THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 2, Issue of January 9, pp. 685–688, 1998

Printed in U.S.A.

Communication

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

(Received for publication, October 6, 1997, and in revised form, November 4, 1997)

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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, β_2-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)[1] facilitates the exchange of bound GDP for GTP on heterotrimeric G proteins, resulting in dissociation of the G protein into active Gα-GTP and Gβγ subunits. The interaction of Gα-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).

GPCR → Gβγ → Tyr kinase → Shc → Grb2-mSos

→ Ras → Raf → MEK → MAPK

REACTION 1

In fibroblasts, endogenous lysophosphatidic acid (LPA) and β_2-adrenergic (β_2-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, β-arrestin1 V53D, dynamin, and dynamin K44A transfected plasmids were verified by immunoblotting of whole cell lysates using commercially available antibodies.

Sequestration Assay—The β_2-AR sequestration was determined by immunofluorescence flow cytometry (15). Cells expressing epitope-tagged β_2-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 [^{3}H]adenine/ml, washed in PBS, and incubated with 1 mM isobutyl-

* This work was supported in part by National Institutes of Health Grant HL16037 (to R. J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a National Institutes of Health Clinical Investigator Development Award.

§ Supported by National Institutes of Health MSTP Grant T32GM-07171.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; MAP, mitogen-activated protein β_2-AR. β_2-adrenergic receptor; RTK, receptor tyrosine kinase; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; ISO, isoproterenol; PMA, phorbol 12-myristate 13-acetate.

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 [^{14}C]cAMP/ml, 2.5% perchloric acid). Cell lysates were neutralized with KOH, and total [^{3}H]cAMP was assayed by anion exchange chromatography (14).

Phosphoinositide Hydrolysis—Cells were metabolically labeled for 16–18 h with 2 μCi of [^{3}H]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, wash buffer (137 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4, 10% glycerol, 1% Nonidet P-40), and kinase buffer (75 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_3VO_4, 30 μM ATP). Raf-1 kinase activity was determined by incubating the resuspended immune complexes in kinase buffer containing 10 μCi of [^{32}P]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 ml 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). Immune complexes 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. 1*A* depicts the effects of wild type and mutant β-arrestin1 and dynamin expression on β_2-AR internalization. Whereas overexpression of the wild type β-arrestin1 protein increases isoproterenol-mediated internalization of the β_2-adrenergic receptor (β_2-AR) modestly, overexpression of wild type dynamin has no effect. In cells overexpressing the dominant suppressor forms of these proteins (β-arrestin1 V53D or dynamin K44A) a dramatic decrease in agonist-mediated internalization of the β_2-AR is observed, in agreement with previous results (6, 19).

