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Role of Arrestins in Endocytosis and Signaling of α_2-Adrenergic Receptor Subtypes*

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Jessica L. DeGraff, Alison W. Gagnon‡, Jeffrey L. Benovic§, and Michael J. Orsini

From the Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

We investigated the role of arrestins in the trafficking of human α_2-adrenergic receptors (α_2-ARs) and the effect of receptor trafficking on p42/p44 MAP kinase activation. α_2-ARs expressed in COS-1 cells demonstrated a modest level of agonist-mediated internalization, with $\alpha_{2c} > \alpha_{2b} > \alpha_{2a}$. However, upon coexpression of arrestin-2 (β-arrestin-1) or arrestin-3 (β-arrestin-2), internalization of the α_{2b} AR was dramatically enhanced and redistribution of receptors to clathrin coated vesicles and endosomes was observed. Internalization of the α_{2c} AR was selectively promoted by coexpression of arrestin-3, while α_{2a} AR internalization was only slightly stimulated by coexpression of either arrestin. Coexpression of GRK2 had no effect on the internalization of any α_2-AR subtype, either in the presence or absence of arrestins. Internalization of the α_{2b} and α_{2c} ARs was inhibited by coexpression of dominant negative dynamin-K44A. However, α_2-AR-mediated activation of either endogenous or cotransfected p42/p44 mitogen-activated protein (MAP) kinase was not affected by either dynamin-K44A or arrestin-3. Moreover, activation of p42/p44 MAP kinase by endogenous epidermal growth factor, lysophosphatidic acid, and β_2-adrenergic receptors was also unaltered by dynamin-K44A. In summary, our data suggest that internalization of the α_{2b}, α_{2c}, and to a lesser extent α_{2a} ARs, is both arrestin- and dynamin-dependent. However, endocytosis does not appear to be required for α_2-adrenergic, epidermal growth factor, lysophosphatidic acid, or β_2-adrenergic receptor-mediated p42/p44 MAP kinase activation in COS-1 cells.

Three human α_2-adrenergic receptor (α_2-AR)[1] subtypes have been identified, known as α_{2a}, α_{2b}, and α_{2c}, which belong to the G protein-coupled receptor (GPCR) superfamily. A fourth subtype, α_{2d}, has been identified in rat, mouse, and bovine tissues and is considered to be the ortholog of α_{2a} (1). Although the subtype-specific pharmacological differences, G protein coupling, and desensitization of the α_2-ARs have been extensively investigated, there is little information regarding their trafficking after agonist stimulation, in particular the role of arrestins and G protein-coupled receptor kinases (GRKs).

Arrestins[2] have been shown to mediate the desensitization and internalization of G protein-coupled receptors (reviewed in Refs. 2 and 3). This is accomplished by binding of arrestins to agonist-activated GPCRs after phosphorylation of receptors by GRKs. Arrestins are recruited to at least 15 different GPCRs after agonist activation, highlighting the critical role of arrestins in receptor turn-off (4). Binding of arrestin results in the physical uncoupling of GPCRs from heterotrimeric G proteins, thus terminating agonist-mediated signaling (5, 6). The α_{2a} AR has been shown to be phosphorylated by GRK2 and GRK3, but not GRK5 or GRK6, whereas the α_{2c} AR does not appear to be a substrate for GRK phosphorylation (7). The α_{2b} AR also undergoes agonist-promoted phosphorylation (8, 9). Similarly, the α_{2a} and α_{2b} ARs undergo rapid agonist-mediated desensitization, whereas the α_{2c} AR does not (7–9).

In several different cell types, the α_{2a} and α_{2b} ARs have been shown to localize initially to the plasma membrane (10, 11). However, a significant proportion of the α_{2c} AR appears to be intracellular, accumulating in both the Golgi apparatus and endoplasmic reticulum (10, 12). In HEK-293 cells, the α_{2b} AR has been shown to traffic to endosomes upon agonist stimulation, similar to the classic pattern of internalization observed with the β_2-adrenergic receptor (β_2-AR) (12). In contrast, the α_{2a} AR appears to undergo little or no agonist-mediated redistribution (12). However, in Chinese hamster ovary cells, internalization of the α_{2a} AR was significantly greater (13), which may be attributed to cell type-specific differences in regulatory proteins or in differences between the methods used to quantitate internalization. Recently, *in vitro* arrestin binding to the third intracellular loop of the α_{2a} AR has been reported, suggesting that arrestins have the ability to interact with the α_{2a} AR (14). Analysis of α_{2c} AR trafficking is complicated by the sizable pool of intracellular receptors. However, using multiple epitope tags and labeling of cell surface receptors, Daunt *et al.* observed agonist-promoted internalization of the plasma membrane-localized α_{2c} AR in HEK-293 cells, but did not detect redistribution of the intracellular pool of α_{2c} AR (12).

In the present study, we investigated the role of arrestins and GRKs in the agonist-mediated trafficking of the different α_2-AR subtypes. Since the different α_2-ARs display clear differ-

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‡ Current address: Cardiovascular Drug Discovery, Rhone-Poulenc Rorer Pharmaceuticals, Collegeville, PA 19426.

§ Established investigator of the American Heart Association. To whom correspondence should be addressed: Thomas Jefferson University, 233 S. 10th Street, Philadelphia, PA 19107. Tel.: 215-503-4607; Fax: 215-923-1098; E-mail: benovic@lac.jci.tju.edu.

[1] The abbreviations used are: α_2-AR, α_2-adrenergic receptor; β_2-AR, β_2-adrenergic receptor; EGF, epidermal growth factor; GPCR, G protein-coupled receptor, GRK, G protein-coupled receptor kinase; LPA, lysophosphatidic acid; p42/p44 MAP kinase, p42/p44 mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; TBST, Tris-buffered saline with Tween 20; HA, hemagglutinin.

[2] Although a variety of names have been used for the various mammalian arrestins, we propose that the following nomenclature be used based on the order of discovery of the arrestins: arrestin-1 (visual arrestin, S-antigen, 48-kDa protein); arrestin-2 (β-arrestin, β-arrestin-1); arrestin-3 (β-arrestin-2, arrestin3, thy-X arrestin); arrestin-4 (C-arrestin, X-arrestin).

