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Dynamin and β-Arrestin Reveal Distinct Mechanisms for G Protein-coupled Receptor Internalization*

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Jie Zhang, Stephen S. G. Ferguson‡, Larry S. Barak§, Luc Ménard, and Marc G. Caron¶

From the Howard Hughes Medical Institute Laboratories and Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

The process of agonist-promoted internalization (sequestration) of G protein-coupled receptors (GPCRs) is intimately linked to the regulation of GPCR responsiveness. Following agonist-mediated desensitization, sequestration of GPCR is presumably associated with the dephosphorylation and recycling of functional receptors. However, the exact mechanisms responsible for GPCR sequestration, even for the prototypic β_2-adrenergic receptor (β_2AR), have remained controversial. We demonstrate here that dynamin, a GTPase that regulates the formation and internalization of clathrin-coated vesicles, is essential for the agonist-promoted sequestration of the β_2AR, suggesting that the β_2AR internalizes via the clathrin-coated vesicle-mediated endocytic pathway. In contrast, internalization of the angiotensin II type 1A receptor ($AT_{1A}R$), another typical GPCR, does not require dynamin. In addition, the $AT_{1A}R$ internalizes independent of the function of β-arrestin, a critical component for β_2AR cellular trafficking, but additional $AT_{1A}R$s are mobilized to the dynamin-dependent pathway upon overexpression of β-arrestin. These findings demonstrate that GPCRs can utilize distinct endocytic pathways, distinguishable by dynamin and β-arrestin, and that β-arrestins function as adaptor proteins specifically targeting GPCRs for dynamin-dependent endocytosis via clathrin-coated vesicles.

G protein-coupled receptor (GPCR)[1] internalization is a phenomenon triggered by agonist stimulation. This rapid internalization, referred to as sequestration (1), follows the agonist-mediated phosphorylation and desensitization of the signaling function of these receptors and is thought to contribute to the resensitization of GPCR responsiveness (2–5). Although the process of sequestration has been documented for many GPCRs, the cellular nature and molecular determinants of sequestration have remained elusive. Recently, however, phosphorylation of GPCRs by specific G protein-coupled receptor kinases has been demonstrated to facilitate agonist-mediated receptor sequestration (6, 7). This receptor phosphorylation serves to increase the affinity of GPCRs for β-arrestin proteins, which, in addition to uncoupling receptor-G protein interactions, can act as adaptor-like molecules for receptor trafficking (8).

Considerable evidence exists that GPCRs internalize via the clathrin-coated vesicle-mediated endocytic pathway (9–12). However, even for the prototypic GPCR, β_2-adrenergic receptor (β_2AR), this issue has remained controversial (9, 10, 13). Dynamin, a 100-kDa GTPase, originally isolated as a nucleotide-dependent microtubule binding protein, has been identified as a major component and marker of the clathrin-mediated endocytic pathway (14–18). Dynamin colocalizes with clathrin (19, 20) and binds to the appendage domain of α-adaptin, a component of the clathrin-coated pits (21). Functionally, dynamin contributes to the early stages of endocytosis by catalyzing a GTP-dependent pinching off of endocytic vesicles from the plasma membrane (22, 23). Dynamin mutants defective in GTP binding cause clathrin-coated pits to accumulate at the plasma membrane (17, 19), and in doing so, specifically block the internalization of some non-G protein-coupled receptors, including those for transferrin and epidermal growth factor (19, 24, 25).

To characterize the endocytic pathways utilized by GPCRs and to assess the specificity of the trafficking function of β-arrestin, we examined the effects of both dynamin and β-arrestin on the agonist-promoted sequestration of two prototypic GPCRs, the β_2AR and the angiotensin II type 1A receptor ($AT_{1A}R$). The results demonstrate both dynamin-dependent and -independent mechanisms of GPCR internalization. In addition, they assign a potential role for β-arrestin in directing GPCRs for dynamin-dependent endocytosis.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and fetal bovine serum were provided by Life Technologies, Inc. Human embryonic kidney cells (HEK 293) and COS-7 cells were from the American Type Culture Collection. Isoproterenol, angiotensin II, and secondary antibodies were purchased from Sigma. Anti-12CA5 monoclonal antibody was obtained from Boehringer Mannheim and anti-FLAG M2 monoclonal antibody from Kodak.

