Stimulation of Mitogen-activated Protein Kinase by G Protein-coupled \( \alpha_2 \)-Adrenergic Receptors Does Not Require Agonist-elicited Endocytosis*

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Agonist-elicited receptor sequestration is strikingly different for the \( \alpha_{2A} \) versus \( \alpha_{2B} \)-adrenergic receptor (\( \alpha_2 \)-AR) subtypes; the \( \alpha_{2B} \)-AR undergoes rapid and extensive disappearance from the HEK 293 cell surface, whereas the \( \alpha_{2A} \)-AR does not (Daunt, D. A., Hurt, C., Hein, L., Kallio, J., Feng, F., and Kobilka, B. K. (1997) Mol. Pharmacol. 51, 711–720; Eason, M. G., and Liggert, S. B. (1992) J. Biol. Chem. 267, 25473–25479). Since recent reports suggest that endocytosis is required for some G protein-coupled receptors to stimulate the mitogen-activated protein (MAP) kinase cascade (Daaka, Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S., Caron, M. G., and Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 605–688; Luttrell, L. M., Daaka, Y., Della Rocca, G. J., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 31648–31656; Ignatova, E. G., Belcheva, M. M., Bohn, L. M., Neuman, M. C., and Coscia, C. J. (1999) J. Neurosci. 19, 56–63), we evaluated the differential ability of these two subtypes to activate MAP kinase. We observed no correlation between subtype-dependent agonist-elicited receptor redistribution and receptor activation of the MAP kinase cascade. Furthermore, incubation of cells with K+-depleted medium eliminated \( \alpha_{2B} \)-AR internalization but did not eliminate MAP kinase activation, suggesting that receptor internalization is not a general prerequisite for activation of the MAP kinase cascade via G\( \alpha \)-coupled receptors. We also noted that neither dominant negative dynamin (K44A) nor concanavalin A treatment dramatically altered MAP kinase activation or receptor redistribution, indicating that these experimental tools do not universally block G protein-coupled receptor internalization.

The three subtypes of \( \alpha_2 \)-adrenergic receptors (\( \alpha_2 \)-AR) signal via the G\( \alpha \)/G\( \beta \) subfamily of G proteins to effect several downstream signaling events (1). Since all three receptor subtypes appear to couple to the same effectors, it is of interest to explore other differences among these subtypes in an effort to understand the functional relevance of subtype diversity. Two \( \alpha_2 \)-AR subtypes manifest differences in agonist-induced receptor redistribution from the cell surface as follows: the \( \alpha_{2B} \)-AR becomes rapidly and extensively internalized following agonist occupancy, whereas the \( \alpha_{2A} \)-AR does not readily redistribute to an intracellular compartment following agonist occupancy (2, 3). The \( \alpha_{2C} \)-AR exists both on the surface and in an intracellular compartment at steady state (2), confounding quantitative assessment of \( \alpha_{2C} \)-AR redistribution.

Recent reports suggest that internalization is required for some G protein-coupled receptors (GPCRs) to stimulate the MAP kinase cascade (4–6). The purpose of the current studies was to determine if the differing profiles of agonist-induced internalization of the \( \alpha_{2A} \)-AR and \( \alpha_{2B} \)-AR subtypes are paralleled by differing rates or extents of MAP kinase activation and whether agents that interfere with agonist-elicited receptor redistribution alter MAP kinase activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

HEK 293 cells were maintained in DMEM containing 10% fetal calf serum at 37 °C in a 5% CO\( _2 \) incubator. Permanent transfectants were generated by LipofectAMINE-mediated co-transfection of the cells with plasmids containing the indicated receptors and a neomycin resistance gene. Cells that survived selection in medium containing 500 \( \mu \)g/ml G418 were screened for expression of the expected receptor by binding assays of the radiolabeled \( \alpha_2 \)-AR antagonist, \[^{3}H\]rauwolscine. Clonal cell lines with varying levels of \( \alpha_2 \)-AR expression were kept for further study. The experiments reported here were performed on an \( \alpha_{2A} \)-AR expressing cell line that contains 7–8 pmol of receptor per mg of protein and an \( \alpha_{2B} \)-AR-expressing cell line that contains 2–4 pmol/mg.

