Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors

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Many G protein-coupled receptors (GPCRs) have been shown to activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade. Recently, it was discovered that for some GPCRs, events associated with receptor internalization and receptor phosphorylation, specifically, it was discovered that for some GPCRs, events associated with receptor internalization and receptor phosphorylation, are required for ERK activation. This suggests that the sensitivity of GPCR-mediated ERK activation might correlate with signaling via EGFR transactivation. That is, we hypothesized that, in cells in which GPCRs activate MAPK via transactivation of the EGFR, ERK activation would be sensitive to inhibitors of endocytosis, regardless of whether the GPCR itself underwent agonist-induced internalization. To test this hypothesis, we have examined the role of clathrin-mediated endocytosis in ERK activation via internalization of β2 ARs and noninternalizing α2A ARs in COS-7 cells, a cell type in which both receptors stimulate MAPK primarily via EGFR transactivation.

Because direct EGF-induced ERK activation has been shown to depend on clathrin-mediated endocytosis (21), we hypothesized that the sensitivity of GPCR-mediated ERK activation might correlate with signaling via EGFR transactivation. That is, we hypothesized that, in cells in which GPCRs activate MAPK via transactivation of the EGFR, ERK activation would be sensitive to inhibitors of endocytosis, regardless of whether the GPCR itself underwent agonist-induced internalization. To test this hypothesis, we have examined the role of clathrin-mediated endocytosis in ERK activation via internalization of β2 ARs and noninternalizing α2A ARs in COS-7 cells, a cell type in which both receptors stimulate MAPK primarily via EGFR transactivation.

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Materials and Methods

Materials. Tyrphostin AG1478 and recombinant EGF were from Calbiochem, monodansylcadaverine (MDC) was from Sigma, and pertussis toxin was from List Biological Laboratories (Campbell, CA). Anti-phospho-MAPK antibodies were from New England Biolabs, the total ERK 1/2 antibody and the EGFR antibodies were from Upstate Biotechnology (Lake Placid, NY), and the anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY). The unlabelled and rhodamine-labeled 12CA5 antibodies were from Roche Biochemicals, and the M2 Flag antibody was from Sigma. The anti-hemagglutinin (HA) affinity beads were from Covance (Princeton, NJ). Secondary antibodies were from Jackson ImmunoResearch (Indianapolis). All other reagents were standard laboratory grade.

Plasmids. HA-α2A was obtained from Brian Kobilka (Stanford Univ.), β-arrestin 1 318–419 from J. L. Benovic (Thomas Jefferson Univ.), EGFR–green fluorescent protein (GFP) from A. Sorkin (University of Colorado Health Sciences Center), and HA-ERK-1 from J. Pouyssegur (Univ. of Nice). All other plasmids were constructed in our laboratory.

Tissue Culture. COS-7 cells were maintained in DMEM containing 10% fetal bovine serum and 100 μg/ml gentamicin. HEK293 cells were maintained in modified Eagle’s medium containing 10% fetal bovine serum and 100 μg/ml gentamicin. Cells were transiently transfected by using Lipofectamine as described (16). Experiments were performed 2–3 days posttransfection, and in all cases, cells were serum starved overnight in medium containing 10 mM Hepes, 0.1% BSA, and 100 μg/ml gentamicin.

Sequestration Assays. COS-7 cells transiently expressing HA epitope-tagged α2A ARs or Flag epitope-tagged β2 ARs were exposed to isoproterenol (10 μM) or UK14304 (10 μM), respectively, for 30 min at 37°C. Cell-surface receptors were labeled with a 12CA5 monoclonal antibody (Roche) or an M2 Flag monoclonal antibody (Sigma) by using FITC-conjugated goat anti-mouse IgG as a secondary antibody. Receptor sequestration was quantified as loss of cell-surface receptors in agonist-treated cells measured by flow cytometry (22).

