginate and ultimately detach from the plasma membrane forming clathrin-coated vesicles. The endocytic machinery comprises two major structural proteins, clathrin and the adapter protein complex AP2, which plays a central role in CP assembly. A number of more recently identified cytosolic or integral-membrane proteins regulate CP formation and the fission of clathrin-coated vesicles. In the conventional view of constitutive endocytosis, readily accessible tyrosine- or di-leucine-based endocytic motifs, present in the cytoplasmic tails of membrane proteins that are to be internalized, interact with the AP2 adapter complex. In the case of ligand-induced endocytosis, these endocytic signals are cryptic and thought to be unmasked upon receptor activation by its cognate ligand (1–3).

G protein-coupled receptors (GPCRs), one of the largest families of membrane receptors (4), represent a different model of ligand-induced endocytosis through CPs due to their use of specific adapter proteins called β-arrestins that form a bridge between activated GPCRs and the clathrin coat (5). Endocytosis of GPCRs plays an important role in the regulation of their signaling cycle (6). Agonist-stimulated GPCRs initiate cell responses by modulating effector molecules via the activation of heterotrimeric G proteins. These receptors are then rapidly phosphorylated by specific kinases. This phosphorylation promotes the binding of cytosolic nonvisual arrestins (β-arrestin1 and β-arrestin2) to GPCRs (7) resulting in their desensitization (8, 9), a transient state during which receptors become refractory to any further stimulation. To recover a full signaling function, GPCRs have to be internalized (10), dephosphorylated in the endosomal compartment (11), and then recycled back to the plasma membrane (12, 13). Previous studies have indicated that nonvisual arrestins play the role of adapter molecules during this process (14). The overexpression of arrestins can rescue an endocytosis-defective mutant of the β2-adrenergic receptor, a member of the GPCR family and the overexpression of dominant negative forms of arrestins inhibits GPCR internalization (15). In addition, activated GPCR-arrestin complexes concentrate in punctate areas of the plasma membrane where they colocalize with clathrin (14, 16) and AP2 (17, 18). The fact that arrestins have been shown to directly interact with both clathrin (14) and AP2 (19) provided a potential mechanism by which GPCRs are internalized through CPs. In this model, arrestins recruit activated GPCRs into CPs by connecting the cytosolic domain of GPCRs to clathrin-coat components. Recent studies confirmed this scenario by showing that mutants of β-arrestin2 lacking the AP2 binding motif are unable to efficiently recruit activated GPCRs into CPs (17).

A commonly proposed paradigm for receptor-mediated endocytosis is that the endocytic machinery is recruited to the inner face of the plasma membrane from the cytoplasm by cytoplasmic tails of membrane proteins (nucleation) (20, 21). In the case of ligand-activated receptors, such as GPCRs, this would occur after stimulation, permitting CP formation at random sites on the plasma membrane through the association of individual coat components. Recent evidence, however, has challenged this model of nucleation and suggests that regulation of CP levels is independent of endocytic signal levels. In these studies transferrin receptors (22) or chimeric integral membrane reporter proteins containing cytoplasmic endocytic signals (23) were expressed to levels that saturated the internalization machinery, but no evidence to suggest a change in CP levels was found. In addition CPs have been shown to exhibit limited mobility within the membrane and form repeatedly at distinct sites or “hot spots” (24). In the model of pre-existing CPs, the receptor would perhaps be mobilized to these sites upon activation. To investigate this issue in a model of GPCR endocytosis we studied arrestin translocation and redistribution of receptor-arrestin complexes in CPs during the early stages of ligand-mediated endocytosis of the rat thyrotropin-releasing hormone receptor (TRHR) (8, 10), a transient state during which receptors become refractory to any further stimulation. To recover a full signaling function, GPCRs have to be internalized (10), dephosphorylated in the endosomal compartment (11), and then recycled back to the plasma membrane (12, 13). Previous studies have indicated that nonvisual arrestins play the role of adapter molecules during this process (14). The overexpression of arrestins can rescue an endocytosis-defective mutant of the β2-adrenergic receptor, a member of the GPCR family and the overexpression of dominant negative forms of arrestins inhibits GPCR internalization (15). In addition, activated GPCR-arrestin complexes concentrate in punctate areas of the plasma membrane where they colocalize with clathrin (14, 16) and AP2 (17, 18). The fact that arrestins have been shown to directly interact with both clathrin (14) and AP2 (19) provided a potential mechanism by which GPCRs are internalized through CPs. In this model, arrestins recruit activated GPCRs into CPs by connecting the cytosolic domain of GPCRs to clathrin-coat components. Recent studies confirmed this scenario by showing that mutants of β-arrestin2 lacking the AP2 binding motif are unable to efficiently recruit activated GPCRs into CPs (17).