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ences with respect to their intracellular localization and trafficking, they constitute an interesting receptor family in which to investigate the role of GRKs and arrestins in these processes. Furthermore, endocytosis has recently been suggested to be important in the GPCR-mediated activation of p42/p44 MAP kinase (15). Therefore, the α_2-AR family should also be useful to further explore a potential link between endocytosis and MAP kinase activation. In the present work, we used COS-1 cells to study the role of GRKs and arrestins in α_2 AR trafficking, since COS cells have been shown to contain low endogenous levels of GRKs and arrestins (16). We show that expression of arrestins, but not GRKs, differentially mediates the trafficking of the different α_2-AR subtypes. However, expression of wild-type arrestins or dominant negative dynamin-K44A had no effect on activation of p42/p44 MAP kinase by either α_2-ARs or endogenous EGF, lysophosphatidic acid (LPA), or β_2-adrenergic receptors in COS-1 cells.

EXPERIMENTAL PROCEDURES

Materials—LPA, isoproterenol, epinephrine, EGF, and pertussis toxin were purchased from Sigma. UK 14,304 was purchased from Research Biochemicals International. Unless otherwise noted, the standard concentrations of these compounds used in this study were: 10 μM UK 14,304, 100 μM epinephrine, 10 μM isoproterenol, 10 ng/ml EGF, and 25 μM LPA.

Plasmid Construction—α_{2a}, α_{2b}, and α_{2c} AR cDNAs in pBC12BI were kind gifts from John Regan (University of Arizona) (17–19). A Flag epitope was created by PCR amplification of a *Hin*dIII/*Nco*I fragment from pcDNA3-Flag-β_2AR. This fragment encodes a 23-amino acid amino-terminal peptide, which permits membrane targeting and processing to expose an epitope that is recognized by both the M1 and M2 Flag antibodies. The amino acid sequence of this region is: MKTIIASYIFCLVFADYKDDDDA. In order to introduce epitope tags at the amino terminus of each of the α_2-ARs, the full-length α_{2a} and α_{2c} ARs were digested with *Nco*I and *Sal*I and ligated with the PCR-amplified *Hin*dIII/*Nco*I fragment (containing the Flag epitope) and pBC (from pBC-Flag-β_2AR) that was digested with *Hin*dIII/*Sal*I. The 5′ region that resulted from the PCR reaction was confirmed by dideoxy sequencing. The Flag-tagged α_{2c} and α_{2a} ARs in pBC were digested with *Sal*I, blunted with Klenow, then digested with *Hin*dIII to release the insert. This insert was then ligated with pcDNA3 that had been digested with *Hin*dIII and *Eco*RV. To construct pcDNA3-Flag-α_{2b}AR, the pBC-based α_{2b} AR, which has three internal *Nco*I sites, was restricted with *Sfi*I, which cleaves at an internal 5′ *Sfi*I site, and *Hin*dIII. A 40-base pair double-stranded overlapping oligonucleotide was then designed to reconstruct the region from the *Nco*I site at the initiating methionine to the *Sfi*I site. The *Hin*dIII/*Nco*I Flag fragment, the 40-base pair *Nco*I/*Sfi*I fragment, and the *Sfi*I/*Hin*dIII α_{2b} AR fragment were then ligated together with *Hin*dIII-digested pBluescript KS (Stratagene). The Flag-tagged α_{2b} AR was then digested with *Hin*dIII to liberate the insert, blunted with Klenow, and subcloned into pcDNA3 that was digested with *Eco*RV. The plasmids pcDNA3-arrestin-2, pcDNA3-arrestin-3, pcDNA3-GRK2-CT (containing residues 495–689 of GRK2), and pcDNA3-dynamin-K44A have been described previously (20–23). pcDNA-HA-p42 was a gift from J. Silvio Gutkind (National Institutes of Health), and RasN17 was a gift from Jonathan Chernoff (Fox Chase Cancer Center).

Cell Culture and Transfection—COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. COS-1 cells were grown in 60-mm or 100-mm dishes (Falcon) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Cells were grown to 80–90% confluence prior to transfection. Cells were transfected using FuGENE-6 reagent (Boehringer Mannheim) according to the manufacturer's protocol. For transfection of 60-mm dishes, 10 μl of FuGENE-6 was used, and 30 μl of FuGENE-6 was used to transfect 100-mm dishes. Cells were incubated with FuGENE-DNA mixture for 5 h, after which the cells were split into 12-well dishes (for p42/p44 MAP kinase assays) or 24-well dishes (for ELISA). All assays were performed 24 h after transfection.

Internalization of Receptors—Internalization of α_2-ARs was assessed by ELISA essentially as described by Daunt *et al.* (12). Briefly, transfected cells in either 60-mm or 100-mm dishes were split into 24-well dishes coated with 0.1 mg/ml poly-L-lysine (Sigma). 24 h after transfection, cells were treated with or without 100 μM (−)-epinephrine in DMEM containing 0.3 mM ascorbate, then fixed for 5 min with 3.7% formaldehyde. The primary anti-Flag antibody M1 (Sigma or IBI) and the secondary antibody, goat anti-mouse conjugated with alkaline phosphatase (Bio-Rad), were used at a 1:1000 dilution. Visualization of antibody binding was performed using an alkaline phosphatase substrate kit (Bio-Rad). Plates were read at 405 nm in a microplate reader driven by Microplate Manager software (Bio-Rad).

Immunofluorescence Microscopy—COS-1 cells in 60-mm dishes were transfected as described above with 3 μg of the different Flag-tagged α_2-ARs and 1 μg of the indicated arrestin-3 construct. Following transfection, cells were split and allowed to adhere overnight on glass coverslips grown in 24-well dishes. To visualize cell surface receptors, cells were incubated with M1 antibody diluted 1:500 for 1 h at 4 °C in DMEM supplemented with 0.1% bovine serum albumin. Cells were then treated with 100 μM (−)-epinephrine for 10 min, washed, fixed with 3.7% formaldehyde, permeabilized with 0.05% Triton X-100 for 10 min, and incubated with goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (1:200 dilution). Coverslips were mounted using Slow-Fade mounting medium (Molecular Probes) and examined by microscopy on a Nikon Eclipse E800 fluorescence microscope using a Plan Fluor 40× objective. Cells expressing the lowest levels of transiently expressed proteins, but clearly above those of nonexpressing cells, were chosen for view. Images were collected using QED Camera software and processed with Adobe Photoshop version 3.0.