Immunoprecipitation. Serum-starved transfected cells were exposed to agonist at 37°C, washed once with ice-cold phosphate-buffered saline, lysed in glycerol lysis buffer [5 mM Hepes, 250 mM NaCl, 10% (vol/vol) glycerol, 0.5% Nonidet P-40, 2 mM EDTA, 100 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin], clarified by centrifugation, and immunoprecipitated by using the appropriate antibodies. HA–ERK-1 was immunoprecipitated by using 20 μl of overnight and exposed to UK14304 (10 μM) or isoproterenol (10 μM), respectively, for 30 min at 37°C. Cell-surface receptors were labeled with an 12CA5 monoclonal antibody or an M2 Flag monoclonal antibody, by using FITC-conjugated goat anti-mouse IgG as the secondary antibody. Receptor sequestration, quantified as the percent loss of cell-surface fluorescence in agonist-treated cells, was measured by using flow cytometry. The data are expressed as the mean ± SEM of four independent experiments performed in triplicate.

Fig. 1. Agonist-promoted α2A AR and β2 AR sequestration and ERK 1/2 phosphorylation in COS-7 cells. (A) COS-7 cells transiently expressing either HA-epitope tagged α2A ARs or Flag epitope-tagged β2 ARs were serum-starved overnight and exposed to UK14304 (10 μM) or isoproterenol (10 μM), respectively, for 30 min at 37°C. Cell-surface receptors were labeled with an 12CA5 monoclonal antibody or an M2 Flag monoclonal antibody, by using FITC-conjugated goat anti-mouse IgG as the secondary antibody. Receptor sequestration, quantified as loss of cell-surface receptors in agonist-treated cells measured by flow cytometry (22).

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anti-HA affinity beads and rotated for 4 hr at 4°C, the immune complexes were washed twice with cold glycerol lysis buffer, denatured in 2× Laemmli sample buffer, and electrophoresed on SDS/PAGE gels. The proteins were transferred to poly(vinylidene difluoride) and probed for both phospho-ERK 1/2 and total ERK 1/2 as described below. Immunoprecipitation and detection of tyrosine phosphorylation of the EGFR was performed as described (16).

**ERK 1/2 Phosphorylation.** Serum-starved transfected cells grown in 12-well dishes were stimulated with agonist for 5 minutes at 37°C, the media aspirated, and the cells lysed in 100 μl of 2× Laemmli sample buffer. The samples were then electrophoresed on SDS/PAGE gels and transferred to poly(vinylidene difluoride). Phospho-ERK 1/2 was detected by using a 1:3,000 dilution of a rabbit polyclonal phospho-ERK 1/2-specific antibody (New England Biolabs), and total ERK 1/2 was detected by using a 1:1,000 dilution of an ERK 1/2 antibody (Upstate Biotechnology). Blots were probed with a 1:7,000 dilution of a donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody. Blots were visualized by using ECL (enhanced chemiluminesence reagent; Amersham Pharmacia) and quantitated by using a scanning laser densitometer.

**Immunofluorescence Microscopy.** HEK-293 cells transiently expressing HA epitope-tagged α2A ARs or β2 ARs together with an EGFR–GFP fusion protein (23) were grown on sterile coverslips. Before stimulation, epitope-tagged receptors were labeled with a 1:100 dilution of a rhodamine-conjugated anti-HA antibody (Roche). Cells were then stimulated for 30 min at 37°C in the absence or presence of UK14304 (10 μM), isoproterenol (10 μM), or EGF (10 ng/ml) and fixed in 4% paraformaldehyde. Confocal microscopy was performed on a Zeiss LSM-510 laser scanning microscope by using a Zeiss 100× oil-immersion lens. Fluorescent signals were collected by using the Zeiss LSM software in the line switching mode by using dual excitation (488, 568 nm) and emission (515–540 nm, 590–610 nm) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.
Results and Discussion

