Communication

Essential Role for G Protein-coupled Receptor Endocytosis in the Activation of Mitogen-activated Protein Kinase*

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The classical paradigm for G protein-coupled receptor (GPCR) signal transduction involves the agonist-dependent interaction of GPCRs with heterotrimeric G proteins at the plasma membrane and the subsequent generation, by membrane-localized effectors, of soluble second messengers or ion currents. Termination of GPCR signals follows G protein-coupled receptor kinase (GRK)- and β-arrestin-mediated receptor uncoupling and internalization. Here we show that these paradigms are inadequate to account for GPCR-mediated, Ras-dependent activation of the mitogen-activated protein (MAP) kinases Erk1 and -2. In HEK293 cells expressing dominant suppressor mutants of β-arrestin or dynamin, β2-adrenergic receptor-mediated activation of MAP kinase is inhibited. The inhibitors of receptor internalization specifically blocked Raf-mediated activation of MEK. Plasma membrane-delimited steps in the GPCR-mediated activation of the MAP kinase pathway, such as tyrosine phosphorylation of Shc and Raf kinase activation by Ras, are unaffected by inhibitors of receptor internalization. Thus, GRKs and β-arrestins, which uncouple GPCRs and target them for internalization, function as essential elements in the GPCR-mediated MAP kinase signaling cascade.

Stimulation of G protein-coupled receptors (GPCRs) facilitates the exchange of bound GDP for GTP on heterotrimeric G proteins, resulting in dissociation of the G protein into active Go-GTP and Gβγ subunits. The interaction of Go-GTP and Gβγ subunits with effectors initiates and accounts for the known signaling events mediated by GPCRs. Exposure of GPCRs to an agonist often results in rapid attenuation of receptor responsiveness, a process termed desensitization. Signal termination is initiated by phosphorylation of agonist-occupied receptors, mediated by the G protein-coupled receptor kinase (GRK) family (1–3). The GRK-mediated phosphorylation of activated GPCRs promotes binding of members of a family of cytosolic proteins, β-arrestins, to the receptor (4, 5). Binding of β-arrestins to phosphorylated receptors serves two functions. First, it uncouples the receptor from its cognate G protein and thus leads to diminished receptor signaling (4, 5). Second, it initiates the process of receptor internalization (also termed sequestration) by targeting the receptor to clathrin-coated pits (6, 7).

G protein-coupled receptors and receptor tyrosine kinases (RTKs) stimulate mitogenesis in part via mitogen-activated protein (MAP) kinase cascades. The mechanism of activation of MAP kinase signaling pathways by GPCRs is poorly understood, although it is becoming evident that signal transduction by certain GPCRs utilizes many of the same intermediates as those activated by RTKs (see Reaction 1).

\[
\text{GPCR} \rightarrow G\beta\gamma \rightarrow \text{Tyr kinase} \rightarrow \text{Shc} \rightarrow \text{Grb2-mSos} \\
\rightarrow \text{Ras} \rightarrow \text{Raf} \rightarrow \text{MEK} \rightarrow \text{MAPK}
\]

**REACTION 1**

In fibroblasts, endogenous lysophosphatidic acid (LPA) and β2-adrenergic (β2-AR) receptors, acting via unknown effectors of Gβγ subunits, stimulate tyrosine phosphorylation of plasma membrane-associated proteins to create tyrosine phosphoprotein scaffolds (8–12). Receptor activation coincides with an increase in tyrosine phosphorylation of the adaptor protein Shc (10–12), and recruitment of Ras guanine exchange factors, such as the Grb2-mSos complex, to the plasma membrane.

Recruitment of mSos facilitates Ras GDP/GTP exchange leading to recruitment of Raf (MAP kinase kinase kinase) into complex with activated Ras. Subsequent signal transduction involves sequential phosphorylation of the MEK (MAP kinase kinase) and MAP kinases (13). Here we demonstrate that, unlike classical GPCR-mediated activation of adenylate cyclase or phospholipase C which occurs entirely within the plasma membrane, MAP kinase activation involves GPCR sequestration.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells, maintained in minimum essential medium supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin were transiently transfected using calcium phosphate coprecipitation (14). Cells were starved overnight in medium containing 10 mM Hepes (pH 7.4) and 0.1% (v/v) bovine serum albumin prior to agonist stimulation. All assays were performed 48 h after transfection. Transient expression of β-arrestin1 V53D, dynamin, and dynamin K44A transfected plasmids were verified by immunoblotting of whole cell lysates using commercially available antibodies.