Analysis of p42/p44 MAP Kinase Activation—COS-1 cells in 100-mm dishes were transfected as described above, then split into 12-well dishes and serum-starved overnight in DMEM containing 0.5% fetal bovine serum. After agonist stimulation, cells were rapidly rinsed once with phosphate-buffered saline and lysed by addition of 100 μl of SDS sample buffer. Samples were heated to 95 °C for 10 min, and proteins (20 μl) were separated on 10% SDS-polyacrylamide gels. The gels were transferred to nitrocellulose and blocked for 30 min in blocking buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 5% nonfat dry milk. To detect p42/p44 MAP kinase activation, blots were incubated in blocking buffer overnight at 4 °C with a polyclonal rabbit antibody (New England Biolabs) that specifically recognizes the amino acids (Thr-202/Tyr-204) that are phosphorylated upon activation of p42/p44 MAP kinase (diluted 1:1000). The following day, blots were washed three times with TBST, then incubated with goat-anti rabbit horseradish peroxidase secondary antibody (Bio-Rad) diluted 1:2000 in blocking buffer for 1 h. Blots were then washed three times in TBST and developed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation and Analysis of HA-tagged ERK-2 (p42)—COS-1 cells were transfected with HA-ERK-2 and the indicated α_2-AR and either dynamin-K44A or pcDNA3. Cells were serum-starved in DMEM containing 0.5% fetal bovine serum overnight. Cells were stimulated with specific agonists as indicated in the figure legends, washed once with phosphate-buffered saline and lysed in 1 ml of ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 μg/ml leupeptin). Cells were scraped off the plates and sonicated twice using a Fisher model 550 sonic dismembrator set at 2 for 15 s each. All subsequent manipulations were performed on ice or at 4 °C with constant rocking on a platform rocker. Cell debris was removed by centrifugation for 10 min at 13,000 rpm in an Eppendorf microcentrifuge. The supernatant was precleared for 1 h to reduce nonspecific binding by addition of 25 μl of protein G-agarose (Boehringer Mannheim). Samples were then centrifuged in an Eppendorf microcentrifuge at 3000 rpm for 2 min and 3 μl of monoclonal anti-HA antibody (unpurified mouse ascites, Babco) was added to the supernatant for 3 h. 50 μl of protein G-agarose was then added, and the samples were incubated overnight. The next day, protein G-antibody-antigen complexes were collected by centrifugation and washed three times with cold lysis buffer. The final pellet was resuspended in 45 μl of SDS sample buffer and boiled for 10 min at 95 °C. 20 μl was electrophoresed on a 10% SDS-polyacrylamide gel, and the gel was transferred to nitrocellulose. Activated HA-p42 was detected using the phosphorylation state-specific p42/p44 antibodies as described above.

RESULTS

Effect of Arrestin and GRK Expression on α_2-AR Internalization—To investigate the role of arrestins and GRKs in trafficking of α_2-ARs, we initially assessed the internalization of the three human α_2-AR subtypes in COS cells. COS cells contain relatively low amounts of arrestins and GRKs in comparison to other cell types such as HEK-293 (16), thus enabling assess-

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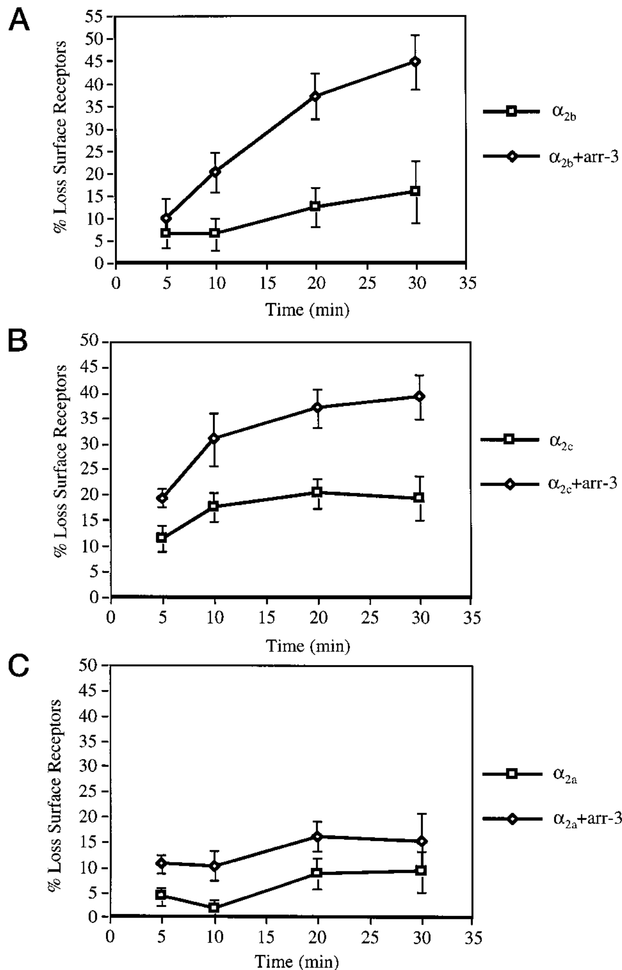

FIG. 1. **Internalization of α_2-ARs in the presence or absence of arrestin-3.** COS-1 cells in 100-mm dishes were transfected with 5 μg of pcDNA-Flag-α_{2b}AR (*A*), 5 μg of pcDNA-Flag-α_{2c}AR (*B*), or 0.3 μg of pcDNA-Flag-α_{2a}AR (*C*) and 5 μg of pcDNA-arrestin-3 or vector. In the case of the α_{2a} AR, an additional 4.7 μg of vector was cotransfected such that all dishes were transfected with a total of 10 μg of DNA. 5 h after transfection, cells were split to 24-well dishes. 24 h after transfection, cells were exposed to 100 μM epinephrine for the indicated times, fixed, and internalization of receptors was quantitated by ELISA as described under "Experimental Procedures." Values shown are the mean ± S.E. of at least four independent experiments performed in triplicate.