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Fig. 1. Time course of TRHR internalization and its colocalization with β-arrestin2-GFP following stimulation. a, HeLa cells transiently cotransfected with VSV-TRHR and β-arrestin2-GFP constructs were subsequently treated with 40 nM [3H]TRH for different times, and surface and internal [3H]TRH binding was measured. Data are expressed as the percentage of surface receptors at 0 min (cells incubated on ice for the entirety of the experimental period) and represent means ± S.D. from three independent experiments performed in triplicate. b–g, HeLa cells transiently cotransfected with VSV-TRHR and β-arrestin2-GFP constructs were fixed and processed for fluorescence microscopy using the P5D4 antibody directed against the VSV epitope and revealed by a Texas Red-labeled secondary antibody. The cells were observed under an epifluorescence microscope attached to a cooled CCD camera. The focus was on the planar plasma membrane adherent to the coverslip. b, d, and f, green fluorescence emitted by GFP in unstimulated cells and in cells stimulated with 10 μM TRH for 2 and 15 min, respectively. c, e, and g, red fluorescence emitted by Texas Red corresponding to VSV-TRHR in unstimulated cells and in cells stimulated with 10 μM TRH for 2 and 15 min, respectively. Insets show higher magnifications of representative areas of the plasma membrane. Bar, 15 μm. In parallel control experiments, TRH had no effect on β-arrestin2 distribution in HeLa cells transfected with β-arrestin2-GFP alone (i.e. without TRHR, data not shown).
hormone receptor-1 (TRHR). In particular, we have conducted dynamic studies in living cells expressing a β-arrestin 2-enhanced green fluorescent protein (GFP) and a chimera between Eps15, a constitutive component of CPs (25, 26), and the red fluorescent protein (RFP) Ds-Red. Our results show that agonist-activated TRHRs do not promote the formation of specific

**Fig. 2. Redistribution of β-arrestin2-GFP to CPs following TRHR stimulation.** HeLa cells transiently cotransfected with VSV-TRHR and β-arrestin2-GFP constructs were fixed, permeabilized, processed for fluorescence microscopy using the AP-6 antibody directed against AP2 or the anti-Eps15 antibody, and revealed by Texas Red-labeled secondary antibodies. a, c, e, and g, green fluorescence emitted by GFP in unstimulated cells and in cells following stimulation with 10 μM TRH for 2 and 15 min, respectively. b, d, and h, red fluorescence emitted by Texas Red corresponding to AP2 complexes in unstimulated cells (b) and in cells following stimulation with 10 μM TRH for 2 (d) and 15 min (h) stimulation. f, red fluorescence emitted by Texas Red corresponding to Eps15 complexes 2 min after stimulation. Insets show higher magnifications of representative areas of the plasma membrane. Bar, 15 μM.
material

coated pits. Instead, receptor-arrestin complexes are recruited to pre-existing sites of constitutive endocytosis.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and fetal bovine serum were from Life Technologies. TRH, neomycin, and 100/2 antibody were purchased from Sigma. FuGENE 6 reagent and monoclonal anti-GFP antibody were from Roche Molecular Biochemicals. Mouse monoclonal antibodies AP.6 (anti-AP2 (27)) and OKT9 (anti-human transferrin receptor (TfR)) were from Sigma. FuGENE 6 reagent and monoclonal anti-GFP antibody were purchased from PerkinElmer Life Science.

Plasmids—The construction of the VSV-TRHR plasmid has been described previously (30) and was a kind gift from Dr G. Milligan. pβ-arrestin2-EGFP was generated by excising the β-arrestin2 cDNA fragment from pβ-arrestin2-YFP (31) (generously provided by S. Angers) using NheI and cloning this into the NheI site of EGFPN1 (CLONTECH). pβ-Arrestin1-EGFP was generated by excising the XhoI/SacII sites of EGFPN1. pRFP-Eps15 was generated by performing PCR of the first 944 bases of Eps15, corresponding to the NH2-terminal 314 amino acids, introducing a BamHI site in the upper primer. The PCR product was digested with BamHI and HindIII and cloned into pEGX5-1.
acetic acid, 0.5 μM NaCl, pH 2.6) followed by 0.5 ml of 0.15 M NaCl wash. Internal [3H]TRH was determined after cell solubilization with 1% (w/v) SDS/1% (v/v) Triton X-100 in 10 mM Tris/HCl, pH 8.0. Samples incubated in the presence of 10 μM cold TRH allowed the determination of nonspecific binding. Results are expressed as a percentage of surface receptors at 0 min (cells incubated on ice for the duration of the experiment).

