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Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$

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ABSTRACT **Stimulation of G_i-coupled receptors leads to the activation of mitogen-activated protein kinases (MAP kinases). In several cell types, this appears to be dependent on the activation of $p21^{ras}$ (Ras). Which G-protein subunit(s) (G_α or the $G_{\beta\gamma}$ complex) primarily is responsible for triggering this signaling pathway, however, is unclear. We have demonstrated previously that the carboxyl terminus of the β-adrenergic receptor kinase, containing its $G_{\beta\gamma}$-binding domain, is a cellular $G_{\beta\gamma}$ antagonist capable of specifically distinguishing G_α- and $G_{\beta\gamma}$-mediated processes. Using this $G_{\beta\gamma}$ inhibitor, we studied Ras and MAP kinase activation through endogenous G_i-coupled receptors in Rat-1 fibroblasts and through receptors expressed by transiently transfected COS-7 cells. We report here that both Ras and MAP kinase activation in response to lysophosphatidic acid is markedly attenuated in Rat-1 cells stably transfected with a plasmid encoding this $G_{\beta\gamma}$ antagonist. Likewise in COS-7 cells transfected with plasmids encoding G_i-coupled receptors (α_2-adrenergic and M2 muscarinic), the activation of Ras and MAP kinase was significantly reduced in the presence of the coexpressed $G_{\beta\gamma}$ antagonist. Ras–MAP kinase activation mediated through a G_q-coupled receptor (α_1-adrenergic) or the tyrosine kinase epidermal growth factor receptor was unaltered by this $G_{\beta\gamma}$ antagonist. These results identify $G_{\beta\gamma}$ as the primary mediator of Ras activation and subsequent signaling via MAP kinase in response to stimulation of G_i-coupled receptors.**

The α_2-adrenergic receptor (α_2-AR) and the receptor for lysophosphatidic acid (LPA) have been shown to transduce cellular signals through G_i proteins, resulting in cell proliferation (1–3). These responses resemble those evoked by growth factor tyrosine kinase receptors, in which the signaling cascade has been more thoroughly delineated. Signaling via growth factors such as epidermal growth factor (EGF) involves phosphorylation of tyrosine residues and a series of protein–protein interactions, mediated via Src homology 2 and 3 (SH2/SH3) domains, leading to serial activation of $p21^{ras}$ (Ras), Raf-1 kinase and mitogen-activated protein kinases (MAP kinases) (reviewed in refs. 4 and 5). Two members of the MAP kinase family are the p44 MAP kinase (extracellular signal-regulated kinase 1, ERK1) and p42 MAP kinase (ERK2). When these two isoforms are activated by phosphorylation catalyzed by Raf-1 kinase or other MAP kinase kinases (MEKs) they in turn catalyze the phosphorylation of a large group of substrates located at the cell membrane, cytoplasm and nucleus (reviewed in ref. 6). Recently, several G_i-coupled receptors, including the α_2-AR, LPA receptor, M2 muscarinic acetylcholine receptor (AChR), and platelet-activating factor (PAF) receptor, have been shown to stimulate MAP kinase activity in various cell types (7–13). The signaling pathways by which G_i-coupled receptors activate MAP kinase, however, remain poorly understood, but there is evidence for both Ras-dependent (7–12) and Ras-independent (5, 13, 14) activation of MAP kinases. The recently discovered MAP kinase kinase kinase (MEK kinase or MEKK) (14) is a possible link in a Ras/Raf-independent G protein-coupled MAP kinase activation cascade.

Agonist activation of G protein-coupled receptors results in the dissociation of heterotrimeric ($\alpha\beta\gamma$) G proteins into activated G_α-GTP and $G_{\beta\gamma}$ subunits (15, 16). It has been established that not only G_α but also $G_{\beta\gamma}$ transduces cellular signals following agonist occupancy of receptors (17, 18). A variety of effectors have now been identified that can be directly regulated by $G_{\beta\gamma}$, including ion channels, adenylyl cyclases, and phospholipases (18). In addition to modulation of these effectors, $G_{\beta\gamma}$ mediates the membrane translocation/activation of the β-adrenergic receptor kinases (βARK1 and βARK2) leading to the phosphorylation and desensitization of agonist-occupied receptors (19, 20). The primary G-protein subunit(s) responsible for G_i-mediated Ras and/or MAP kinase activation has not been identified, but two recent reports (21, 22) have implicated $G_{\beta\gamma}$ as an activator of overexpressed p44 MAP kinase (ERK1) in COS-7 cells. However, a direct role for $G_{\beta\gamma}$ in Ras activation or in the regulation of endogenous MAP kinase activity has not been established.