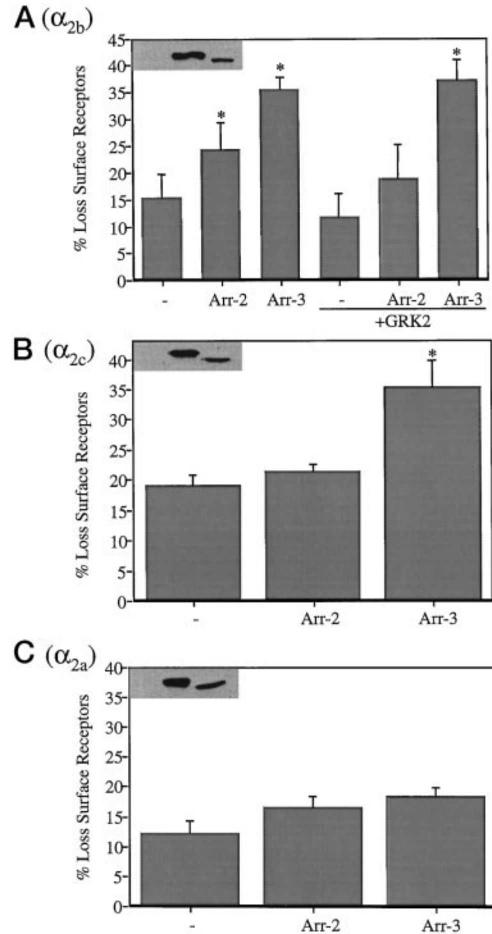

FIG. 2. **Effects of arrestins and GRKs on α_2-AR internalization.** COS-1 cells in 60-mm dishes were transfected with 3 μg of pcDNA-Flag-α_{2b}AR (*A*), 3 μg of pcDNA-Flag-α_{2c}AR (*B*), or 0.1 μg of pcDNA-Flag-α_{2a}AR (*C*) and 1 μg of pcDNA-arrestin-2, pcDNA3-arrestin-3, pcDNA-GRK2, or vector. In the case of α_{2a}, an additional 0.7–1.7 μg of vector was cotransfected such that all dishes were transfected with a total of 4 μg (arrestins alone) or 5 μg (arrestins + GRK2) of DNA. 5 h after transfection, cells were split to 24-well dishes. 24 h after transfection, cells were exposed to 100 μM epinephrine for 30 min, and internalization of receptors was quantitated by ELISA. Values shown are the mean ± S.E. of at least four independent experiments performed in triplicate. *Asterisks* (*) indicate the result of statistical analysis by paired t test with $p < 0.05$ sufficient to reject the null hypothesis. *Insets*, immunoblot analysis of arrestin expression from a representative experiment in cells cotransfected with vector alone (*left*), arrestin-2 (*middle*), or arrestin-3 (*right*).

ment of the relative contributions of GRKs and arrestins to receptor trafficking. COS-1 cells were transfected with plasmids expressing Flag epitope-tagged α_{2a}, α_{2b}, and α_{2c} ARs in the presence or absence of cotransfected arrestin-3, and receptor internalization was assayed by ELISA. In order to achieve equivalent expression of all three α_2-AR subtypes (4–5 pmol/mg), it was necessary to transfect 10-fold lower amounts of the α_{2a} AR construct (data not shown). In the absence of coexpressed arrestin-3, the α_{2b} AR displayed a slow rate of internalization, which reached 15% after 30 min (Fig. 1*A*). Internalization of the α_{2c} AR occurred at a faster rate, reaching 20% after 20 min (Fig. 1*B*). In contrast, there was no significant internalization of the α_{2a} AR (Fig. 1*C*). Internalization of both the α_{2b} and α_{2c} ARs was significantly promoted upon coexpression of arrestin-3 (Fig. 1, *A* and *B*). Both the rate and total amount of receptor internalization was increased in the presence of arrestin-3. In contrast, internalization of the α_{2a} AR was only modestly promoted by arrestin-3 coexpression (Fig. 1*C*).

We next compared the ability of arrestin-2 and arrestin-3 to promote α_2-AR internalization. We have previously shown that transfection of arrestin-2 or arrestin-3 results in approximately 20–30-fold overexpression compared with endogenous arrestin levels (21, 22). Both arrestin-2 and arrestin-3 promoted internalization of the α_{2b} AR, with arrestin-3 promoting a significantly higher level (Fig. 2*A*). Coexpression of GRK2, in either the presence or absence of arrestins, had no effect on α_{2b} AR internalization, suggesting that GRK2 is not limiting for α_{2b} AR internalization in COS-1 cells (Fig. 2*A*). Expression of GRK2 also did not promote internalization of either the α_{2a} or α_{2c} ARs even at times earlier than 30 min (data not shown). Interestingly, α_{2c} AR internalization was significantly promoted by arrestin-3, but not by arrestin-2 (Fig. 2*B*) even though expression of arrestin-2 was more efficient. These data suggest that either arrestin-2 or arrestin-3 can promote α_{2b} AR internalization, but that the α_{2c} AR may preferentially respond to arrestin-3. In contrast, arrestin-2 or arrestin-3 (Fig. 2*C*) only modestly promoted α_{2a} AR internalization.

To further characterize the arrestin-mediated internalization of α_2-ARs, we transfected cells with either the α_{2b} or α_{2c} AR

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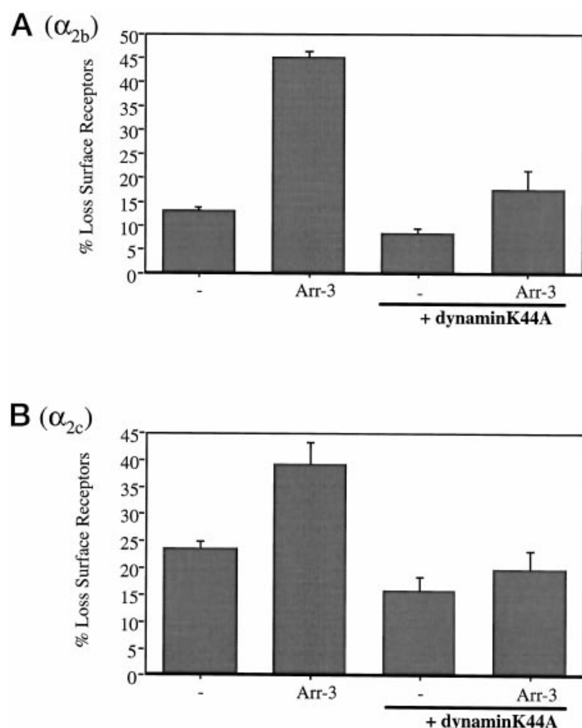

FIG. 3. **Effect of dominant negative dynamin-K44A on α_2-AR internalization.** COS-1 cells in 60-mm dishes were transfected with 3 μg of pcDNA-Flag-α_{2b}AR (*A*) or 3 μg of pcDNA-Flag-α_{2c}AR (*B*), 1 μg of pcDNA3-arrestin-3 or vector, and 1 μg of pcDNA-dynamin-K44A. 5 h after transfection, cells were split to 24-well dishes. 24 h after transfection, cells were exposed to 100 μM epinephrine for 30 min, and internalization of receptors was quantitated by ELISA. Values shown are the mean ± S.E. of three or four independent experiments performed in triplicate.