RESULTS AND DISCUSSION

To investigate the issue of whether ligand-activated GPCRs can initiate the formation of new CPs or whether they are recruited to pre-existing CPs, we utilized a system that would result in a high density of internalized GPCR following receptor activation. To this end, we chose to study HeLa cells overexpressing a vesicular stomatitis virus epitope-tagged form of TRHR (VSV-TRHR). Previous studies have demonstrated that the TRHR is endocytosed by a β-arrestin- (33, 34) and clathrin-dependent (35, 36) mechanism. This GPCR was chosen, as in preliminary experiments using real-time confocal microscopy it was found to be one of the best GPCR translocators of β-arrestins to the plasma membrane among several GPCRs tested including the β2-adrenergic receptor, the receptor for the chemokine receptors CXCR4 and CCR5 (data not shown). In addition, the TRHR also belongs to the recently described class of GPCRs, which forms stable complexes with both β-arrestins1 and -2 and traffics together with β-arrestins in endocytic vesicles (37), allowing β-arrestins to act as markers of GPCRs during the entire endocytic process. VSV-TRHR was cotransfected in combination with a chimeric protein, consisting of the rat β-arrestin2 cDNA fused to the NH2-terminal end of GFP (β-arrestin2-GFP). In this system the endocytosis rate of the TRHR (t1/2 of ~10 min, Fig. 1a) is similar to that obtained in rat pituitary GH3C1 cells expressing endogenous TRHR (38). The cellular distribution of TRHR and β-arrestin2 was analyzed by immunofluorescence: VSV-TRHR was detected using the P5D4 monoclonal antibody specific to the VSV-tag, and β-arrestin2-GFP was directly visualized by the green fluorescence emitted by GFP. This approach therefore allowed us to observe a TRH-dependent membrane recruitment of β-arrestin2 as described previously in other cell systems.

**Traffic of TRHR in HeLa Cells**—Previous studies have reported that β-arrestin-GFP chimeras maintain their function in receptor endocytosis (39) and may be used as markers of GPCR endocytosis (37, 40, 41). To determine whether β-arrestin and TRHR traffic together as a complex after receptor stimulation in our model, their relative cellular distributions were studied by immunofluorescence microscopy. The images shown focus on the plasma membrane of cells that are adherent to the coverslip as described previously (32). Under basal conditions, β-arrestin2 displayed a characteristic diffuse cytosolic distribution (39) (Fig. 1b), whereas the TRHR was evenly distributed at the cell surface (Fig. 1c). Following TRHR stimulation by TRH for 2 min, β-arrestin2 (Fig. 1d) translocated to the plasma membrane as a punctate staining. The TRHR also displayed a punctate staining at the plasma membrane (Fig. 1e) that completely colocalized with β-arrestin2. Following longer term stimulation (15 min), β-arrestin2 (Fig. 1f) and TRHR (Fig. 1g) were found colocalized in internal endosomal structures, demonstrating their continued presence in a shared endocytic pathway. β-Arrestin2 could therefore be used as a marker of TRHR in this...
model to follow its cell trafficking upon activation, as reported previously by Groarke et al. (34), in HEK-293 cells. This approach was preferred, in this study, instead of the direct labeling of TRHR with GFP, because it avoids any potential artifact that could arise from the fusion of GFP to the COOH-terminal tail of the receptor.