We have previously identified the region within the carboxyl-terminal domain of βARK that physically interacts with $G_{\beta\gamma}$ (23). When the carboxyl terminus of βARK1 is expressed as a fusion protein and purified from *Escherichia coli*, it can effectively inhibit the $G_{\beta\gamma}$-mediated *in vitro* translocation of βARK to rhodopsin-enriched rod outer-segment membranes (24). In a rat olfactory ciliary preparation, the purified carboxyl terminus of βARK2, as well as a peptide from its $G_{\beta\gamma}$-binding domain, inhibited desensitization of the cAMP response generated by activation of odorant receptors (25). In addition to the inhibition of βARK activity, we have shown that when the $G_{\beta\gamma}$-binding domain of βARK1 is expressed in COS-7 cells, it significantly attenuates phospholipase C β and type II adenylyl cyclase signaling through G_i-coupled receptors, which are known to be $G_{\beta\gamma}$-mediated processes (26). In these whole-cell experiments, signaling through G_α subunits was not affected by the expressed βARK1 carboxyl-terminal polypeptide, verifying its utility as a specific $G_{\beta\gamma}$ antagonist.

As reported here, we have employed this specific $G_{\beta\gamma}$ antagonist (the carboxyl terminus of βARK1) to investigate

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Abbreviations: AR, adrenergic receptor; AChR, acetylcholine receptor; βARK, β-AR kinase; PTX, pertussis toxin; MAP kinase, mitogen-activated protein kinase; LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; SH, Src homology; PH, pleckstrin homology.

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the role of $G_{\beta\gamma}$ in G protein-coupled receptor activation of both Ras and MAP kinase. Endogenous Ras and p42 MAP kinase (ERK2) responses through several G_i-coupled receptors were studied in both transiently transfected COS-7 cells and stably transfected Rat-1 fibroblasts. Our results directly demonstrate that $G_{\beta\gamma}$ released by the stimulation of G_i-coupled receptors stimulates the activity of MAP kinase by activation of Ras.

MATERIALS AND METHODS

DNA Constructs and Cell Culture. The construction of the plasmid encoding the carboxyl-terminal βARK1 polypeptide $G_{\beta\gamma}$ antagonist pRK-βARK1-(Gly^{495}–Leu^{689}) has been described (26). The βARK1-(495–689) DNA was stably transfected into Rat-1 fibroblasts by calcium phosphate precipitation (27). Transient transfections were done in COS-7 cells by a standard DEAE-dextran method (23, 26).

Determination of MAP Kinase Phosphorylation. The phosphorylation/activation of p42 MAP kinase (ERK2) was determined by an electrophoretic mobility-shift assay similar to that described by Alblas *et al.* (11). Stably transfected Rat-1 cells or transiently transfected COS-7 cells were plated at equal densities on six-well dishes, grown to confluence, and then serum-starved for 18–24 hr. Cells were then exposed to agonists, rinsed with ice-cold phosphate-buffered saline, and lysed in RIPA buffer [150 mM NaCl/50 mM Tris·HCl, pH 8.0/5 mM EDTA/1% (vol/vol) Nonidet P-40/0.5% (wt/vol) sodium deoxycholate/0.1% SDS/10 mM NaF/10 mM disodium pyrophosphate/0.1 mM phenylmethanesulfonyl fluoride containing leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), benzamide (10 μg/ml), aprotinin (5 μg/ml), and pepstatin A (1 μg/ml)]. Proteins were electrophoresed in an SDS/12% polyacrylamide gel and transferred to nitrocellulose for Western blot analysis using an ERK2 monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) and ^{125}-labeled anti-mouse IgG (Amersham). The nonphosphorylated ERK2 and the slower-migrating activated, phosphorylated ERK2 were then quantitated with a Molecular Dynamics PhosphorImager.

