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G_o-protein α-Subunits Activate Mitogen-activated Protein Kinase via a Novel Protein Kinase C-dependent Mechanism*

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Mitogen-activated protein kinase (MAPK) is activated in response to both receptor tyrosine kinases and G-protein-coupled receptors. Recently, G_i-coupled receptors, such as the α_{2A} adrenergic receptor, were shown to mediate Ras-dependent MAPK activation via a pathway requiring G-protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) and many of the same intermediates involved in receptor tyrosine kinase signaling. In contrast, G_q-coupled receptors, such as the M_1 muscarinic acetylcholine receptor (M_1AChR), activate MAPK via a pathway that is Ras-independent but requires the activity of protein kinase C (PKC). Here we show that, in Chinese hamster ovary cells, the M_1AChR and platelet-activating factor receptor (PAFR) mediate MAPK activation via the α-subunit of the G_o protein. G_o-mediated MAPK activation was sensitive to treatment with pertussis toxin but insensitive to inhibition by a $G_{\beta\gamma}$-sequestering peptide (βARK1ct). M_1AChR and PAFR catalyzed G_o α-subunit GTP exchange, and MAPK activation could be partially rescued by a pertussis toxin-insensitive mutant of $G_{o\alpha}$ but not by similar mutants of G_i. G_o-mediated MAPK activation was insensitive to inhibition by a dominant negative mutant of Ras (N17Ras) but was completely blocked by cellular depletion of PKC. Thus, M_1AChR and PAFR, which have previously been shown to couple to G_q, are also coupled to G_o to activate a novel PKC-dependent mitogenic signaling pathway.

Mitogen-activated protein kinase (MAPK)[1] can be activated by a variety of extracellular stimuli, including those mediated by receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) (1–3). The mitogenic signaling pathway mediated by the epidermal growth factor RTK involves a cascade of protein-protein interactions, leading to Ras-dependent MAPK activation (4, 5). Agonist binding to the epidermal growth factor RTK leads to receptor dimerization and autophosphorylation, resulting in a phosphotyrosine-dependent association with Shc. The subsequent interaction between Tyr(P)-phosphorylated Shc and the Grb2 adaptor protein causes a translocation of the Grb2-SOS complex to the membrane, where SOS mediates guanine nucleotide exchange on Ras (6).

Recently, $\beta\gamma$ subunits derived from PTX-sensitive heterotrimeric G-proteins were also shown to mediate Ras-dependent MAPK activation (7–10). Release of $G_{\beta\gamma}$ promotes the tyrosine phosphorylation of Shc and its subsequent association with Grb2-SOS. Both RTK- and $G_{\beta\gamma}$-mediated MAPK activation are completely blocked by the expression of dominant negative mutants of mSOS1 and Ras, demonstrating that RTKs and $G_{\beta\gamma}$ activate MAPK via a common signaling pathway involving Shc, Grb2, SOS, and Ras (7).

MAPK activation via G_i-coupled receptors, such as α_{2A}AR and the lysophosphatidic acid receptor, is sensitive to inhibition by the C-terminal fragment of βARK1 (βARK1ct), a competitive inhibitor of $G_{\beta\gamma}$-mediated signals (10). However, not all GPCRs mediate MAPK activation exclusively via receptor-catalyzed release of $\beta\gamma$ subunits. For example, in COS-7 cells, MAPK activation via receptors coupled to members of the PTX-insensitive $G_{q/11}$ family, such as M_1AChR and the α_1 adrenergic receptor (α_1AR), is insensitive to the $G_{\beta\gamma}$-sequestrant βARK1ct peptide (11). Instead, MAPK activation occurs predominantly via a PKC-dependent pathway. The GTP-bound α-subunit of the $G_{q/11}$ protein activates phosphoinositide hydrolysis (12) and protein kinase C (PKC). Once activated, PKC stimulates MAPK activity via a poorly understood mechanism involving the activation of Raf kinase (13, 14).