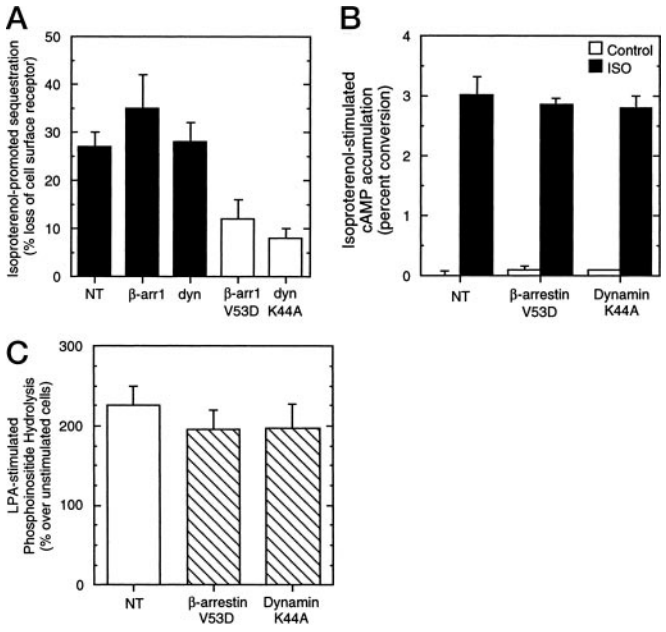

FIG. 1. **Effect of wild type and mutant β-arrestin1 and dynamin proteins on β_2-AR sequestration and receptor-G protein coupling.** *A*, effect on sequestration. Flag-tagged β_2-ARs were transfected into HEK293 cells together with plasmids expressing the following: empty pRK5 vector (*NT*), β-arrestin1 (*β-arr1*), dynamin (*dyn*), β-arrestin1 V53D (*β-arr1 V53D*), or dynamin K44A (*dyn K44A*). Cells were exposed to 10 μM isoproterenol for 30 min at 37 °C and analyzed for their plasma membrane content of the β_2-AR by flow cytometry. Values shown are expressed as percent loss of agonist-promoted cell surface receptor. *B*, β_2-AR-mediated accumulation of cAMP. Transfected cells were metabolically labeled with [^{3}H]adenine and exposed to medium with (*black bars*) or without (*open bars*) isoproterenol (*ISO*). Percent conversion is expressed as [^{3}H]cAMP produced/total [^{3}H]adenine uptake. Control cell values for percent conversion of ^{3}H into cAMP were 0.21 ± 0.08 (basal) and 3.3 ± 0.6 (isoproterenol-treated). *C*, LPA-induced phosphoinositide hydrolysis. HEK293 cells were metabolically labeled with [^{3}H]inositol and stimulated with LPA (10 μM). For cells stimulated with lysophosphatidic acid (*LPA*), phosphoinositide hydrolysis ranged from 200 to 250% relative to unstimulated cells. The percent incorporation of ^{3}H into inositol phosphates was 0.56 ± 0.1 for unstimulated cells and 1.34 ± 0.03 for LPA-treated cells. Data shown represent means ± S.E. from three independent experiments done in triplicate and are expressed as fold increase over unstimulated cells.

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. 1*B*, cells expressing β-arrestin1 V53D or dynamin K44A exhibit normal β_2-AR coupling efficiency to G_s, as measured by the accumulation of intracellular cAMP. Similarly, coupling of the LPA receptor to G_i, as measured by the accumulation of intracellular inositol phosphates, was unaffected by expression of β-arrestin1 V53D or dynamin K44A proteins (Fig. 1*C*). 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; β_2-AR) in HEK293 cells induces a 6- to 8-fold increase in phosphorylated MAP kinase (Erk1/2) levels (Fig. 2*A*). Activation of MAP kinases by the β_2-AR in HEK293 cells, like in Cos-7 cells (20), is c-Src- and Ras-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. 2*A*). 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-

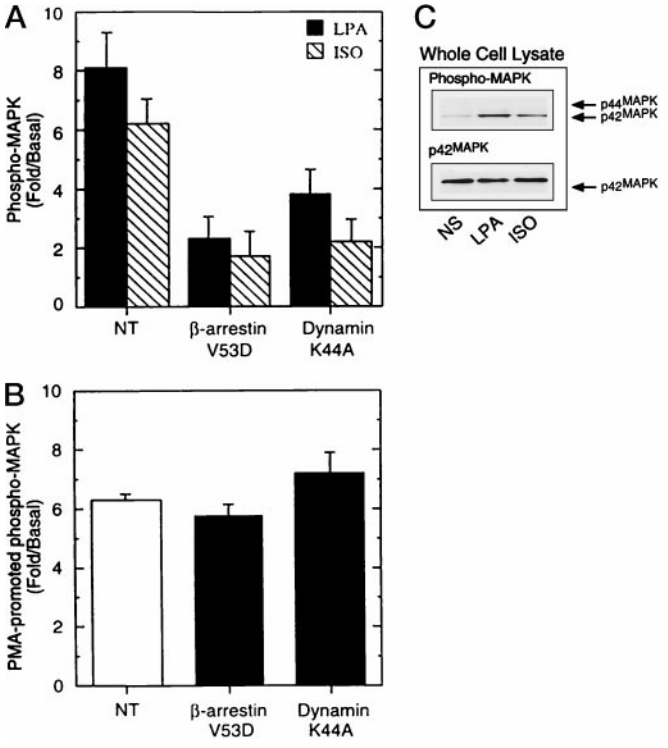

FIG. 2. **Mutant β-arrestin1 and dynamin proteins attenuate GPCR-mediated phosphorylation of MAP kinase.** Cells were transfected with empty pRK5 vector (*NT*), β-arrestin1 V53D, or dynamin K44A and serum-starved overnight prior to activating the endogenous lysophosphatidic acid (*LPA*) or β_2-adrenergic (*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 $p42^{MAPK}$ and $p44^{MAPK}$ (*upper*) and $p42^{MAPK}$ 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.