Activation of Ras. Cells plated on six-well dishes were serum-starved and preincubated with [^{32}P]orthophosphate for 2 hr. Cells were treated with agonists, rinsed with phosphate-buffered saline, and solubilized with 1% Nonidet P-40 in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/25 mM $MgCl_2$. Ras was immunoprecipitated with monoclonal antibody Y13-259 (Oncogene Science) and protein-G Sepharose 4 Fast Flow (Pharmacia) [75 μl of a 20% (vol/vol) slurry in lysis buffer]. Ras-bound GDP and GTP were then separated by TLC on polyethyleneimine-cellulose plates in 0.75 M KH_2PO_4 (pH 3.4) as described (28). After autoradiography, the labeled GDP and GTP species were quantitated with a Molecular Dynamics PhosphorImager.

RESULTS AND DISCUSSION

Studies in Rat-1 Cells. In quiescent Rat-1 fibroblasts and various other cell types, the phospholipid LPA stimulates DNA synthesis and promotes cell proliferation (1–3). This response is mediated through a pertussis toxin (PTX)-sensitive G protein-coupled receptor, and signaling is independent of other known G_i-mediated effectors (1). LPA effects on cell proliferation involve the activation of MAP kinase and are dependent on the activation of Ras (7–10). However, the mechanism of activation of the Ras–MAP kinase pathway by LPA is unknown, and the G-protein subunit ($G_{i\alpha}$ or $G_{i\beta\gamma}$) responsible for mediating this signal transduction pathway has not been identified.

In order to investigate which G_i protein subunit(s) is involved in the cellular proliferative effects of LPA, we created stably transfected Rat-1 cell lines expressing the βARK1-(495–689) polypeptide, which we have previously shown to be a specific cellular $G_{\beta\gamma}$ antagonist (26). We studied the effect of $G_{\beta\gamma}$ inhibition on the ability of LPA to activate p42 MAP kinase (ERK2) by stimulating serum-starved wild-type Rat-1 and βARK1-(495–689)-expressing cells with 10 μM LPA for various times (Fig. 1). Following agonist incubation, activated ERK2 was assayed by Western blotting using anti-ERK2 serum and measuring the appearance of a form of ERK2 with retarded gel mobility due to phosphorylation of ERK2 on both tyrosine and threonine residues (11). LPA-stimulated phosphorylation of ERK2 (represented by the slower-migrating, upper band) peaked in ≈5 min and was 5-fold greater than the basal level (Fig. 1). In two cell lines expressing βARK1-(495–689), the phosphorylation of ERK2 was markedly inhibited at 5 min compared with control Rat-1 cells, and only minor amounts of the slower-migrating ERK2 species were detectable after 10 min of LPA exposure (Fig. 1). The activation of ERK2 by EGF, which occurs via the tyrosine kinase receptor pathway, was greater than the LPA response and was unaffected by βARK1-(495–689) expression (Fig. 1). The level of inhibition of LPA-induced ERK2 activation seen with βARK1-(495–689) expression was the same as that resulting from pretreating wild-type Rat-1 cells with PTX (Fig. 1), suggesting that MAP kinase signaling by LPA via G_i is solely through the βγ subunit complex.

The ability of βARK1-(495–689) expression to inhibit LPA-mediated ERK2 activation was also studied in Rat-1 cells by labeling serum-starved cells with [^{32}P]orthophosphate and immunoprecipitating activated ERK2 after agonist exposure. Virtually identical results were obtained with this assay as with the gel mobility-shift assay (data not shown). Thus, two independent methods show that Rat-1 cells stably