MAPK activation in CHO cells stably transfected with PAFR cDNA has been reported to be sensitive to PTX and independent of Ras (15). We have studied MAPK activation by GPCRs in COS-7 and CHO cells and find that the mechanism of M_1AChR- and PAFR-mediated MAPK activation varies between cell types. Our data demonstrate the existence of a novel PKC-dependent mitogenic signaling pathway, which is mediated by the α-subunit of the PTX-sensitive G_o-protein and which is independent of Ras activation.

EXPERIMENTAL PROCEDURES

DNA Constructs—Hemagglutinin-tagged $p44^{MAPK}$ ($p44^{HA\text{-}MAPK}$) cDNA was provided by J. Pouysségur; the dominant negative mutant $p21^{N17Ras}$ cDNA was provided by D. Altschuler and M. Ostrowski; the PTX-insensitive $G_{o\alpha}$ ($G_{o\alpha}$PT) cDNA was provided by R. Taussig; the PTX-insensitive mutants of $G_{i1\alpha}$, $G_{i2\alpha}$, and $G_{i3\alpha}$ ($G_{i1\alpha}$PT, $G_{i2\alpha}$PT, and $G_{i3\alpha}$PT) cDNAs were provided by S. Senogles. All PTX-insensitive G_α-subunits were created by a mutation of the C-terminal cysteine, thereby removing the site of ADP-ribosylation by PTX. PAFR cDNA was provided by R. Snyderman; M_1AChR cDNA was provided by E. Peralta (16); G_β and G_γ cDNAs were provided by M. Simon; α_{2A}AR cDNA and

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; GPCR, G-protein-coupled receptor; α_{2A}AR, α_{2A} adrenergic receptor; PTX, pertussis toxin; M_1AChR, M_1 muscarinic acetylcholine receptor; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; α_1AR, α_1 adrenergic receptor; PKC, protein kinase C; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; MBP, myelin basic protein.

α_{1B}AR cDNA were cloned in our laboratory.

Cell Culture and Transfection—COS-7 and CHO-K1 cells were maintained as described (11). Transient transfection of both cell types was performed using LipofectAMINE (Life Technologies, Inc.) as described previously (17). Cells were treated with PTX or phorbol ester (PMA) 24 h after transfection, where indicated. Assays were performed 48 h after transfection.

Measurement of MAPK Activity—Agonist-stimulated activation of $p44^{HA\text{-}MAPK}$ was determined as described previously (17) using myelin basic protein (MBP) as an exogenous substrate. [^{32}P]ATP-labeled MBP was detected and quantitated after electrophoresis using a Molecular Dynamics PhosphorImager.

Immunoblotting—The expression of endogenous G_α subunits was assayed by immunoblotting whole cell lysates using standard methods and anti-$G_{o\alpha}$ rabbit polyclonal antibody, anti-$G_{q\alpha}$ rabbit polyclonal antibody, or anti-$G_{i3\alpha}$ rabbit polyclonal antibody (Upstate Biotechnology Inc.).

Photoaffinity Labeling of Plasma Membrane G Proteins—[α-^{32}P]GTP azidoanilide was made from [α-^{32}P]GTP and purified by polyethyleneimine cellulose chromatography as described previously (18). Two or three days after transfection, cells were washed three times with Ham's F-12 medium and then permeabilized with streptolysin-O (10 units/5 ml × 30 min). Cells were rinsed three times with medium and once with photolabel buffer (25 mM Hepes, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 5 μg/ml soybean trypsin inhibitor) and then incubated with the same buffer (500 μl) containing [α-^{32}P]GTP azidoanilide (≈10 μCi) and 3 μM GDP at 37 °C for 10 min. Agonist or vehicle was added for a further 10-min incubation. Cells were rinsed twice with ice-cold photolabel buffer containing 1 mM dithiothreitol, placed on ice, and illuminated with UV light for 4 min. After exposure to UV light, cells were washed rapidly twice with unsupplemented photolabel buffer, scraped into 250 μl of solubilization buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% cholate), and incubated on ice for 1 h with frequent vortexing. The suspension was centrifuged at 500,000 × *g* for 20 min. The supernatants were harvested and incubated for 1 h with 5 μl of anti-α_o antibody (Upstate Biotechnology Inc., Lake Placid, NY) and 25 μl of a 50% slurry of protein A-Sepharose. Tubes were centrifuged and supernatants discarded, and pellets were washed three times with ice-cold phosphate-buffered saline and then boiled for 5 min in Laemmli sample buffer. Samples were run under reducing conditions on SDS-polyacrylamide gel electrophoresis and were subjected to autoradiography. Relative densities of the G protein bands were determined with a model GS-670 imaging densitometer and Molecular Analyst™/PC software (Bio-Rad).