Plasmid Construction—All recombinant DNA procedures were carried out following standard protocols. 12CA5 epitope-tagged $AT_{1A}R$ was constructed as described previously (26). Briefly, the 9-amino acid sequence (YPYDVPDYA) recognized by the anti-12CA5 monoclonal antibody was inserted at the amino terminus of the receptor by polymerase chain reaction. The dynamin GTPase domain mutant (K44A) was described previously (24) and was generated by polymerase chain reaction to mutate codon AAG (lysine) to GCC (alanine). Positive clones were isolated, and the integrity of the coding sequences as well as the mutation was confirmed by dideoxy DNA sequencing.

Cell Culture and Transfection—HEK 293 and COS-7 cells were cultured in Eagle's minimal essential medium with Earle's salt and Dulbecco's modified Eagle's medium respectively, supplemented with heat-inactivated fetal bovine serum (10%, v/v). HEK 293 cells were seeded at a density of 2.5×10^6 cells/100-mm dish and COS-7 cells 1.0×10^6 cells/dish. The cells were transiently transfected with a modified calcium phosphate method (27). Following transfection (~18 h), the cells

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¶ To whom correspondence should be addressed: Duke University Medical Center, Box 3287, Durham, NC 27710. Tel.: 919-684-5433; Fax: 919-681-8641.

[1] The abbreviations used are: GPCR, G protein-coupled receptor; β_2AR, β_2-adrenergic receptor; $AT_{1A}R$, angiotensin II type 1A receptor; HEK 293 cells, human embryonic kidney 293 cells.

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were incubated with fresh medium and allowed to recover 7–9 h, before being reseeded in either 6- or 12-well dishes (Falcon), and allowed to grow an additional 15–18 h.

Receptor Expression—β_2AR expression was assessed by ^{125}I-pindolol binding studies done at 30 °C for 30 min (6). Bound ligand was separated on glass fiber filters (Whatman, GF/C) by vacuum filtration. The filters were washed four times with 4 ml of cold wash buffer (50 mM Tris and 120 mM NaCl (pH 7.2)) and counted in a γ-counter. Protein concentrations were determined with a Bio-Rad assay kit with bovine serum albumin as the standard. The level of β_2AR expression was between 1.0 and 1.5 pmol/mg of whole cell protein for all the experiments. $AT_{1A}R$ expression was adjusted to be equivalent to that of the β_2AR in each experiment, judged by anti-12CA5 monoclonal antibody binding.

Sequestration—Receptor sequestration was assessed by immunofluorescence microscopy and flow cytometry as described previously (5). In brief, sequestration was defined as the fraction of total cell surface receptors which, after exposure to agonist, are removed from the plasma membrane and thus are not accessible to antibodies from outside the cell. The cells were exposed to 10 μM isoproterenol (β_2AR agonist) or 100 nM angiotensin II for 30 min at 37 °C before antibody staining.

Data Analysis—Mean and the standard error of the mean are expressed for values obtained from the number of separate experiments indicated. Statistical significance was determined by analysis of variance.

RESULTS

The role of dynamin in GPCR internalization was examined by testing the ability of wild-type rat dynamin I and mutant dynamin I-K44A constructs to influence the sequestration of cotransfected β_2ARs in HEK 293 cells. Immunofluorescence microscopy and flow cytometry were used to assess changes in cell surface receptor number by measuring anti-12CA5 monoclonal antibody binding to the β_2AR, which was epitope-tagged on the amino terminus (5). In the absence of cotransfected dynamin, agonist stimulation resulted in rapid internalization of β_2ARs, directly observable as a loss of cell surface fluorescence by immunofluorescence microscopy (Fig. 1*A*). Quantitation by flow cytometry indicated that 53 ± 5% of cell surface receptors were internalized in response to a 30-min agonist stimulation (Fig. 1*B*). In contrast, in cells cotransfected with the dynamin GTPase domain mutant, K44A, agonist-promoted β_2AR internalization was abolished, as shown by the comparable fluorescence intensity on the surface of agonist-treated and -untreated cells (Fig. 1*A*). This dynamin mutant exhibits diminished guanine nucleotide binding affinity and when overexpressed in mammalian cells blocks endocytosis of transferrin and epidermal growth factor receptors (19, 24). In HEK 293 cells, dynamin-K44A inhibited β_2AR sequestration in an expression-dependent manner by as much as 86 ± 8% (Fig. 1*B*). However, overexpression of wild-type dynamin neither inhibited nor stimulated β_2AR sequestration in these experiments, indicating that endogenous dynamin expression was not rate-limiting in these cells. Similar results were obtained when β_2AR sequestration was assessed by radioligand binding. Taken together, these results provide strong evidence that functional dynamin is required for the internalization of the β_2AR.