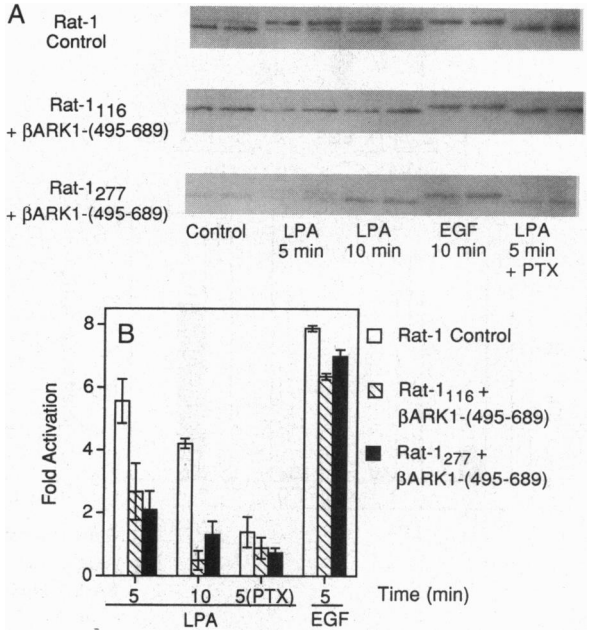

FIG. 1. Effect of the $G_{\beta\gamma}$ antagonist βARK1-(495–689) on LPA-induced ERK2 activation in Rat-1 cells. Parental Rat-1 cells and cell lines stably expressing the $G_{\beta\gamma}$ antagonist βARK1-(495–689) polypeptide (Rat-1_{116} and Rat-1_{277}) were serum-starved and subjected to agonist treatment [LPA (10 μM) or EGF (100 ng/ml)] for the times indicated. Some cells were treated with PTX (100 ng/ml) ≥16 hr before LPA treatment. Endogenous ERK2 MAP kinase was detected by Western blotting. (*A*) A representative Western blot done in duplicate. (*B*) The slower-migrating ERK2 was quantitated, and the data represent the mean ± SEM of at least four independent experiments done in duplicate.

expressing the βARK1-(495–689) polypeptide have attenuated endogenous MAP kinase responses to LPA activation of its endogenous G_i-coupled receptor, demonstrating that this process is mediated by $G_{i\beta\gamma}$. The activation of MAP kinase by LPA does not occur through the activation of phospholipase C by $G_{i\beta\gamma}$, since LPA-induced increases in phosphoinositol accumulation in Rat-1 cells has been shown to be PTX-insensitive (1). Consistent with this in the Rat-1 cell lines expressing βARK1-(495–689), LPA-induced inositolphospholipid accumulation was unaltered and was much greater than levels stimulated by other G_i-coupled receptor agonists (data not shown). These observations are also consistent with previous findings suggesting that LPA couples to more than one G protein in Rat-1 cells (presumably G_q as well as G_i) (1).

Since the activation of MAP kinases by LPA in cells such as Rat-1 fibroblasts has been shown to be mediated by activation of Ras (7–10), we next examined Ras·GTP accumulation in wild-type Rat-1 and βARK1-(495–689)-expressing cells. Serum-starved cells were labeled with [^{32}P]orthophosphate, and the ratio of Ras-bound guanine nucleotides was measured following agonist exposure. Both LPA and EGF activated Ras as determined by increased percent GTP over the basal level (Fig. 2). The increased Ras·GTP due to LPA (≈70% over basal) peaked in 2 min and remained elevated at 3 min (Fig. 2*B*) and then declined rapidly at 5 and 10 min of agonist exposure (data not shown). In the Rat-1_{277} cell line, expressing the βARK1-(495–689) polypeptide, virtually no LPA-induced Ras·GTP accumulation was seen at any time period tested (Fig. 2*B*). PTX also inhibited Ras activation by LPA, as previously shown (7–10), consistent with our MAP kinase data above. The activation of Ras by EGF was more robust (2- to 3-fold over basal), reaching a maximum of 80–90% Ras·GTP, and this level of activation was not significantly altered in cells expressing βARK1-(495–689) (Fig. 2). A second cell line (Rat-1_{345}) stably expressing βARK1-(495–689) produced identical results (data not shown).