Sequestration Assay—The β2-AR sequestration was determined by immunofluorescence flow cytometry (15). Cells expressing epitope-tagged β2-AR at 300–400 fmol/mg of whole cell protein were exposed to 10 µM isoproterenol for 30 min at 37 °C prior to addition of antibodies. Sequestration is defined as the fraction of total cell surface receptors which are removed from the plasma membrane (and thus are not accessible to antibodies added to the cells) following agonist treatment.

**cAMP Production**—Cells were metabolically labeled with 1 µCi of [3H]adenosine/m, washed in PBS, and incubated with 1 mM isobutyl-
methylxanthine for 25 min at 37 °C. Agonist was added for 5 min followed by the addition of 1 ml of stop solution (0.1 mM CAMP, 4 nCi of [3H]cAMP per ml, 2.5% perchloric acid). Cell lysates were neutralized with KOH, and total [3H]cAMP was assayed by anion exchange chromatography (14).

**Phosphoinositide Hydrolysis**—Cells were metabolically labeled for 16–18 h with 2 μCi of [3H]inositol/ml and washed in PBS containing 20 mM LiCl alone (basal) or with agonist for 5 min at 37 °C. Reactions were terminated by the addition of an equal volume of 0.8 M perchloric acid, and total inositol phosphates were assayed by anion exchange chromatography (14).

**MAP Kinase Assay**—Agonist-treated cells were lysed by direct addition of Laemmli sample buffer. Aliquots were resolved by SDS-PAGE, and phosphorylated MAP kinases on nitrocellulose filters were detected using a phosphospecific MAP kinase IgG (Promega). Bands corresponding to MAP kinase were visualized with enzyme-linked chemiluminescence (ECL; Amersham Corp.) and quantitated by scanning laser densitometry.

**Shc Phosphorylation**—Agonist-treated HEK293 cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 0.5% deoxycholate) and clarified by centrifugation. Shc proteins were immunoprecipitated using rabbit polyclonal anti-Shc antibodies (Transduction Laboratories) and resolved by SDS-PAGE. Phosphorylated Shc proteins on nitrocellulose were detected using anti-phosphotyrosine antibodies (RC20H, Transduction Laboratories), visualized with ECL, and quantitated by scanning laser densitometry.

**Raf Kinase Assay**—Lysates were prepared in RIPA buffer from cells treated with agonists for 5 min at 37 °C. Raf-1 was immunoprecipitated with 0.5 μg of anti-Raf-1 polyclonal antibody (C-12, Santa Cruz Biotechnology). Immunocomplexes were washed with cold RIPA, washed buffer (137 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM Na3VO4, 10% glycerol, 1% Nonidet P-40), and kinase buffer (75 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM Na3VO4, 30 μM ATP). Raf-1 kinase activity was determined by incubating the resuspended immune complexes in kinase buffer containing 1 μCi of [γ-32P]ATP and 0.5 μg of MEK at ambient temperature for 15 min. Reactions were terminated by the addition of SDS sample buffer, and the phosphorylated substrate bands were resolved by SDS-PAGE and quantitated by phosphorimaging.