Similar to the β_2AR, the $AT_{1A}R$ undergoes rapid internalization within minutes following agonist stimulation (28–30). It is generally assumed that the $AT_{1A}R$ and β_2AR internalize via the same cellular machinery. However, recent studies suggest that the $AT_{1A}R$ and β_2AR have different structural requirements governing their internalization (5, 30–32). The dissimilarity between these two receptors indicated that the $AT_{1A}R$ might represent a good candidate for testing whether dynamin plays a more general role in GPCR internalization. When tested in HEK 293 cells, the magnitudes of agonist-promoted β_2AR and $AT_{1A}R$ internalization were similar: 53 ± 5% *versus* 52 ± 4%. However, in contrast to the results obtained for the

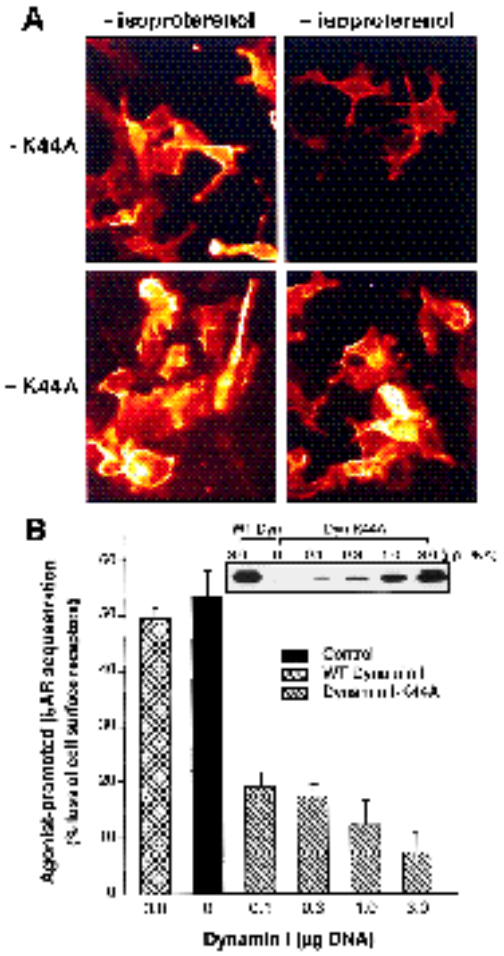

FIG. 1. **Effect of wild-type and mutant dynamin overexpression on the agonist-promoted sequestration of β_2ARs as assessed by indirect immunofluorescence (*A*) and flow cytometry (*B*) using the anti-12CA5 monoclonal antibody.** 12CA5 epitope-tagged β_2ARs (5) were transiently expressed in HEK 293 cells in a pcDNA1-Amp expression vector together with 3 μg of empty pCMV5 vector (control), 3 μg of pCB1 rat dynamin I, or 0.1–3 μg of rat dynamin I-K44A as indicated. For indirect immunofluorescence monitoring (*A*), cells were transfected with 3 μg of dynamin I-K44A. Expression of mutant and wild-type dynamin was monitored by immunoblot using an antibody for dynamin I (37). The data represent the mean ± S.E. of four independent experiments.