As shown in Fig. 1, when expressed in COS-7 cells, the $\beta_2$ AR undergoes agonist-driven internalization, whereas the $\alpha_2A$ AR does not. These data are consistent with previous studies that demonstrated that the $\alpha_2A$ AR exhibits little or no agonist-induced sequestration in either HEK293 (9, 24) or COS-1 (8) cells. In addition, as shown in Fig. 1B, activation of MAPK by both receptors is significantly dependent on the activation of pertussis toxin-sensitive G proteins. Because both the $\alpha_2A$ AR- and the $\beta_2$ AR-mediated activation of ERK is pertussis toxin-sensitive, but the $\beta_2$ AR internalizes whereas the $\alpha_2A$ AR does not, this is an ideal system to examine the requirement for clathrin-mediated endocytosis in ERK activation. Our previous studies have suggested that for the $\beta_2$ AR, inhibitors of clathrin-mediated endocytosis block MAPK downstream of $\beta_2$ AR internalization (2). Moreover, in addition to a role for clathrin-mediated endocytosis in GPCR activation by GPCRs, Vieira et al. (21) have suggested that clathrin-mediated endocytosis is involved in ERK activation mediated by the EGFR. Thus, we tested whether ERK activation by the $\alpha_2A$ AR, the $\beta_2$ AR, and the EGFR was sensitive to inhibitors of clathrin-mediated endocytosis. The effects of three mechanistically distinct inhibitors of clathrin-mediated endocytosis, MDC, Y231F Y597F dynamin I, and $\beta$-arrestin 1 318–419, were determined. MDC inhibits clathrin-mediated endocytosis by stabilizing clathrin cages and has been shown to inhibit insulin-like growth factor-I (25) as well as LPA-mediated ERK activation (3). Y231F Y597F dynamin I is a dominant inhibitory form of dynamin I that cannot be phosphorylated by c-src (26), and $\beta$-arrestin 1 318–419 is a truncated form of $\beta$-arrestin 1 that interferes with GPCR sequestration through interactions with clathrin (27). MDC, Y231F Y597F dynamin I, and $\beta$-arrestin 1 318–419 all inhibited agonist-stimulated internalization of the $\beta_2$ AR and the EGFR by 45–75% measured either by flow cytometry (\(\beta_2\) AR) or by I251-labeled EGF-induced EGFR internalization (data not shown). As shown in Fig. 2, MDC inhibited ERK 1/2 phosphorylation by the $\alpha_2A$ AR (Left), the $\beta_2$ AR (Center), and the EGFR (Right). Similarly, the dominant inhibitory forms of both dynamin I and of $\beta$-arrestin 1 significantly attenuated $\alpha_2A$ AR (Fig. 2B, Left), $\beta_2$ AR (Fig. 2B, Center) and EGFR-mediated (Fig. 2B, Right) MAPK activation. Thus, even though the $\alpha_2A$ AR itself does not internalize, the activation of MAPK by UK14304, like the activation by isoproterenol, is sensitive to all three inhibitors of clathrin-mediated endocytosis. Although consistent with a role for clathrin-mediated endocytosis in GPCR-mediated ERK activation, these data clearly dissociate sequestration of the GPCR from ERK activation. They also suggest that the G<sub>i</sub>-dependent ERK signaling cascades used by the $\beta_2$ and $\alpha_2A$ ARs as well as the pathway used by
the EGFR in COS-7 cells all depend on clathrin-mediated endocytosis.

One pathway by which many GPCRs have been shown to activate ERK is via transactivation of RTKs, including the EGFR. For instance, the ET-1, LPA, and thrombin receptors in Rat-1 cells (16, 18) the LPA receptor, and the β2 adrenergic receptors (28) in COS-7 cells each activate MAPK via transactivation of RTKs. To establish whether, in COS-7 cells, the α2A AR and β2 AR–mediated activation of ERK 1/2 proceeds via a transactivation-dependent mechanism, we performed two experiments. First, we measured the ability of UK14304 and isoproterenol to stimulate increased tyrosine phosphorylation of the EGFR in cells expressing the α2A AR or the β2 AR. As previously reported for several GPCRs including the α2A AR, the LPA receptor, and the thrombin receptor (16), UK14304, isoproterenol, and EGF stimulation each increases tyrosine phosphorylation of the EGFR (Fig. 3). Second, we measured the ability of tyrphostin AG1478, a selective EGFR inhibitor, to block α2A AR–, β2 AR–, and EGFR-induced ERK 1/2 phosphorylation. As shown in Fig. 3B, in appropriately transfected cells, tyrphostin AG1478 pretreatment attenuates the UK14304–, isoproterenol–, and EGF-induced ERK 1/2 phosphorylation. These data suggest that, in COS-7 cells, activation of the MAPK cascade by the α2A AR, the β2 AR, and the EGFR proceeds via a common mechanism, involving both clathrin-mediated endocytosis and activation of the EGFR.