**Subcellular Fractionation**—Cells stimulated with or without 10 μM isoproterenol were washed with ice-cold PBS, scraped into 5% (w/v) sucrose in buffer A (10 mM Tris, pH 7.4, 1 mM EDTA, and disrupted by Dounce homogenization (16). Nuclei and cell debris were removed by centrifugation at 500 × g for 10 min. The supernatant was loaded on a sucrose cushion (4 μl of 35% sucrose in buffer A) and centrifuged at 150,000 × g for 90 min at 4 °C. The 35% (vesicle) sucrose interface fractions were collected, diluted with buffer A, and pelleted. The pellets were resuspended in SDS sample buffer; 25 μg for each protein sample was analyzed. The presence of clathrin and Raf was detected by protein immunoblotting. Clathrin was detected using a 1:500 dilution of a monoclonal anti-clathrin IgM (ICN), and Raf-1 was detected using a 1:1000 dilution of a rabbit polyclonal anti-Raf-1 IgG (Santa Cruz Biotechnology). Immunocomplexes on nitrocellulose were detected using the appropriate horseradish peroxidase-conjugated secondary antibody and visualized by ECL. Binding assays were performed exactly as described (17).

**RESULTS AND DISCUSSION**

To determine the role of GPCR internalization in signal transduction we employed dominant suppressor mutants of β-arrestin1 and dynamin. Dominant suppressor β-arrestin1 V53D prevents GPCR targeting to clathrin-coated pits, while the dominant suppressor form of dynamin (K44A) inhibits fission of the budding vesicle from the plasma membrane (18). Fig. 1A depicts the effects of wild type and mutant β-2-AR on MAP kinase. Chloroquine-digesting clathrin was detected using a 1:500 dilution of a monoclonal anti-clathrin IgM (ICN), and Raf-1 was detected using a 1:1000 dilution of a rabbit polyclonal anti-Raf-1 IgG (Santa Cruz Biotechnology). Immunocomplexes on nitrocellulose were detected using the appropriate horseradish peroxidase-conjugated secondary antibody and visualized by ECL. Binding assays were performed exactly as described (17).

**The β-arrestin1 V53D mutant inhibits sequestration of the receptor by 50%**, and the dynamin K44A mutant inhibits sequestration by 70%.

As shown in Fig. 1B, cells expressing β-arrestin1 V53D or dynamin K44A exhibit normal β2-AR coupling efficiency to Gαs, as measured by the accumulation of intracellular cAMP. Similarly, coupling of the LPA receptor to Gαs, as measured by the accumulation of intracellular inositol phosphates, was unaffected by expression of β-arrestin1 V53D or dynamin K44A proteins (Fig. 1C). Thus, neither inhibitor of receptor sequestration significantly impaired classical receptor-G protein-effector-mediated generation of soluble second messengers.

**Stimulation of the endogenous receptors for lysophosphatidic acid (LPA) and isoproterenol (ISO; β2-AR) in HEK293 cells induces a 6- to 8-fold increase in phosphorylated MAP kinase (Erk1/2) levels (Fig. 2A). Activation of MAP kinases by the β2-AR in HEK293 cells, like in COS-7 cells (22), is Src-dependent (21). Phosphorylation of MAP kinase reflects the enzymatic activation of MEK (13). Expression of β-arrestin1 V53D and dynamin K44A mutants impaired the ability of these receptors to activate MAP kinase (Fig. 2A). β-Arrestin1 V53D inhibited LPA- and ISO-mediated phosphorylation of MAP kinase by 56% and 63%, respectively. Similarly, the dynamin K44A protein inhibited LPA- and ISO-stimulated phos-
GPCR-mediated phosphorylation of MAP kinase. Cells were transfected with empty pRK5 vector (NT), β-arrestin1 V53D, or dynamin K44A and serum-starved overnight prior to activating the endogenous lysophosphatidic acid (LPA) or β2-adrenergic (ISO) receptors for 5 min at 37 °C. A, stimulated monolayers were lysed in Laemmli sample buffer, and levels of phosphorylated MAP kinases were determined as described. C, a representative Western blot showing tyrosine-phosphorylated p42MAPK and p44MAPK (upper) and p42MAPK immunoreactivity (lower). B, PMA-promoted phospho-MAP kinase accumulation. Cells were exposed to 1 μM PMA for 5 min prior to determining active MAP kinase levels. Values represent means ± S.E. of three independent experiments performed in duplicate. NS, unstimulated cells.