β_2AR, dynamin-K44A overexpression did not affect the maximal extent of $AT_{1A}R$ sequestration in response to agonist stimulation (Fig. 2*A*), nor did overexpression of wild-type dynamin. The same results were obtained when FLAG epitope-tagged β_2AR and 12CA5 epitope-tagged $AT_{1A}R$ were cotransfected into the same HEK 293 cells with or without dynamin-K44A and the internalization of the β_2AR and $AT_{1A}R$ were measured individually using anti-FLAG and -12CA5 antibodies, respectively (Fig. 2*B*). Therefore, unlike for the β_2AR, dynamin does not appear necessary for $AT_{1A}R$ internalization, suggesting that the $AT_{1A}R$ and β_2AR utilize distinct pathways for internalization.

Recently we demonstrated that β-arrestins serve as trafficking molecules participating in agonist-promoted β_2AR internalization (8). In addition, substitution of valine 53 for an aspartic acid in β-arrestin-1 produced a sequestration-specific dominant-negative β-arrestin (8). In the present experiments, overexpression of β-arrestin-1-V53D inhibited β_2AR sequestration by 50 ± 6%, whereas it did not affect $AT_{1A}R$ internalization (Fig. 3), indicating that, like dynamin, β-arrestins were not necessary for the internalization of the AT_{1A} receptor under

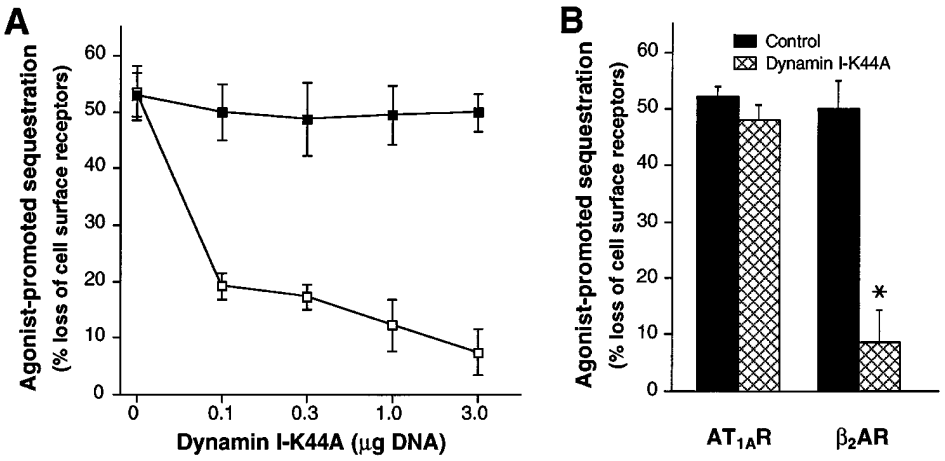

FIG. 2. **Differential effects of the dynamin mutant-K44A on the agonist-promoted sequestration of β_2ARs and AT_{1A}Rs.** *A*, HEK 293 cells were transfected transiently with pcDNA1-Amp containing the cDNA for either 12CA5 epitope-tagged β_2AR (□) or AT_{1A}R (■) together with increasing amount of pCB1 rat dynamin I-K44A and tested for agonist-promoted sequestration by flow cytometry. The expression levels of the β_2AR and AT_{1A}R were equivalent in these experiments. Results are expressed as the mean ± S.E. of three to four different experiments. *B*, FLAG epitope-tagged β_2AR and 12CA5 epitope-tagged AT_{1A}R were cotransfected into the same HEK 293 cells in the absence (control) or presence of 3 μg of pCB1 rat dynamin I-K44A. After being exposed to the corresponding agonist for 30 min, sequestration of the β_2AR and AT_{1A}R was assessed by flow cytometry using monoclonal anti-FLAG or -12CA5 antibody, respectively. The data represent the mean ± S.E. of four separate experiments. *, $p < 0.05$ compared with control values.