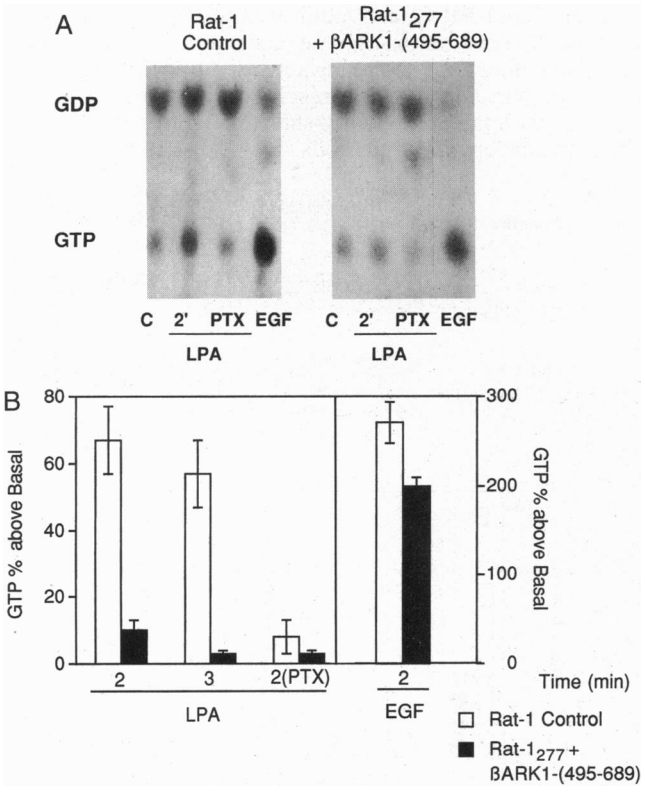

FIG. 2. Effect of the $G_{\beta\gamma}$ antagonist βARK1-(495–689) on LPA-induced Ras activation in Rat-1 cells. Control Rat-1 cells and cells stably expressing the βARK1-(495–689) polypeptide (line 277) were serum-starved, metabolically labeled with [^{32}P]orthophosphate, and stimulated with control diluent or agonists [LPA (10 μM) or EGF (100 ng/ml)] for 2 min (*A*) or for 2 or 3 min (*B*). Some cells were pretreated with PTX (100 ng/ml) for ≥16 hr. Guanine nucleotides bound to Ras were quantitated from immunoprecipitated Ras. *A* shows a representative TLC plate and *B* displays the increase in the ratio of Ras·GTP vs. total guanine nucleotides bound to Ras as percent above the basal (untreated control, C) ratio. Data represent the mean ± SEM of at least three independent experiments done in duplicate.

These data support the hypothesis that in Rat-1 cells, LPA activates MAP kinase through a Ras-dependent pathway which is initially triggered by the βγ subunit complex of a PTX-sensitive G protein. The inhibition of G_i-mediated Ras activation by the carboxyl terminus of βARK may lead to the attenuation of Ras-mediated cellular proliferation. That G_i-mediated Ras activation in Rat-1 cells occurs via $G_{\beta\gamma}$ is somewhat surprising, since mutationally activated $G_{\alpha i2}$ (gip2) is a known transforming agent in Rat-1 cells (29). Perhaps it is the unnatural activated state of this mutant protein which produces these effects, since the role of the endogenous wild-type $G_{\alpha i}$ in Rat-1 cells is not fully understood and the data in this report indicate that it is not involved in the LPA-induced Ras response.

Studies in COS-7 Cells. Since previous G protein-coupled Ras–MAP kinase signaling results appeared to be cell specific, we employed our $G_{\beta\gamma}$ antagonist to investigate MAP kinase and Ras activation in a second cell type by transiently transfecting several receptors into COS-7 cells. The α_2-AR and M2 muscarinic AChR are two G_i-coupled receptors which have been shown in stably transfected Rat-1 cells to activate the Ras–MAP kinase pathways (11, 12). Recently, Faure *et al.* (21) and Crespo *et al.* (22) have shown that agonist-occupied G_i-coupled receptors can stimulate cotransfected p44 MAP kinase (ERK1) in COS-7 cells and that this overexpressed MAP kinase can be activated by combinations of co-overexpressed $G_{\beta\gamma}$ subunits. Transfected wild-type $G_{\alpha i}$ did not, by itself, activate overexpressed ERK1 in COS-7 cells (21), and experiments using a dominant negative mutant of Ras (22) suggested that $G_{i\beta\gamma}$-linked activation of overexpressed ERK1 is mediated through Ras activation, correlating with our results shown above for LPA in Rat-1 cells. We therefore assessed the effect of $G_{\beta\gamma}$ inhibition on Ras and endogenous p42 MAP kinase (ERK2) activation in COS-7 cells through G_i as well as by receptors coupled to other G proteins.

In COS-7 cells transiently transfected with either α_2-AR or M2-AChR, agonist exposure produced a 2- to 3-fold increase of phosphorylated ERK2 over a time course of 2–10 min as determined by the gel mobility assay, with peak phosphorylation occurring at 5 min (Fig. 3). When COS-7 cells were cotransfected with plasmid DNAs encoding either of these two G_i-coupled receptors (expressing 1–2 pmol of receptor per mg of membrane protein) and βARK1-(495–689), the activation of ERK2 was largely blocked (Fig. 3).