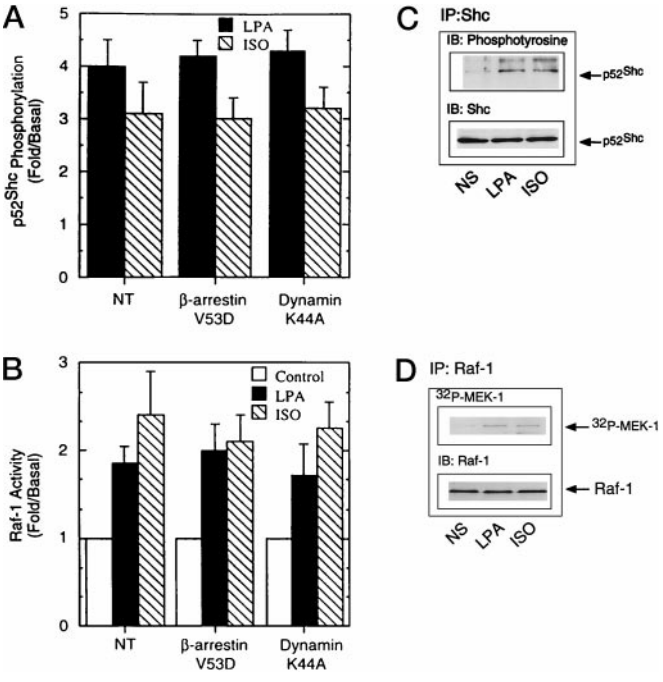

FIG. 3. **Effect of mutant β-arrestin1 and dynamin proteins on endogenous lysophosphatidic acid and β_2-adrenergic receptors-mediated Shc phosphorylation (*A*) and Raf-1 enzymatic activity (*B*).** Cells were transiently transfected with empty pRK5 vector (*NT*), β-arrestin1 V53D, or dynamin K44A and serum-starved prior to stimulation with agonists. *A*, immunoprecipitates of Shc were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. Shown are quantitations of the effects of the mutant β-arrestin1 V53D or dynamin K44A on agonist-stimulated tyrosine phosphorylation of $p52^{shc}$, expressed as fold increase over unstimulated cells. *C*, a representative Western blot showing tyrosine-phosphorylated $p52^{shc}$ (*upper*) and total $p52^{shc}$ 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 quantitations of the effects of β-arrestin1 V53D and dynamin K44A on Raf-1-mediated phosphorylation of MEK. *D*, a representative autoradiogram showing ^{32}P-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.

phorylation of MAP kinase by 60% and 55%, respectively. Other known inhibitors of GPCR sequestration, concanavalin A, monodansylcadavarine, and low temperature (22–24), all inhibited LPA- and isoproterenol-stimulated MAP kinase phosphorylation by approximately 70% (data not shown). In contrast, as shown in Fig. 2*B*, phorbol ester (PMA)-stimulated MAP kinase phosphorylation in HEK293 cells, which is not receptor-mediated, is not affected by the presence of β-arrestin1 V53D or dynamin K44A proteins. Together, these data demonstrate that inhibition of receptor sequestration attenuates activation of MAP kinase without affecting early, plasma membrane-delimited, signaling events such as receptor coupling to G proteins.