Because transactivated EGFRs serve as an intermediate for α2A AR and β2 AR–mediated ERK activation, our data support the hypothesis that endocytosis of the EGFR or of another downstream effector accounts for the sensitivity of the GPCR signals to inhibitors of clathrin-mediated endocytosis. To examine whether stimulation of the α2A AR and the β2 AR leads to internalization of transactivated EGFRs, we used confocal immunofluorescence microscopy to examine the localization of each of these receptors after agonist treatment. Fig. 4 shows that in unstimulated cells transfected with the β2 AR and the EGFR, both the β2 AR and the EGFR localize primarily to the cell surface (a–c). Isoproterenol treatment of these cells leads to an increase in the intracellular localization of both the β2 AR and the EGFR (d–f). EGFR treatment of these cells, however, leads to an increase in EGFR localized inside the cells, whereas the β2 AR remains on the cell surface (g–i). As shown in Fig. 5, treatment of cells expressing both the α2A AR and the EGFR with either UK14304 (d–f) or EGF (g–i) leads to increased intracellular localization of the EGFR, whereas the α2A AR remains localized on the cell surface. Thus, although activation of the β2 AR leads to internalization of both the transactivated EGFR and the β2 AR, UK14304 treatment of cells expressing the α2A AR leads to internalization of the transactivated EGFR but not the α2A AR. Our data suggest that GPCR-mediated transactivation of an RTK can lead to internalization of either the RTK alone (as is the case for the α2A AR) or both the RTK and the GPCR (as is the case for the β2 AR).

Recently Whistler and von Zastrow (7) reported that MAPK activation by the noninternalizing µ-opioid receptor is attenuated by a dominant inhibitory form of dynamin I. Their interpretation was that dynamin plays a unique signal transduction role distinct from its role in clathrin-mediated endocytosis. However, an alternative possibility is that the µ-opioid receptor, like the α2A AR and the β2 AR, mediates an endocytosis-dependent signal via EGFR transactivation. Our data, which indicate that several mechanistically distinct inhibitors of clathrin-mediated endocytosis block GPCR-mediated ERK activation, are consistent with a more general role for the clathrin-mediated endocytic machinery in signal transduction.

RTK transactivation is but one mechanism of many by which GPCRs can activate the ERK cascade. We have previously demonstrated that the same GPCR can activate MAPK via multiple pathways and that the cellular context in which a receptor is expressed can determine the mechanism of GPCR-mediated MAPK activation (20). In addition to MAPK activation that

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proceeds via the transactivation pathway, a second major pathway involves calcium and the tyrosine phosphorylation of the focal adhesion kinase (FAK)-like scaffolding protein, PYK2. Depending on the cell type, the contribution of transactivation (17) to ERK activation varies dramatically (20). In some cells, such as Rat-1 fibroblasts, the transactivation-dependent pathway is the major pathway to ERK activation, whereas in other cells such as PC12 cells, the PYK2 pathway is the major pathway. For instance, LPA receptor-mediated ERK activation can range from completely EGFR-dependent in Rat-1 cells to completely EGFR-independent in PC-12 cells (20). In HEK 293 cells, ERK 1/2 activation via both endogenous LPA receptor activation (20) and transiently expressed α2A AR activation (data not shown) is only partially sensitive to tyrphostin AG1478. In these cells, the α2A AR primarily activates ERK via a calcium-dependent signal that is blocked by a dominant-inhibitory mutant of the calcium-activated FAK family tyrosine kinase PYK2 (29). Such heterogeneity in GPCR signaling among cell types may account for the variable effects of clathrin-mediated inhibitors on MAPK that have recently been reported (2–14).

Taken together, our data suggest a model in which MAPK activation that proceeds via EGFR transactivation involves engagement of the clathrin-mediated endocytic machinery. What remains to be determined is whether endocytosis of a multireceptor complex including the EGFR and Raf is essential for transactivation-dependent MAPK activation or whether instead clathrin-coated endocytic pits serve some other function such as that of a specialized microdomain wherein signaling occurs.

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