**MAP Kinase Stimulation**

Permanent transfectants of HEK 293 cells were plated on 60-mm dishes and allowed to multiply until they reached a density of 60–80%. The cells were then serum-deprived overnight. On the day of the experiment, the medium was replaced with fresh serum-free DMEM, DMEM supplemented with 250 \( \mu \)g/ml concanavalin A, or K+-depleted medium as described below to block clathrin-mediated endocytosis. After the pretreatment, the indicated drugs were added directly to the medium on the cells and were swirled to mix. After the indicated times, the cells were washed once with Dulbecco’s phosphate-buffered saline containing 1 mM MgCl\(_2\) and 0.5 mM CaCl\(_2\), (PBS/Ca\(^{2+}\)⁄Mg\(^{2+}\) 1) and then lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% w/v SDS; 10% glycerol; 50 mM dithiothreitol) supplemented with 1 mM sodium orthovanadate (Sigma), 10 units/ml leupeptin (Sigma), and 10 units/ml aprotinin (Bayer Corp., Kankakee, IL). The lysates were transferred to an Eppendorf tube on ice. When all samples were collected, they were bath sonicated for 30–40 s, then placed in a heating block at 90 °C, allowed to warm to 95 °C, and incubated at 95 °C for 5 min. The lysates were then spun in a microcentrifuge at room temperature for 5 min to remove debris. The supernatants were assayed in Bio-Rad’s protein assay for relative protein concentration, and equivalent amounts of protein were loaded on a 10% SDS-polyacrylamide gel for electrophoresis. The gel was run for 160 mA-h and then transferred overnight onto a nitrocellulose membrane.
nitrocellulose in transfer buffer (20% methanol; 0.19 g glycine; 25 mM Tris base) at 33 mV.

MAP kinase activation was evaluated using an antibody that recognizes dually phosphorylated Thr747/Tyr747 MAP kinase (Promega catalog number V6617) or with total MAP kinase using an antibody that recognized MAP kinase (8). On gel transfer (New England Biolabs catalog number 9102), to assess activated MAP kinase content, the nitrocellulose blot was incubated in blocking buffer (1× TBS; 0.1% Tween 20; 5% w/v nonfat dry milk) for 1 h at room temperature and then probed with rabbit polyclonal antibody (from Promega) to dually phosphorylated MAP kinase, diluted 1:500 in blocking buffer for 1 h at room temperature. The blot was washed three times for 5 min each with TBST (2.42 g/liter Tris base; 8.0 g/liter sodium chloride; 0.1% Tween 20; pH 7.6) and then probed with donkey anti-rabbit horseradish peroxidase-linked secondary antibody (1/2000 dilution) in blocking buffer (Amersham Pharmacia Biotech) for 1 h at room temperature. The wash protocol was repeated, and the immunoreactive bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). The bands were then stripped with stripping buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 100 mM 2-mercaptoethanol) for 30 min at 65 °C and reprobed with antibody to total MAP kinase (New England Biolabs) at a 1/500 dilution in blocking buffer overnight at 4 °C, followed by donkey anti-rabbit secondary antibody as described above.

To semi-quantify MAP kinase activation, the enhanced chemiluminescence (ECL) images were scanned into Adobe Photoshop with a UMAX Astra 600 scanner, and the band intensities were measured using NIH Image software. Background pixel density was subtracted from each band's pixel density. The corrected pixel density for active MAP kinase was divided by the corrected pixel density for total MAP kinase to obtain the normalized levels of activated MAP kinase reported in the figures as “active/total.”