Fig. 2. Mutant β-arrestin1 and dynamin proteins attenuate GPCR-mediated phosphorylation of MAP kinase. Cells were transfected with empty pRK5 vector (NT), β-arrestin1 V53D, or dynamin K44A and serum-starved overnight prior to activating the endogenous lysophosphatidic acid (LPA) or β2-adrenergic (ISO) receptors for 5 min at 37 °C. A, stimulated monolayers were lysed in Laemmli sample buffer, and levels of phosphorylated MAP kinases were determined as described. C, a representative Western blot showing tyrosine-phosphorylated p42MAPK and p44MAPK (upper) and p42MAPK immunoreactivity (lower). B, PMA-promoted phospho-MAP kinase accumulation. Cells were exposed to 1 μM PMA for 5 min prior to determining active MAP kinase levels. Values represent means ± S.E. of three independent experiments performed in duplicate. NS, unstimulated cells.

Fig. 3. Effect of mutant β-arrestin1 and dynamin proteins on endogenous lysophosphatidic acid and β2-adrenergic receptors-mediated Shc phosphorylation (A) and Raf-1 enzymatic activity (B). Cells were transiently transfected with empty pRK5 vector (NT), β-arrestin1 V53D, or dynamin K44A and serum-starved prior to stimulation with agonists. A, immunoprecipitates of Shc were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. Shown are quantifications of the effects of the mutant β-arrestin1 V53D or dynamin K44A on agonist-stimulated tyrosine phosphorylation of p52shc, expressed as fold increase over unstimulated cells. C, a representative Western blot showing tyrosine-phosphorylated p52shc (upper) and total p52shc immunoreactivity (lower) following agonist stimulation. B, Raf-1 kinase activity from lysophosphatidic acid (LPA) and isoproterenol (ISO) stimulated cells were determined as described. Shown are quantifications of the effects of β-arrestin1 V53D and dynamin K44A on Raf-1-mediated phosphorylation of MEK. D, a representative autoradiogram showing 32P-MEK (upper) and Western blot showing Raf-1 immunoreactivity (lower). Data represent means ± S.E. from three experiments and are expressed as fold increase over unstimulated cells. NS, unstimulated cells.

In HEK293 cells, stimulation with LPA or isoproterenol results in an increase of 4.2- or 3-fold, respectively, in tyrosine phosphorylation of Shc relative to unstimulated cells (Fig. 3A). Expression of neither β-arrestin1 V53D nor dynamin K44A proteins had any effect on LPA- or ISO-stimulated Shc phosphorylation (Fig. 3A). Stimulation of the endogenous receptors for LPA or isoproterenol increases Raf-1 enzymatic activity 2-fold relative to unstimulated cells, in agreement with results reported recently in rat hepatocytes (25). In cells expressing β-arrestin1 V53D or dynamin K44A mutant proteins Raf-1 activity was similar to that in wild type cells following stimulation of these endogenous receptors (Fig. 3B). Thus, the initial steps in the GPCR-mediated MAP kinase cascade, including tyrosine phosphorylation of adaptors and activation of Raf, are unaffected by inhibitors of receptor sequestration. These data demonstrate that the process of vesicle-mediated endocytosis is required for mitogenic signaling initiated by G protein-coupled receptors. Inhibition of endocytosis blocks phosphorylation of MAP kinase, but does not affect plasma membrane-delimited processes such as receptor coupling to G proteins, tyrosine phosphorylation of Shc, or Raf activation. Rather, inhibition of endocytosis impairs signal transduction between activated Ras-bound Raf and the cytosolic MEK kinase. Regulation of Raf kinase activity has been shown to be complex; dependent, in part, upon its translocation to the
TrkA (nerve growth factor receptor) internalization was found to block the nerve growth factor-mediated phosphorylation of the transcription factor CREB (28). Likewise, in endocytosis-defective HeLa cells, epidermal growth factor receptor-mediated activation of phospholipase Cγ is unaffected, but phosphorylation of MAP kinase is impaired (29). Taken together, these data demonstrate yet another emerging analogy between RTKs and GPCRs in mitogenic signal transduction, a requirement for receptor internalization via clathrin-coated pits.

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