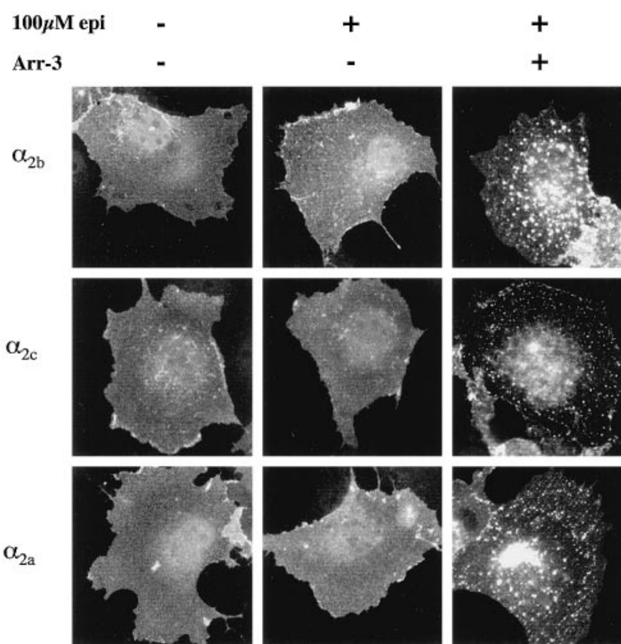

FIG. 4. **Immunofluorescence analysis of α_2-AR internalization.** COS-1 cells in 60-mm dishes were transfected with 3 μg of pcDNA-Flag-α_{2b}AR, 3 μg of pcDNA-Flag-α_{2c}AR, or 0.1 μg of pcDNA-Flag-α_{2a}AR and 1 μg of pcDNA-arrestin-3 or vector. In the case of α_{2a} AR, an additional 0.7 μg of vector was cotransfected such that all dishes were transfected with a total of 4 μg of DNA. 5 h after transfection, cells were split onto glass coverslips in 24-well dishes. 24 h after transfection, cells were incubated for 1 h at 4 °C with M1 anti-Flag antibody to label cell surface receptors, then washed to remove unbound antibody and warmed to 37 °C. Where indicated, cells were exposed to 100 μM epinephrine for 10 min. Cells were then fixed, incubated with goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody, and imaged as described under "Experimental Procedures."

and arrestin-3, with or without dynamin-K44A, a dominant negative mutant of dynamin-I which does not bind GTP (24). Arrestin-mediated internalization of the α_{2b} and α_{2c} ARs was completely inhibited by coexpression of dynamin-K44A (Fig. 3, *A* and *B*). Coexpression of dynamin-K44A in the absence of arrestin-3 also revealed a modest inhibition of internalization of both receptors. These data suggest that agonist-induced internalization of both α_{2b} and α_{2c} ARs in both the presence and absence of arrestin-3 is a dynamin-dependent process.

Immunofluorescence Analysis of α_2-AR Trafficking—We next examined the redistribution of α_2-ARs by immunofluorescence. In permeabilized cells, the α_{2a} and α_{2b} ARs were predominantly localized to the cell surface. In contrast, a significant amount of the α_{2c} AR was localized to the Golgi apparatus and endoplasmic reticulum (Ref. 12 and data not shown). In order to follow the redistribution of receptors present on the cell surface, cells were preincubated with Flag antibody at 4 °C prior to agonist exposure. In this way, only the trafficking of receptors initially present on the cell surface would be detected. Cells were transfected with α_2-ARs in the absence or presence of arrestin-3. In the absence of agonist and arrestin, each α_2-AR subtype was clearly detected on the cell surface (Fig. 4, *left panels*). When cells were stimulated with agonist for 10 min in the absence of coexpressed arrestin-3, little or no redistribution of α_2-ARs was detected (Fig. 4, *middle panels*). In the presence of coexpressed arrestin-3, the α_{2a}, α_{2b}, and α_{2c} ARs demonstrated agonist-dependent redistribution to clathrin-coated pits and endosomes (Fig. 4, *right panels*). No redistribution of receptors was observed in arrestin-3-expressing cells in the absence of agonist and internalization was significantly inhibited by coexpression of dynamin-K44A (data not shown). These results suggest that each α_2-AR subtype internalizes in an arrestin-dependent manner. Taken together with the results of the ELISA, however, internalization of the α_{2a} AR appears to be significantly less efficient than either the α_{2b} or α_{2c} ARs.

Role of Endocytosis in α_2-AR-mediated Activation of p42/p44 MAP Kinase—Recently, it was reported that arrestin-mediated internalization of GPCRs in HEK-293 cells plays a critical role in their ability to activate p42/p44 MAP kinase (15). Therefore, we examined the possibility that internalization may be required for α_2-AR-mediated MAP kinase activation. Moreover, the differences between the α_2-AR subtypes with respect to their internalization suggested that they may constitute an ideal model for studying the role of endocytosis in p42/p44 MAP kinase activation. Initially, we examined the ability of each α_2-AR to activate p42/p44 MAP kinase in the absence of arrestin overexpression. Although little internalization of the α_2-ARs was observed in the absence of arrestins (Figs. 1 and 4), each α_2-AR subtype rapidly activated p42/p44 MAP kinase to a similar extent after agonist addition, whereas no activation of p42/p44 was observed in untransfected cells (Fig. 5*A*). α_2-AR-mediated p42/p44 MAP kinase activation was maximal between 5 and 30 min after agonist addition and slowly declined thereafter. The activation of p42/p44 MAP kinase by α_2-ARs was inhibited by both dominant negative Ras (RasN17, Fig. 5*B*) and pertussis toxin treatment (Fig. 5*C*). Thus, activation of p42/p44 MAP kinase by all three α_2-AR subtypes appears to proceed via activation of $G_{i/o}$ and Ras, in agreement with previous studies on the α_{2a} AR (25, 26).