The specificity of the observed βARK1-(495–689) polypeptide inhibition was also investigated. As shown in Fig. 3, no inhibition of agonist-stimulated ERK2 phosphorylation with the $G_{\beta\gamma}$ antagonist was seen in COS-7 cells coexpressing α_1-AR, which is coupled to G_q. These data demonstrate that under these test conditions in COS-7 cells, only G_i-coupled receptors activate endogenous MAP kinase via $G_{\beta\gamma}$. These data and the results described above indicate that $G_{i\beta\gamma}$ can activate MAP kinase in more than one cell type and suggest that this type of $G_{\beta\gamma}$ signaling may be a general phenomenon for G_i-coupled receptors. These results are also in agreement with the recently reported G_i activation of overexpressed ERK1 in COS-7 cells (21, 22), suggesting a common mechanism of activation for these two MAP kinase isoforms.

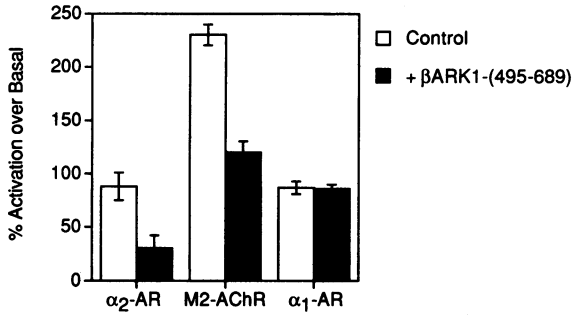

FIG. 3. Effect of $G_{\beta\gamma}$ antagonist βARK1-(495–689) expression on MAP kinase activation by coexpressed G protein-coupled receptors in COS-7 cells. α_2-AR, α_{1B}-AR, and M2-AChR DNAs subcloned into the eukaryotic expression vector pRK5 were transfected into COS-7 cells with either empty pRK5 vector (control) or βARK1-(495–689) DNA. The transfected cells were serum-starved and exposed to receptor-specific agonists (1 μM UK-14304 for α_2-AR, 10 μM epinephrine for α_{1B}-AR, and 100 μM carbachol for M2-AChR) for 5 min. Activated endogenous ERK2 MAP kinase was determined as in Fig. 1. Data shown represent the mean ± SEM of three independent experiments done in duplicate.

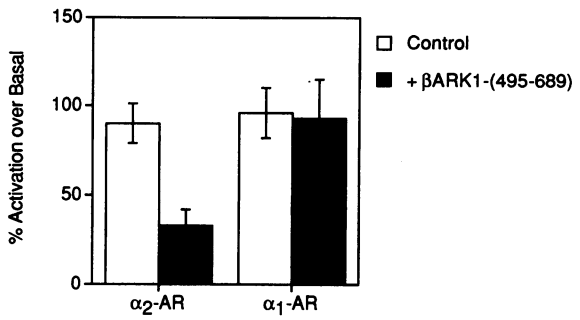

FIG. 4. Effect of $G_{\beta\gamma}$ antagonist βARK1-(495–689) expression on Ras activation by coexpressed α-ARs in COS-7 cells. COS-7 cells were transfected with α_2-AR or α_{1B}-AR DNA and either empty pRK5 vector DNA (control) or βARK1-(495–689) DNA as in Fig. 3. Activation of Ras was determined as in Fig. 2. Data displayed are expressed as the increase in the ratio Ras·GTP/(Ras·GTP + Ras·GDP) as percent above the basal ratio and represent the mean ± SEM of at least three independent experiments done in duplicate.