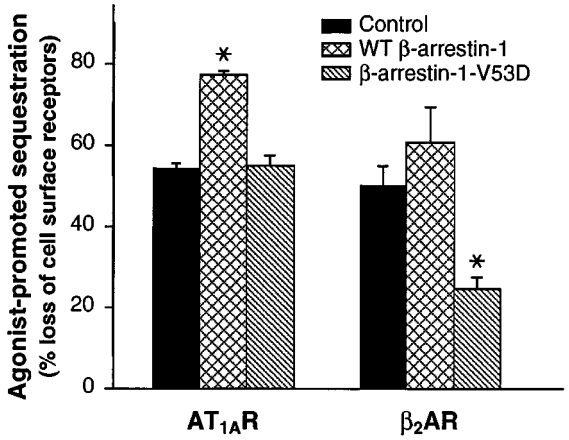

FIG. 3. **Effect of wild-type and mutant β-arrestin overexpression on the agonist-promoted sequestration of β_2ARs and AT_{1A}Rs.** 12CA5 epitope-tagged β_2ARs or AT_{1A}Rs were transfected into HEK 293 cells together with 5 μg of each of the following: empty pCMV5 vector (control), pCMV rat β-arrestin-1, or pcDNA1-Amp rat β-arrestin-1-V53D (8). The data represent the mean ± S.E. of four separate experiments in duplicate. *, $p < 0.05$ compared with control values.

normal conditions in HEK 293 cells. Although β-arrestin-1-V53D did not inhibit AT_{1A}R sequestration, overexpression of wild-type β-arrestin-1 did increase the magnitude of AT_{1A}R sequestration by 42 ± 2% relative to the normal value (Fig. 3). This observation suggested that, when overexpressed, β-arrestins mobilize AT_{1A}Rs for internalization by a mechanism similar to that utilized by the β_2AR. If so, like β_2AR sequestration, this increment of AT_{1A}R internalization in response to β-arrestin overexpression might be dynamin-dependent and inhibitable by overexpression of the mutant dynamin-K44A. As expected, when dynamin-K44A was cotransfected with β-arrestin-1 in HEK 293 cells, the β-arrestin-induced increment in AT_{1A}R sequestration was abolished (Fig. 4*A*). This suggests that β-arrestins specifically target GPCRs for dynamin-dependent endocytosis.

The extent of β_2AR sequestration appears to exhibit some dependence on cell type (6, 33). Therefore, to confirm the above observations in cells other than HEK 293, we examined the agonist-promoted internalization of both AT_{1A}R and β_2AR in COS-7 cells. In COS-7 cells, the β_2AR internalized poorly, whereas AT_{1A}R internalization was equivalent to that observed in HEK 293 cells (Fig. 4, *A* and *B*). Overexpression of β-arrestin-1 enhanced β_2AR internalization toward levels observed in HEK 293 cells and also increased AT_{1A}R sequestration by 52 ± 5% relative to the normal value. Co-expression of dynamin-K44A completely abolished β_2AR sequestration and inhibited the β-arrestin-induced increase in AT_{1A}R internalization in COS-7 cells (Fig. 4*B*). This is consistent with the results obtained in HEK 293 cells and supports the idea that β-arrestins direct GPCRs to the dynamin-dependent endocytic pathway.

DISCUSSION

Our findings provide the first evidence for the involvement of dynamin in GPCR internalization and suggest that, at least in the cell types tested here, β_2AR sequestration is mediated by the clathrin-coated vesicle endocytic pathway. Moreover, the ability of the dynamin dominant-negative mutant to reveal differences in the dependence of β_2AR and AT_{1A}R internalization on dynamin indicates that GPCR internalization can occur via distinct pathways. This idea is corroborated by the observation that the β-arrestin sequestration dominant-negative mutant did not affect AT_{1A}R internalization. The exact cellular mechanism(s) by which the AT_{1A}R normally internalizes still remains undetermined, but possibilities include the caveolae-mediated pathway or a novel non-clathrin-coated vesicle pathway (18).