Since the α_2-ARs exhibited minimal agonist-mediated internalization in the absence of coexpressed arrestins, we next examined the effect of expressing either wild-type arrestin-3 or dominant negative dynamin-K44A on α_2-AR-mediated p42/p44 MAP kinase activation. α_2-AR-mediated activation of p42/p44

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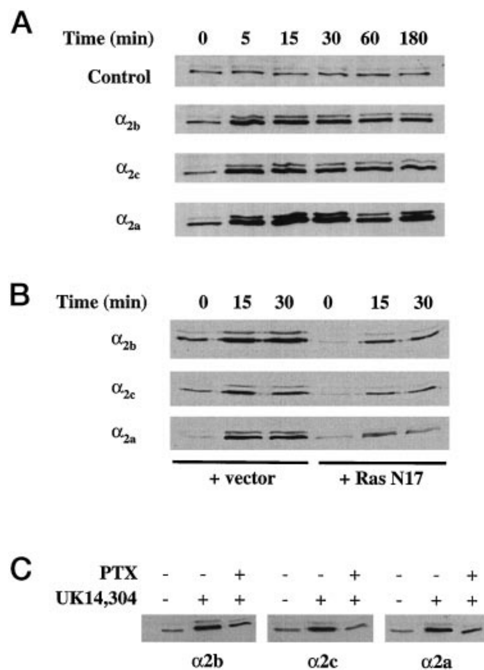

FIG. 5. **Activation of p42/p44 MAP kinase by α_2-ARs.** *A*, COS-1 cells in 100-mm dishes were transfected with 5 μg of pcDNA3-Flag-α_{2b}AR, 5 μg of pcDNA3-Flag-α_{2c}AR, or 0.3 μg of pcDNA3-Flag-α_{2a}AR. Activation of endogenous p42/p44 was measured at the indicated times after addition of 10 μM UK 14,304 by immunoblot using a polyclonal rabbit antibody specific to the phosphorylated form of p42/p44. The data shown are representative of three independent experiments. *B*, COS-1 cells were transfected with α_2-ARs as described in *A* together with 5 μg of pcDNA (*vector*), or 5 μg pcDNA-RasN17 (*RasN17*), and stimulated with 10 μM UK 14,304. Endogenous p42/p44 MAP kinase activation was determined as described in *A*. *C*, Activation of endogenous p42/p44 was determined in COS-1 cells transfected with α_2-ARs as described in *A*. Where indicated, cells were treated with 10 μM UK 14,304 for 15 min in the presence or absence of 100 ng/ml pertussis toxin added to cells 16 h prior to stimulation and present for the duration of the experiment.

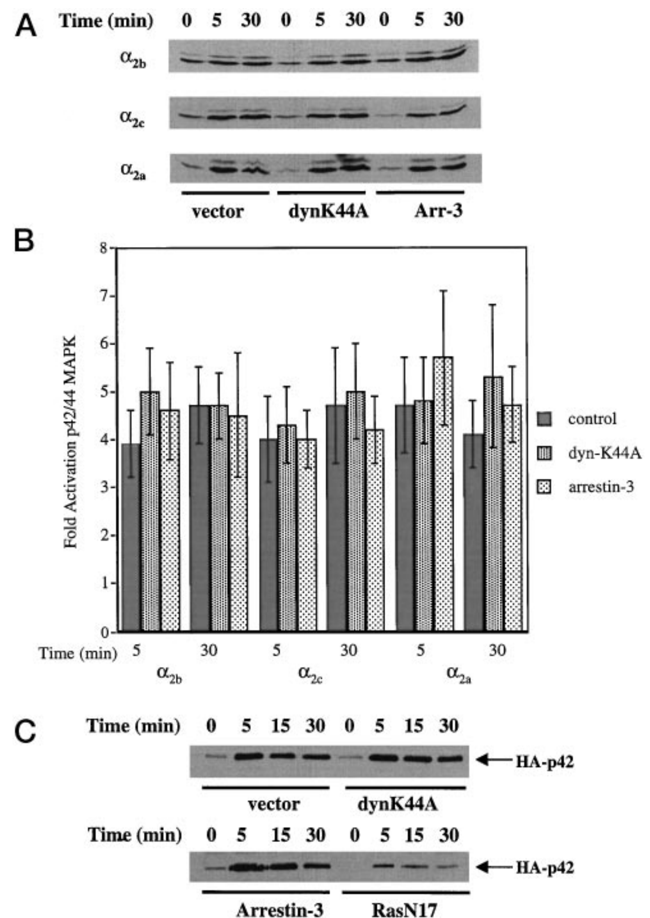

FIG. 6. **Effect of dynamin-K44A and wild-type arrestin-3 on α_2-AR-mediated p42/p44 MAP kinase activation.** *A*, COS-1 cells were transfected with 5 μg of pcDNA-Flag-α_{2b}AR, 5 μg of pcDNA-Flag-α_{2c}AR, or 0.3 μg of pcDNA-Flag-α_{2a}AR together with 5 μg of vector, dynamin-K44A, or wild-type arrestin-3. Cells were stimulated with 10 μM UK 14,304 for the indicated times, and p42/p44 activation was determined as described in Fig. 5. In all experiments, overexpression of dynamin K44A or arrestin-3 was confirmed by immunoblot. *B*, the experiment described in *A* was repeated and p42/p44 activation was quantitated by densitometric scanning of immunoblots. Values shown are the mean ± S.E. of six independent experiments. *C*, COS-1 cells were transfected with 5 μg of pcDNA3-Flag-α_{2b}AR together with 5 μg of vector, dynamin-K44A, wild-type arrestin-3, or RasN17 and 5 μg of HA-p42. The total amount of DNA transfected was held constant at 15 μg. Cells were stimulated with 10 μM UK 14,304 for the indicated times and lysed, and HA-p42 was immunoprecipitated with a mouse monoclonal antibody directed against the HA epitope. Activation of HA-p42 was determined by immunoblot with a polyclonal rabbit antibody specific to phosphorylated p42/p44. Immunoprecipitation of HA-p42 was assessed in parallel immunoblots using a phosphorylation state-independent antibody directed against p42. This experiment was repeated twice with similar results.

was not significantly altered by coexpression of either arrestin-3 or dynamin-K44A (Fig. 6, *A* and *B*). We also attempted to assess the effect of receptor internalization on p42/p44 MAP kinase activation by treating cells with concanavalin A, hypotonic sucrose, or monodansylcadaverine, which each inhibit receptor internalization albeit by different mechanisms (27). Monodansylcadaverine, which inhibited α_2-AR internalization as assessed by ELISA, had no effect on α_2-AR-stimulated p42/p44 activation (data not shown), supporting our dynamin-K44A results. In contrast, we observed that concanavalin A and sucrose directly activated p42/p44 in COS cells (*i.e.* in the absence of receptor and agonist) and were therefore not suited for this analysis.