If the $G_{i\beta\gamma}$ activation of MAP kinase occurs in COS-7 cells by the same mechanism as in Rat-1 cells, then Ras should be activated following G_i-coupled receptor stimulation and should be inhibited by coexpression of the βARK1-(495–689) polypeptide. Therefore, we studied the effects of $G_{\beta\gamma}$ inhibition on Ras activation mediated by G protein-coupled receptors in COS-7 cells. COS-7 cells were transfected with plasmid DNA encoding the α_1- or α_2-AR in the absence or presence of βARK1-(495–689) DNA. The cells were then serum-starved and labeled with [^{32}P]orthophosphate. The ratio of bound GTP to total bound guanine nucleotides following agonist stimulation was then determined as in the experiments with Rat-1 cells above. In COS-7 cells transfected with receptor DNA alone (expressing 1–2 pmol of receptor per mg membrane protein), both ARs were found to activate Ras ≈2-fold over the basal level after 2 min of agonist exposure (Fig. 4). As in the MAP kinase assays above, no significant decrease in the α_1-AR G_q response was seen in the presence of the $G_{\beta\gamma}$ antagonist, whereas a substantial decrease in percent GTP bound to Ras was seen in cells coexpressing α_2-AR and the βARK1-(495–689) polypeptide. The agonist-stimulated fold increase in percent GTP bound to Ras in COS-7 cells coexpressing α_2-AR and $G_{\beta\gamma}$ antagonist was only 30–40% over the basal level, compared with an ≈100% increase in Ras·GTP in cells expressing only this G_i-coupled receptor (Fig. 4). Similar Ras inhibition was observed in COS-7 cells expressing both the G_i-linked M2-AChR and βARK1-(495–689) polypeptide (data not shown). The G_s-coupled D_{1A} dopamine receptor was also tested but did not produce any observable Ras activation when expressed alone under these conditions (data not shown). This result is not surprising, since cAMP accumulation, which would occur subsequent to G_s activation, leads to a decrease in activated MAP kinase due to an inhibition at the level of Raf-1 (30–32). Thus, it appears that only G_i activation leads to $G_{\beta\gamma}$-mediated Ras activation.

Our findings show that MAP kinase activation via G_i-coupled receptors is mediated by $G_{\beta\gamma}$ and occurs as a direct result of Ras activation. Activated Ras can then act as a molecular switch causing the cascade to continue through Raf-1 activation, subsequently leading to the stimulation of the MAP kinases. A scheme for this signaling cascade through which G_i-coupled receptors activate the Ras–MAP kinase pathway is depicted in Fig. 5; this has now been demonstrated in Rat-1 fibroblasts as well as COS-7 cells. The tyrosine kinase growth factor receptor cascade is also shown to illustrate how these two distinct signaling pathways converge at Ras activation.

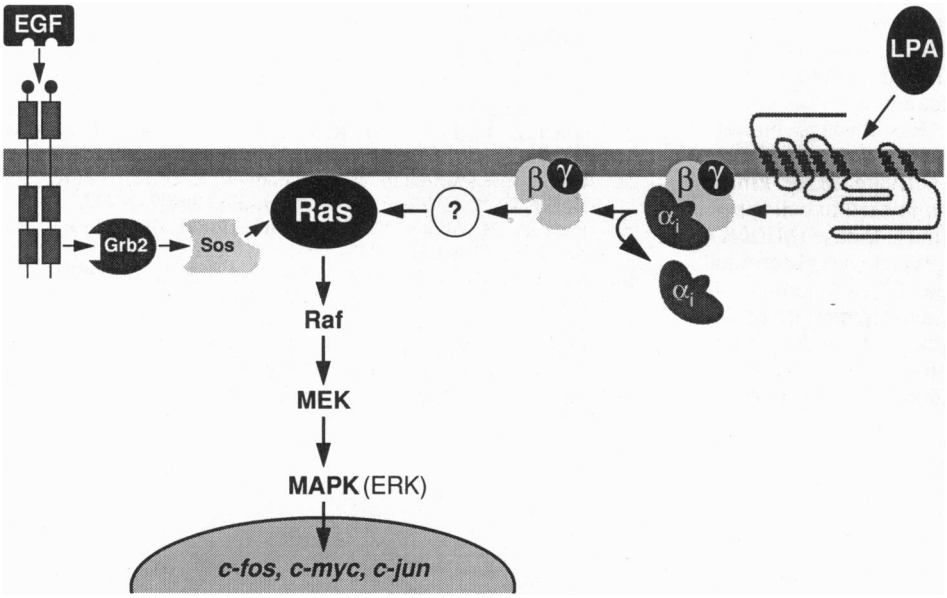

FIG. 5. Scheme of the activation of the Ras–MAP kinase signal transduction pathway through growth factor and G_i-coupled receptors. G_i-coupled receptor activation (e.g., LPA) of Ras is mediated by the $G_{\beta\gamma}$ subunit complex resulting from receptor-mediated dissociation of the heterotrimeric G_i protein. $G_{\beta\gamma}$ alone or through the recruitment of one or more cytoplasmic or membrane-associated factors [possibly by pleckstrin homology (PH) domain-directed binding], leads to Ras activation. Growth factor receptor activation (e.g., EGF) activates Ras through the recruitment (directed by SH2/SH3 domains) of the cytoplasmic proteins Grb2 and Sos1. The convergence of these two different surface receptor signaling pathways on Ras activation, in turn, leads to the sequential activation of the Raf-1–MAP kinase cascade, producing activation of nuclear genes and cellular responses, including growth and differentiation.