Cell Surface Receptor Quantitation via Intact Cell ELISA

The introduction of the HA epitope into the amino terminus of α2-ARs expressed in HEK 293 cells provided a tool for quantitation of receptors expressed at the cell surface using a cell surface ELISA, as described previously (2, 7). On day 1, HEK 293 cells stably expressing the receptors of interest were plated on poly-l-lysine-coated 96-well culture plates at a density of 20,000 cells/well. On day 2, the cells were serum-starved overnight. On the morning of day 3, the cells were treated in the absence or presence of agonist as indicated in the figure legends and then washed twice in PBS/Ca2+/Mg2+ to stop the drug treatment and fixed in 4% paraformaldehyde containing 0.12 mM sucrose for 20 min at room temperature. The wells were washed three times with PBS/Ca2+/Mg2+ and then incubated for 30 min at 37 °C in DMEM containing 10% sheep serum to block nonspecific antibody binding in subsequent incubations. The 12CA5 antibody was directed against the HA epitope and diluted 1/100 in blocking buffer. The cells were then washed three times for 5 min each in PBS/Ca2+/Mg2+ at room temperature. The sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech) was applied at a 1/2500 dilution in blocking buffer. Reactive proteins were visualized by ECL (Amersham Pharmacia Biotech). Bands were quantified by scanning and NIH Image software as described above. To calculate percent internalization, the density of the band from control cells was subtracted from the density of the band from biotinylated cells to determine the difference in density. The ratio of the density of the band from biotinylated cells to the density of the band from control cells was multiplied by 100 to calculate percent internalization.

Potassium Depletion—The potassium depletion protocol for blocking receptor endocytosis was adapted from previously published methods (9, 10). Clonal cell Lines Expressing either α2-AR subtype were incubated at 37 °C for 5 min in hypotonic shock solution (serum-free DMEM and distilled water in a 1:1 ratio). Then they were rinsed once in K+ depletion buffer (100 mM NaCl; 50 mM HEKES, pH 7.4; 1 mM CaCl2; 1 mM MgCl2) and then incubated in the same buffer for 1 h at 37 °C. (Control cells were treated with PBS instead of potassium depletion buffer.) MAP kinase stimulation or biotinylation then proceeded as described above.

Concanavalin A Pretreatment—Clonal cell lines expressing each α2-AR subtype were incubated with 250 μg/ml concanavalin A (Sigma), made fresh daily in serum-free DMEM, for 30 min at 37 °C, as described previously (11). MAP kinase stimulation or biotinylation then proceeded as described above.

Hypertonic Sucrose Treatment—Clonal cell lines expressing each α2-AR subtype were washed once and then incubated for 1 h at 37 °C with DMEM containing 0.45 mM sucrose, as described previously (9). Drug treatment and analysis then proceeded as described.

Co-expression of K44A Dominant Negative Dynamin—Parental HEK 293 cells were plated on 6-well plates or 35-mm culture dishes and transiently transfected with cDNAs encoding the HA-tagged α2-AR and either dominant negative dynamin cDNA (kindly provided by Mark Caron) or “empty” pCMV4 vector as a control. FuGENE 6 transfection reagent (Roche Molecular Biochemicals) was used according to package.
Endocytosis, α2-ARs, and MAP Kinase

RESULTS

α2-AR Subtypes Possess Differing Profiles of Agonist-induced Receptor Redistribution—Two independent experimental strategies were used to examine agonist-elicted receptor redistribution of α2-AR subtypes. An intact cell ELISA performed on HEK 293 cells stably expressing HA-tagged mouse α2A-AR and α2B-AR (see "Experimental Procedures") was used to assess loss of receptor from the cell surface (Fig. 1A). Cell surface ELISAs corroborate earlier morphological findings (2, 3) that the α2A-AR and α2B-AR show differing profiles of agonist-induced internalization. Whereas the α2A-AR demonstrates negligible loss of surface receptors following exposure to maximal agonist concentrations, the α2B-AR subtype demonstrates a 30–40% loss of surface-accessible epitope over the same time.

Receptor internalization also can be examined by quantifying the fraction of surface receptors that moves in a time-dependent fashion to an inaccessible compartment. For these studies, we exploited a reversible biotinylating agent, sulfo-NHS-SS-biotin (Fig. 1B). Biotin remaining on the surface at the end of a particular treatment protocol was removed by treatment with MESNA, a non-permanent reducing agent that cleaves the disulfide bond and liberates the biotinylating reagent from proteins still accessible at the cell surface. MESNA reversal immediately after biotinylation (Fig. 1B; time 0, +) provides an assessment of the amount of biotinylated receptor that is accessible to this reversal reagent at time 0. Further incubation of the cells at 37 °C in the absence or presence of agonist before MESNA reversal of surface biotinylation permits evaluation of agonist-independent versus agonist-accelerated receptor redistribution. As shown in Fig. 1B, the α2B-AR is not extensively redistributed to a MESNA-inaccessible compartment at 37 °C, even in the presence of the agonist epinephrine, whereas the α2B-AR is rapidly internalized (i.e. removed from MESNA accessibility), and this internalization is enhanced by agonist stimulation.