In the experiments shown in Fig. 6 (*A* and *B*), both α_2-ARs and either arrestin-3 or dynamin-K44A were cotransfected. Thus, it is unlikely that the expression of arrestin-3 or dynamin-K44A would be limiting with respect to their ability to modulate the activation of endogenous p42/p44. Moreover, cotransfection of dominant negative Ras significantly inhibited activation of endogenous p42/p44 MAP kinase (Fig. 5*B*). However, to address the possible limitations of transient transfection on p42/p44 activation, we cotransfected HA-tagged p42 MAP kinase together with the α_{2b} AR and either dynamin-K44A, arrestin-3 or RasN17. The α_{2b} AR was chosen since its internalization was the most responsive to arrestin-3 and was completely inhibited by dynamin-K44A (Figs. 2 and 3). The coexpression of either wild-type arrestin-3 or dynamin-K44A did not affect α_{2b} AR-mediated activation of transfected HA-p42 MAP kinase (Fig. 6*C*). In contrast, cotransfection of RasN17 resulted in almost complete inhibition of α_{2b} AR-mediated HA-p42 activation. Taken together, these results strongly suggest that internalization of the α_2-ARs is not required for activation of p42/p44 MAP kinase.

Role of Endocytosis in Activation of p42/p44 MAP Kinase by Endogenous β_2-Adrenergic, LPA, and EGF Receptors—We next addressed the question of whether the lack of effect of arrestin or dynamin-K44A on α_2-AR-mediated p42/p44 MAP kinase activation was specific to the α_2-ARs or a general property of receptor function in COS-1 cells. Therefore, we examined the effect of dynamin-K44A expression on activation of p42/44 by endogenous LPA, β_2-adrenergic, and EGF receptors in COS-1 cells. The dynamin-dependent internalization of the β_2-AR has been well documented, and internalization of the EGF receptor

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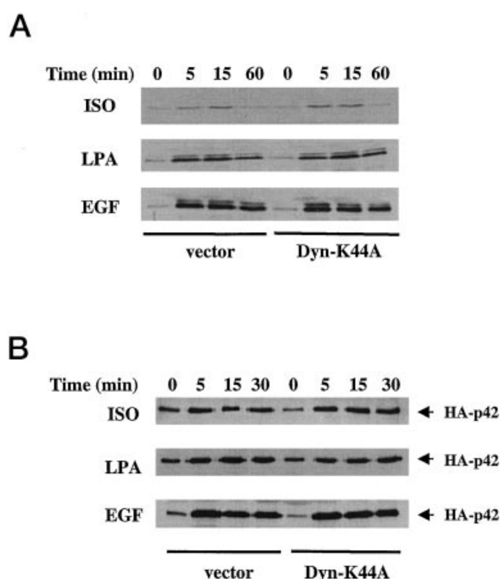

FIG. 7. **Effect of dynamin-K44A on p42/p44 activation by EGF, β_2-adrenergic, and LPA receptors.** *A*, COS-1 cells were transfected with 5 μg of vector or dynamin-K44A and stimulated for the indicated times with 10 μM (−)-isoproterenol, 25 μM lysophosphatidic acid, or 10 ng/ml EGF. Activation of p42/p44 was determined as described in Fig. 5*A*. *B*, COS-1 cells were transfected with 5 μg of vector or dynamin-K44A and 5 μg of HA-p42. Cells were stimulated as described in *A* and then lysed, and HA-p42 was immunoprecipitated with a mouse monoclonal antibody directed against the HA epitope. Activation of HA-p42 was determined by immunoblot using a polyclonal rabbit antibody specific to phosphorylated p42/p44. For each experiment, overexpression of HA epitope-tagged dynamin-K44A was determined by stripping and reprobing of blots with an antibody directed against the HA tag and immunoprecipitation of HA-p42 was monitored in parallel immunoblots using a phosphorylation state-independent antibody directed against p42. These experiments were repeated twice for each receptor with similar results.

has also been reported to be mediated by clathrin-coated pit endocytosis (28, 29). The role of arrestin or dynamin in the trafficking of the LPA receptor has not been reported, although it has been observed that dynamin-K44A and a dominant negative arrestin-2 mutant inhibit LPA-mediated activation of p42/p44 in HEK-293 cells (15). We found that overexpression of dynamin-K44A had no significant effect on the ability of isoproterenol, EGF, or LPA to activate p42/p44 (Fig. 7*A*). β_2-AR-mediated activation was specifically inhibited by the β-antagonist alprenolol, and LPA-mediated p42/p44 activation was sensitive to overexpression of GRK2-CT, which sequesters G$\beta\gamma$ subunits (data not shown). However, it was possible that transient transfection of dynamin-K44A would be insufficient to detect an effect on activation of both endogenous p42/p44 MAP kinase and receptors present in all cells. Therefore, this experiment was repeated by cotransfecting both dynamin-K44A and HA-tagged p42 MAP kinase. HA-p42 was immunoprecipitated, and its activation was assessed by immunoblot using a phosphorylation state-specific p42/p44 antibody. Activation of HA-p42 MAP kinase by EGF, LPA, and isoproterenol was unaltered by overexpression of dynamin-K44A (Fig. 7*B*). Taken together, these data suggest that endocytosis is not required for receptor-mediated activation of p42/p44 MAP kinase in COS-1 cells.

DISCUSSION

The role of arrestins in the trafficking and signaling of the α_2-adrenergic receptors has not been investigated. In this study, we show that arrestins significantly promote internalization of the human α_{2b} and α_{2c} ARs. Internalization of the α_{2c} AR was promoted to a significantly greater extent by arrestin-3 compared with arrestin-2. In contrast, internalization of the α_{2a} AR was only modestly promoted by coexpression of arrestins. However, endocytosis of α_2-ARs does not appear to be required for their ability to activate p42/p44 MAP kinase. Furthermore, EGF, LPA, and β_2-adrenergic receptor-mediated activation of p42/p44 MAP kinase was also insensitive to overexpression of dynamin-K44A, a GTP binding-deficient mutant that has been shown to inhibit both clathrin coated vesicle- and caveolae-mediated receptor internalization (24, 30).