The effect of β-arrestin overexpression on AT_{1A}R internalization clearly indicates that the cellular environment in which GPCRs are expressed (*i.e.* protein composition) plays an important role in determining the mechanism by which these receptors can internalize. Additionally, it is likely that receptor-specific determinants also regulate GPCR internalization. This is certainly the case for the β_2AR and AT_{1A}R. For example, the sequestration of the β_2AR is abolished by mutating tyrosine residue 326 to an alanine, whereas mutation of the equivalent residue in the AT_{1A}R has no effect (5, 30). Similarly, removal of the carboxyl-terminal tail eliminates agonist-promoted internalization of the AT_{1A}R, but not the β_2AR (8, 31, 32). Therefore, the pathway by which a given GPCR internalizes in response to agonist-stimulation is not only a function of cellular environment, but is an intrinsic property governed by receptor struc-

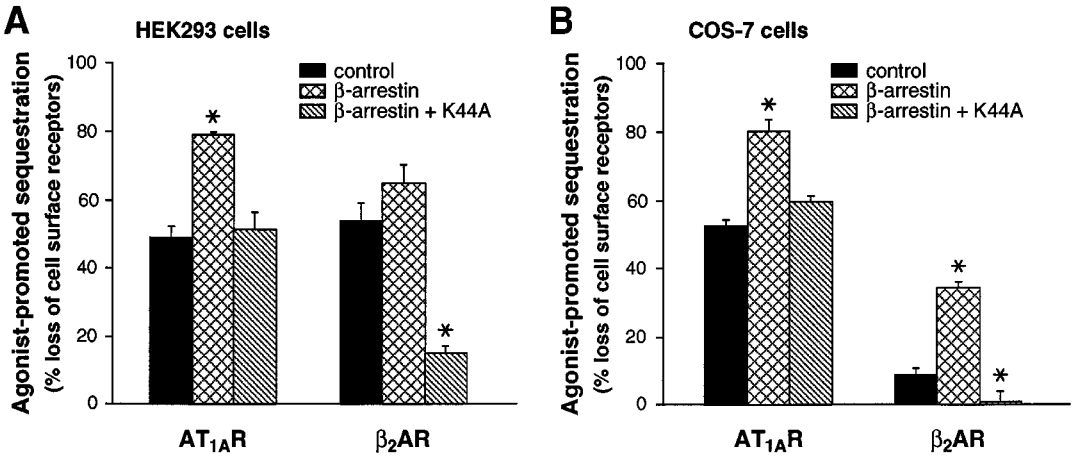

FIG. 4. **Inhibition of β-arrestin-dependent sequestration of β_2ARs and AT_{1A}Rs by dynamin mutant.** HEK 293 cells (*A*) or COS-7 cells (*B*) were transiently transfected with pcDNA1-Amp containing the cDNA for either 12CA5 epitope-tagged β_2AR or AT_{1A}R together with empty pcDNA1-Amp vector (control), 5 μg of pCMV rat β-arrestin-1, or 5 μg of pCMV rat β-arrestin-1 with 3 μg of pCB1 rat dynamin I-K44A. The data represent the mean ± S.E. of three to five separate experiments. *, $p < 0.05$ compared with control values.

ture. This might explain why several studies have provided conflicting evidence for the mechanism by which GPCRs internalize (9–13, 34). While the functional relevance of distinct pathways for GPCR internalization remains unclear, it is probable that these differences have implications for the regulation of signaling, recycling, and down-regulation of these receptors.

We propose that for the GPCRs of which the β_2AR is prototypic, the agonist-dependent step (35) for receptor internalization requires β-arrestin binding to agonist-activated phosphorylated receptors (36). In turn, β-arrestins serve as cellular trafficking molecules by specifically targeting these GPCRs to clathrin-coated vesicles. Dynamin, acting as part of the endocytic machinery analogous to its role in mediating the constitutive recycling of low density lipoprotein- and transferrin-receptors, is implicated in the agonist-independent step of GPCR recycling. In addition, our results establish that GPCRs can internalize by two distinct pathways, even in the same cell. The preferred pathway for the β-arrestin-dependent β_2AR internalization is dynamin-dependent and thus involves clathrin-coated vesicles, whereas agonist-promoted AT_{1A}R internalization proceeds independent of both dynamin and β-arrestin. Moreover, the ability of β-arrestin overexpression to increase the fraction of AT_{1A}Rs internalizing in a dynamin-dependent manner suggests plasticity in the choice of endocytic pathways utilized by GPCRs and that this choice can be influenced by the cellular milieu.

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