To determine whether the punctate areas where β-arrestin2 and TRHR colocalize at early times of receptor stimulation correspond to CPs, we revealed the localization of AP2 complexes using the anti-AP-2, AP6 antibody. The cellular distribution of β-arrestin2 relative to AP2 under basal conditions and after TRHR stimulation was thus monitored. In untreated cells, AP2 displayed a punctate staining (Fig. 2b) characteristic of plasma membrane CPs (18, 23, 32, 42), and β-arrestin2 demonstrated a diffuse cytoplasmic staining (Fig. 2a). There was no colocalization of the two different proteins under these conditions. Two minutes after TRHR activation, however, β-arrestin2 (Fig. 2c) was found to be completely colocalized with AP2 (Fig. 2d). Similar results were also observed with other specific CP markers, Eps15 (Fig. 2, e and f) and clathrin (data not shown). In an attempt to quantify this colocalization, the number of β-arrestin2-GFP and AP2 immunofluorescent dots in a defined area in the same cells 2 min after TRHR stimulation were counted. We counted more than 250 AP2 dots from four different preparations and found no significant difference in the number of β-arrestin2 dots. In addition, each β-arrestin2 dot was found to correspond to an AP2 dot. These results indicate that following TRHR stimulation, β-arrestin2 and TRHR are mobilized to CPs and that all CPs contained β-arrestin2 in agreement with studies investigating β-adrenergic receptor redistribution (16). In contrast, at later time points (15 min; Fig. 2, g and h) there was a loss of β-arrestin2-GFP colocalization with AP2 due to movement of the TRHR/β-arrestin2 complex to the endosomal compartment (see Fig. 3, lower panel).

To determine whether the areas that β-arrestin2 was mobilized to upon TRHR stimulation were areas of the plasma membrane undergoing active endocytosis, we compared the distribution of cell surface transferrin receptors (TIR), which undergo constitutive endocytosis, and β-arrestin2. In control cells, the TIR displayed a punctate staining due to its concentration in CPs (Fig. 3b), and β-arrestin2 demonstrated a diffuse cytosolic staining (Fig. 3a). Following activation of TRHR, β-arrestin2 was found to colocalize with most TIR clusters, indicating that the TRHR/β-arrestin2 complex mobilizes to the same areas of the membrane where TIR are undergoing constitutive endocytosis. This is in agreement with studies investigating the GPCR protease-activated receptor-1 in HeLa cells, which indicate a greater than 90% colocalization of protease-activated receptor-1 with TIR following short term stimulation of protease-activated receptor-1 (43). The localization of β-arrestin following longer TRHR stimulation was also investigated using Alexa-594-labeled transferrin to stain the early/recycling endosomal compartment. At these later times β-arrestin was colocalized with transferrin in perinuclear regions of the cell (Fig. 3, lower panel, 15 min TRH, yellow color) in agreement with results obtained in HEK-293 cells (33).

TRHR-β-Arrestin2 Complex Is Recruited to Pre-existing CPs—Significant cytosolic pools of clathrin and AP2 exist in cells that are often equivalent to or exceed membrane-bound forms (23, 44–46). In the nucleation model of GPCR endocytosis, agonist activation of receptor would promote CP formation, and this would be expected to result in an increase of total CPs. This would result in a recruitment of AP2 from the cytosolic to the plasma membrane pool giving an increase in AP2 levels at the membrane. We used three different approaches to investi-
surrounding areas of exocytosis (48). The fact that GPCRs do not promote the nucleation of "de novo" CPs may explain why their endocytosis is saturable in some cell types even in the presence of overexpressed β-arrestin (49), the limiting factor being the amount of constitutive CPs where GPCRs may be recruited through β-arrestin. In conclusion, therefore, the current study provides novel insights into the regulation of TRHR endocytosis, which directly demonstrates for the first time that a GPCR-β-arrestin complex is targeted to pre-existing CPs. Since the majority of GPCRs studied so far undergo ligand- and β-arrestin-dependent endocytosis, this model is of potential interest for other GPCRs.

**Fig. 5.** β-Arrestin-GFP is targeted to pre-existing areas of CP formation following TRHR stimulation. Real-time visualization of β-arrestin2- and β-arrestin1-GFP translocation to CPs in live HeLa cells, transiently expressing VSV-TRHR, RFP-Eps15, and either β-arrestin2- or β-arrestin1-GFP, in response to stimulation with 10 μM TRH. Experiments were performed on a heated microscope stage set at 37 °C. Upper panel, representative confocal microscopic images of β-arrestin2-GFP and RFP-Eps15 fluorescence are shown before and 0–75 s after addition of TRH to the medium. Bar, 5 μm. Lower panel, representative confocal microscopic images of β-arrestin1-GFP and RFP-Eps15 fluorescence are shown before and 0–30 s after addition of TRH to the medium. Bar, 5 μm. Higher magnifications show colocalization at 0, 15, and 30 s after TRHR activation.
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