We observed significant differences between the α_2-AR subtypes with respect to their cellular localization and their ability to internalize in response to arrestins. Similar to previous reports using different cell types (10, 12), both the α_{2a} and the α_{2b} ARs were initially localized to the cell surface. In contrast, a significant proportion of the α_{2c}AR was localized to the Golgi apparatus and endoplasmic reticulum. The α_{2b}AR internalized to a greater extent in the presence of either arrestin-2 or arrestin-3 compared with the α_{2a} or the α_{2c} ARs and arrestin-3 promoted α_{2b}AR internalization more effectively than arrestin-2. Internalization of the α_{2a}AR was only slightly promoted by either arrestin-2 or arrestin-3. However, the proportion of the α_{2a} AR that did internalize was localized to clathrin-coated pits and endosomes. The agonist-mediated internalization of all three α_2-AR subtypes in the absence of arrestin overexpression, although modest, was only slightly inhibited by expression of dynamin-K44A. Thus, it is possible that internalization of receptor under these conditions may be at least partially dynamin-independent, similar to that previously observed with the angiotensin 1A receptor (31). Although agonist-activated α_{2a} and α_{2b} ARs have been reported to be substrates for GRK phosphorylation (7, 8), cotransfection of GRK2 did not promote internalization of the α_2-ARs or enhance arrestin-promoted internalization. However, internalization of both the α_{2b} and α_{2c} ARs in HEK-293 cells has been shown to occur in the absence of cotransfected GRKs and arrestins (12), and HEK-293 cells possess higher endogenous levels of both GRKs and arrestins (16). In COS cells, internalization of the β_2-AR was shown to be promoted by expression of arrestins, but not by GRK2 (16). Thus, it is likely that trafficking of α_2-ARs, as well as other GPCRs, will be highly dependent upon the complement of GRKs and arrestins in a given cell type, as well as the affinity of these proteins for a given GPCR.

Interestingly, internalization of the α_{2c} AR was promoted to a much larger extent by arrestin-3 compared with arrestin-2. To our knowledge, this is the first reported observation of significant preference of a GPCR for either arrestin-2 or arrestin-3. Since both the ELISA and the immunofluorescence studies exclusively measured surface α_{2c} AR, the intracellular pool of receptor did not confound our results. Studies performed in HEK-293 cells also showed that cell surface α_{2c} ARs can internalize in response to agonist, whereas no redistribution of the intracellular pool of receptor was observed (12). However, α_{2c} ARs do not appear to be a substrate for GRK phosphorylation (7), and we observed that coexpression of GRK2 had no effect on α_{2c}AR internalization. It is possible that internalization of the α_{2c}AR receptor may occur in a phosphorylation-independent manner. Indeed, internalization of β_2-AR mutants that are deficient in agonist-mediated phosphorylation can be rescued by overexpression of arrestins (32). More recently, phosphorylation-independent but arrestin- or dynamin-dependent internalization has been described for both the follitropin and δ opioid receptors (33, 34). The exquisite specificity of visual arrestin for rhodopsin has been well characterized (35). However, the precise determinants that account for this specificity, either within the receptor binding domain of arrestin or the cytoplasmic loops of rhodopsin or other GPCRs are not known.

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The subtype-specific differences noted here between the α_2-ARs may provide a suitable model to address the question of specificity of GPCR-arrestin interaction.

Several observations support our conclusion that endocytosis of α_2-ARs is not a prerequisite for their activation of p42/p44 MAP kinase. First, agonist-induced activation of all three α_2-AR subtypes resulted in activation of p42/p44 MAP kinase under conditions in which little or no receptor endocytosis was observed. Second, overexpression of arrestins, which significantly promoted endocytosis, did not affect the magnitude of p42/p44 activation. Conversely, expression of dynamin-K44A, which ablated arrestin-mediated internalization, did not inhibit activation of p42/p44 even in the absence of coexpressed arrestin. Third, in experiments in which receptor, dynamin-K44A, or wild-type arrestin-3 and HA-tagged p42 MAP kinase were transfected into cells, we observed no effect of either dynamin-K44A or arrestin-3 on α_2-AR-mediated HA-p42 activation. Thus, it is unlikely that the limitations imposed by transfection efficiency account for the lack of effects of either dynamin-K44A or arrestin-3 on activation of endogenous p42/p44 MAP kinase. Moreover, cotransfection of dominant negative RasN17 inhibited activation of endogenous p42/p44 MAP kinase and transfected HA-p42 MAP kinase by all three α_2-AR subtypes. Interestingly, expression of dynamin-K44A did not inhibit p42/p44 activation by the β_2-adrenergic, EGF, or LPA receptors. Dynamin-dependent endocytosis of both the β_2-AR (31) and the EGF receptor (29) has been reported, although evidence of dynamin-dependent trafficking of the LPA receptor is lacking. Therefore, our results suggest that the lack of effect of mediators of receptor internalization on p42/p44 MAP kinase activation is not unique to α_2-ARs, but extends to endogenous receptors in COS-1 cells as well.

Our results contrast with those of Daaka *et al.* (15), who reported that activation of p42/p44 MAP kinase by endogenous β_2-adrenergic and LPA receptors in HEK-293 cells is inhibited by cotransfection of either dynamin-K44A or dominant negative arrestin-2 (V53D). It was proposed that inhibition of p42/p44 MAP kinase occurred at a step downstream from Raf, since second messenger generation, Shc phosphorylation, and Raf activity were not affected by either dominant negative dynamin or arrestin. The differences between our studies might be accounted for by the use of different cell lines and their respective levels of arrestins and signaling intermediates or by differences in their endocytic pathways. Other GPCRs have been reported to activate p42/p44 MAP kinase in the absence of detectable receptor internalization. Activation of the μ-opioid receptor by morphine activates p42/p44 (36), but fails to induce receptor internalization (37). Furthermore, cell-type and agonist-specific activation of κ-opioid receptors also promotes p42/p44 MAP kinase activation independent of receptor internalization (38). Recently, endocytosis of the insulin-responsive glucose transporter GLUT4 has been reported to be sensitive to expression of dynamin-K44A, whereas insulin signaling, including MAP kinase activation, was unaffected (39). In HeLa cells made defective for receptor-mediated endocytosis by conditional expression of dynamin-K44A, EGF-mediated cell proliferation was enhanced (40). In contrast, both EGF receptor and p42/p44 phosphorylation appeared to be reduced, while Shc phosphorylation was increased. Whether reduced MAP kinase activation was a consequence of reduced activation of the EGF receptor or other signaling intermediates in the MAP kinase pathway or reduced EGF receptor internalization *per se* remains unclear. A recent report also suggests that activation of p42/p44 MAP kinase may be involved in the phosphorylation and desensitization of μ-opioid receptor (36). Thus, there is likely to be a complex interplay between signal transduction molecules and regulators of receptor endocytosis such as GRKs and arrestins that ultimately determines the extent and duration of responses to different stimuli. Analysis of the interaction of molecules that facilitate receptor desensitization and internalization with components of the GPCR and receptor tyrosine kinase signaling pathways should greatly enhance our understanding of how cells control responses to extracellular stimuli.

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Role of Arrestins in Endocytosis and Signaling of α_2-Adrenergic Receptor Subtypes

Jessica L. DeGraff, Alison W. Gagnon, Jeffrey L. Benovic and Michael J. Orsini

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