Signal transduction between receptors on the plasma membrane and MAP kinase in the cytosol requires a series of events leading first to the assembly of an activated Ras-Raf complex on the membrane, followed by initiation of the cytosolic MEK-MAP kinase cascade. To determine the point in the signaling cascade at which receptor internalization is required, we assayed the effects of β-arrestin1 V53D and dynamin K44A on two intermediate steps in the pathway: tyrosine phosphorylation of the Shc adaptor protein and activation of the Raf kinase. Activation of protein-tyrosine kinases by GPCRs and RTKs is obligatory for signal transduction to MAP kinases (8–13; see Reaction 1). Coincident with the increase in protein-tyrosine kinase activity is the tyrosine phosphorylation of the adaptor protein Shc, thought to play an important role in the nucleation and assembly of the multiprotein, Ras activation complex (11, 12). 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. 3*A*). Expression of neither β-arrestin1 V53D nor dynamin K44A proteins had any effect on LPA- or ISO-stimulated Shc phosphorylation (Fig. 3*A*). 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. 3*B*). 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

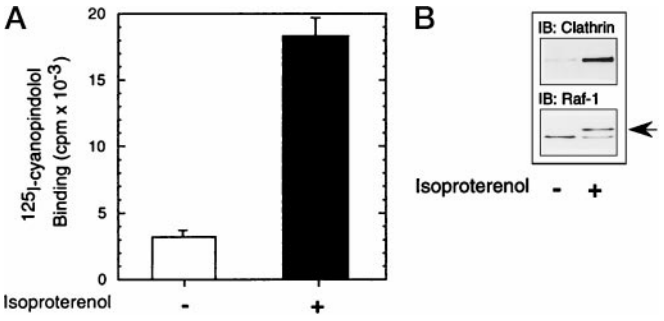

FIG. 4. **Agonist-stimulated increase in β_2-ARs (*A*) and Raf and clathrin (*B*) in the vesicle compartment in HEK293 cells.** HEK293 cells transfected with β_2-AR cDNA were incubated with or without isoproterenol, and crude membranes were subjected to sucrose gradient fractionation. *A*, isoproterenol-induced internalization of the β_2-AR. Vesicle fractions were assayed for ^{125}I-cyanopindolol binding. Data represent means ± S.E. from three experiments. *B*, β_2-AR-dependent redistribution of Raf protein to intracellular vesicles. Vesicle fractions from *A* were examined for their content of clathrin or Raf-1 proteins. Shown is a representative immunoblot from three independent experiments.

plasma membrane and binding to Ras and 14-3-3 proteins, serine and tyrosine phosphorylation, and oligomerization (26, 27). Our data suggest an additional mechanism for regulating the biological actions of Raf; targeting the activated form of the enzyme to an intracellular compartment wherein its substrate, MEK, resides.

Since both β-arrestin V53D and dynamin K44A exert their inhibitory effects downstream of Raf, why does inhibition of *receptor* internalization (by β-arrestin V53D) impair the mitogenic signaling cascade initiated by the GPCRs? A likely possibility is that the agonist-occupied, β-arrestin-bound receptor actually comprises part of a multicomponent signaling complex, assembled at the plasma membrane, which includes not only the receptor, but also various intermediates in the pathway up to and including Raf. Stimulation of HEK293 cells with isoproterenol results in the internalization of 25–35% of the receptor into a vesicular compartment (17). In addition, agonist treatment induces recruitment of soluble clathrin onto the plasma membrane where it forms cage-like structures that enclose a membrane vesicle (18). Following fission of the vesicle from the plasma membrane, clathrin rapidly dissociates from the internalized vesicle into the cytosolic compartment. Fig. 4 shows the agonist-mediated translocation of β_2-AR (4.2-fold), clathrin (3.7-fold), and Raf (2.4-fold) proteins to the internalized vesicles. These data support the notion that agonist stimulates the assembly of a multiprotein signaling complex, including Raf, which is internalized by the clathrin-coated vesicle pathway. These results, however, do not exclude the possibility that Raf internalization, independent of receptor, is required for signal transduction.

A high degree of parallelism exists among GPCR- and RTK-mediated mitogenic signal transduction pathways to MAP kinases. Both receptor types utilize many of the same intermediates in this signaling cascade (13). Recently, inhibition of 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.

Acknowledgments—We thank Dr. T. Sudhof for the cDNA encoding dynamin and Dr. P. Dent for purified $(His)_6$-MEK. We thank Dr. D. Ginty for sharing data prior to publication, Dr. A. Howe for help with the Raf assay, and Drs. J. Pitcher, W. Wetzel, and D. Luttrell for helpful discussion and critical reading of the manuscript. We also thank M. Holben and D. Addison for excellent secretarial assistance.

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