The findings in this report support a previously unappreciated role of $G_{\beta\gamma}$ in cellular signaling. The activation of Ras by $G_{i\beta\gamma}$ leads to the activation of MAP kinases in at least the two cell types studied here. Since our studies used only endogenous G proteins and signaling components (excluding receptors), it appears that indeed $G_{\beta\gamma}$ can activate Ras, which can now be added to the increasing number of known $G_{\beta\gamma}$-mediated effector systems. This study also adds the inhibition of cell proliferation to the growing list of potential applications of the βARK1-(495–689) polypeptide as a specific $G_{\beta\gamma}$ antagonist. The use of this reagent to study $G_{\beta\gamma}$ signaling provides further evidence for the importance of this G-protein subunit complex. Cell-specific differences in $G_{i\beta\gamma}$ activation of MAP kinases through Ras activation may still occur, since the G_i-coupled receptor for platelet-activating factor can activate MAP kinase independently of Ras in CHO cells (13). It will be interesting to see whether MAP kinase activation by this G_i-coupled receptor also involves $G_{\beta\gamma}$.

What is not known at this time is the mechanism by which $G_{\beta\gamma}$ activates Ras. Evidence is lacking to make a strong case for any single hypothesis, but several possible $G_{\beta\gamma}$ targets exist which may result in the activation of Ras. One possible effector of $G_{\beta\gamma}$ would be a novel tyrosine kinase. There is some evidence supporting the idea of G_i-induced tyrosine phosphorylation prior to MAP kinase activation (8, 10). In this scenario, $G_{\beta\gamma}$ may bind to and translocate a tyrosine kinase to the membrane much like the membrane targeting of βARK (19, 20). The membrane-bound $G_{\beta\gamma}$-tyrosine kinase complex may then resemble an activated growth factor receptor, recruiting a Grb2-like adapter molecule through SH2/SH3 domains and activating Ras by subsequent binding to a Sos-like protein (Fig. 5).

Another possibility arises from the recent discovery that the carboxyl terminus of βARK contains a PH domain, sharing sequence homology with several proteins involved in cellular signal transduction (24, 33). Interestingly, several proteins involved in Ras regulation also have PH domains, including Ras GRF (guanine-nucleotide releasing factor), Ras GAP (GTPase-activator protein), and Sos. Fusion proteins of several of these PH domains can bind to purified $G_{\beta\gamma}$ subunits (24). Thus, the possibility exists that $G_{\beta\gamma}$ can bind directly to a guanine nucleotide-exchange factor such as Sos, which can directly activate Ras, or $G_{\beta\gamma}$ may first bind to a novel adapter protein via a PH domain and proceed down the cascade at this point. Alternatively, $G_{\beta\gamma}$ may interact with specific GAPs through a PH domain, thereby altering their direct interaction with steady-state Ras. This could possibly alter the equilibrium of activated Ras and produce MAP kinase activation and subsequent cell proliferation. As shown in Fig. 5, we hypothesize that in an analogous fashion G_i-coupled receptors act as Ras activators primarily through protein–protein interactions involving $G_{\beta\gamma}$ and PH domains, whereas growth factor receptors (e.g., EGF) activate Ras through interactions involving SH2/SH3 domains.

It is also possible that $G_{i\beta\gamma}$ can activate MAP kinase independent of its effects on Ras. Thus in certain cell types, $G_{\beta\gamma}$ may interact with a MAP kinase kinase kinase (MEKK), or other pathways may exist. By using the carboxyl-terminal domain of βARK1 as a specific cellular $G_{\beta\gamma}$ antagonist, we have demonstrated the key role of $G_{\beta\gamma}$ in the activation of Ras through G_i-coupled receptor activation in both Rat-1 and COS-7 cells. Further experimentation will be required to elucidate the primary $G_{\beta\gamma}$ effector responsible for Ras–MAP kinase activation.

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