α2A-AR and α2B-AR Subtypes Evoke Similar Profiles of MAP Kinase Stimulation—Previous studies in heterologous systems have demonstrated α2-AR-elicited activation of MAP kinase (12–16). Recent findings suggest that G protein-coupled receptors may require internalization to activate MAP kinase, particularly via pertussis toxin-sensitive pathways (4, 5). To assess whether receptor internalization is required for MAP kinase activation by α2-ARs, we explored the differential internalization profiles of the α2A-AR and α2B-AR subtypes and compared their ability to activate MAP kinase in permanent transfectants of HEK 293 cell lines. As shown in Fig. 2, the extent of MAP kinase activation by these two receptor subtypes is similar when measured by the appearance of dually phosphorylated Erk 1 and Erk 2. Stimulation by both subtypes was sensitive to pertussis toxin pretreatment (data not shown), indicating that α2A-AR and α2B-AR are activating MAP kinase via a G coupled pathway, as observed previously (17). We are confident that the stimulation of MAP kinase by epinephrine demonstrated in Fig. 2 is due to activation of the heterologously expressed α2A-AR or α2B-AR in the HEK 293 cells, as activation of MAP kinase is also elicited by the α2-AR agonist UK 14,304 and is blocked by the α2-AR-specific antagonists yohimbine and RX 821002 (data not shown). Furthermore, parental HEK 293 cells did not demonstrate activation by epinephrine prior to introduction of the α2-AR-encoding cDNAs. Unlike the properties of some clonal HEK 293 cell lines reported in the literature (4), we have found no evidence for β-AR stimulation of MAP kinase in our HEK 293 cells, as MAP kinase is not activated by isoproterenol and the response to epinephrine was not blocked by the β-AR antagonist, propranolol.

Blockade of α2B-AR Internalization Does Not Block MAP Kinase Activation—The observation that both α2A-AR and α2B-AR subtypes stimulate MAP kinase suggests that receptor
internalization, which is highly efficient for the α2B-AR but not for α2A-AR subtype, is not required for MAP kinase activation. Consistent with this interpretation is the realization that maximal MAP kinase activation occurs at 2 min, a time when agonist-elicited receptor loss from the surface, even for α2B-AR, is just beginning (see Fig. 1A).

To assess directly the impact of internalization on MAP kinase activation, we utilized four independent experimental manipulations previously demonstrated to block internalization by clathrin-coated pit pathways as follows: hypertonic sucrose (9), pretreatment with concanavalin A (11), exposure to K+-depleted medium (9), and co-expression of dominant negative (K44A) dynamin (18, 19). Hypertonic sucrose proved a non-viable strategy in our HEK 293 cell lines, as it caused MAP kinase activation. However, it is also important to note that interpretations from previous studies relied at least in part on transient expression of receptors (4) or on transient expression of dominant negative structures of dynamin or arrestin to block internalization as a mechanism of desensitization of MAP kinase signaling.

**DISCUSSION**

The present studies provide two lines of evidence that α2-ARs do not require internalization to evoke MAP kinase activation, in contrast to recent reports implicating internalization as a prerequisite for stimulating the MAP kinase cascade by G-mediated GPCR signaling. First, the α2A-AR and α2B-AR subtypes both elicit MAP kinase activation (Fig. 2), despite the greater efficiency of α2B-AR in leaving the surface (Fig. 1A) and appearing in an inaccessible (presumably internal) compartment (Fig. 1B). Second, incubation of HEK 293 cells permanently expressing the α2B-AR subtype in K+-depleted medium eliminates receptor internalization (Fig. 4A) but does not eliminate MAP kinase activation (Fig. 4B).

The reason for the discrepancy between our findings and those previously reported for other G protein-coupled receptors is not certain. The simplest interpretation is that different GPCRs have different mechanistic requirements to stimulate MAP kinase. However, it is also important to note that interpretations from previous studies relied at least in part on transient expression of receptors (4) or on transient expression of dominant negative structures of dynamin or arrestin to block internalization of heterologous or endogenous receptors (4). Our findings suggest that agonist-elicited redistribution of the α2B-AR does not require dynamin-dependent mechanisms (Fig. 3), and thus dynamin K44A does not provide a diagnostic reagent for evaluating the role of endocytosis in α2B-AR-mediated MAP kinase activation. Why different pathways would be utilized by different GPCRs for internalization is not known, although a lack of reliance on dynamin for agonist-elicited endocytosis has also been observed for dopaminergic and muscarinic receptors (21, 22). It may be that, in previous studies examining the impact of dynamin K44A on receptor-mediated MAP kinase activation, expression of mutant dynamin structures may have altered molecular events in addition to those involved in receptor association with clathrin-coated pits (23) or in pinching of clathrin-coated vesicles from the surface (19), and it is these events that play a role in MAP kinase activation.
Our findings provide strong evidence that G protein-coupled receptor internalization is not a general prerequisite for activation of the MAP kinase cascade via Gi-coupled receptors (Fig. 4). Similar results have been obtained previously with chemokine receptors (24). Our results also suggest that MAP kinase activation by epinephrine was evaluated in the absence (upper panel) or presence (middle panel) of dynamin K44A and was quantified for four independent experiments (mean ± S.E.), as shown in the bottom panel.

by some, but clearly not all, GPCRs.

Fig. 3. Dynamin K44A does not block endocytosis of α2-AR or α2-AR activation of MAP kinase. The α2-AR was transiently expressed in HEK 293 cells with or without dominant negative dynamin (K44A) as described under "Experimental Procedures." A, receptor redistribution to a MESNA-inaccessible compartment was evaluated as in Fig. 1B (see "Experimental Procedures") and quantified for three independent experiments (mean ± S.E.). In these experiments, internalization was assessed at the 20-min time point, because internalization was undetectable at the 5-min time point in transiently transfected cells. B, MAP kinase activation by epinephrine was evaluated as in Fig. 1B (see "Experimental Procedures") and quantified for three independent experiments (mean ± S.E.), as shown in the bottom panel.

Fig. 4. Blockade of α2-AR internalization in K+-depleted medium does not block epinephrine stimulation of MAP kinase. HEK 293 cells permanently expressing the α2-AR were incubated in K+-depleted medium prior to and during stimulation by epinephrine (see "Experimental Procedures"). A, receptor redistribution to a MESNA-inaccessible compartment was measured in control cells (upper panel) or cells exposed to K+-depleted medium (middle panel) following surface biotinylation with the reversible reagent, sulfo-NHS-SS-biotin (see "Experimental Procedures"), as in Fig. 1B. Reversal of biotinylation was performed immediately after biotinylation or after incubation for 5 min at 37 °C with or without the agonist epinephrine. Lower panel, quantification of internalization, mean ± S.E. for three independent experiments (see "Experimental Procedures"). B, MAP kinase activation by epinephrine in the absence (upper panel) or presence (middle panel) of K+-depleted medium. Stimulation was performed for the indicated times, in minutes. Normalized level of activated MAP kinase (active/total) was calculated as described previously and is plotted in the lower panel for the maximal stimulation (2-min time point) and for the point at which desensitization has usually occurred (30-min time point) (mean ± S.E. for three independent experiments).
activation may be terminated by internalization, since activation of MAP kinase by the α_{2A}-AR persists longer than that by the α_{2B}-AR (Fig. 2), and incubation in K^{+}-depleted medium also parallels sustained activation of the enzyme (Fig. 3B). Future studies will resolve whether different molecular events dictate the duration of receptor-elicited MAP kinase signaling and other Gi-mediated phenomena, such as inhibition of adenylyl cyclase, activation of K^{+} currents, or suppression of Ca^{2+} currents.

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