RESULTS

Effects of Pertussis Toxin Treatment and the $G_{\beta\gamma}$ Sequestrant βARK1ct Peptide on MAPK Activation in COS-7 and CHO Cells—Fig. 1 depicts the effects of PTX and βARK1ct, an inhibitor of $G_{\beta\gamma}$-mediated signaling (10, 19), on MAPK activation by GPCRs and overexpressed $G_{\beta\gamma}$ subunits in COS-7 and CHO cells. In COS-7 cells, α_1AR- and M_1AChR-mediated MAPK activation was insensitive both to PTX treatment and the expression of the βARK1ct peptide (Fig. 1*A*). The G_i-coupled α_{2A}AR activated MAPK in a PTX- and βARK1ct-sensitive manner, while MAPK activity mediated by transiently transfected $G_{\beta\gamma}$ subunits was blocked by βARK1ct. Agonist treatment of COS-7 cells transiently expressing PAFR did not result in detectable MAPK activation. These data are consistent with two distinct pathways. One, employed by α_1AR and M_1AChR, is primarily mediated by the α-subunits of PTX-insensitive G proteins; the other, employed by the α_{2A}AR, is primarily dependent upon $G_{\beta\gamma}$ subunits derived from PTX-sensitive G proteins.

In CHO cells, three patterns emerged. First, the PTX-insensitive α_1AR-mediated signal remained PTX-insensitive, as found in COS-7 cells. Similarly, the $G_{\beta\gamma}$-dependent signals, mediated by either α_{2A}AR or by transfected $G_{\beta\gamma}$, remained sensitive to βARK1ct. In contrast, stimulation of PAFR-transfected CHO cells mediated a 5-fold increase in MAPK activity. Moreover, MAPK activation via M_1AChR and PAFR was abolished by PTX treatment but remained insensitive to $G_{\beta\gamma}$ sequestration by βARK1ct expression (Fig. 1*B*). Thus, M_1AChR

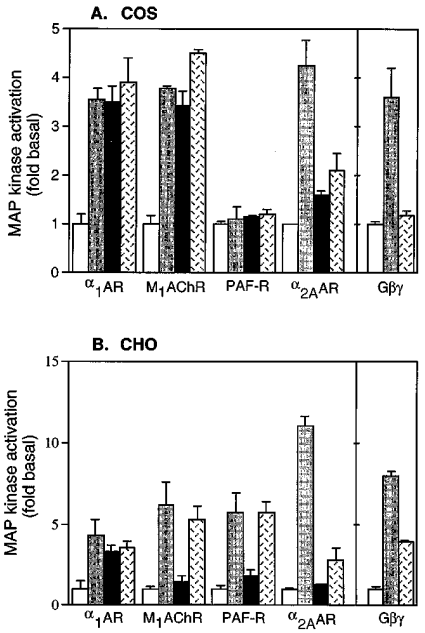

FIG. 1. **Comparison of the effects of PTX treatment and βARK1ct peptide on MAPK activation in COS-7 and CHO cells.** *A*, COS-7 cells were transiently co-transfected with $p44^{HA\text{-}MAPK}$ plus the indicated cDNAs with or without the βARK1ct peptide cDNA. The effects of PTX treatment (100 ng/ml, 20 h) and βARK1ct peptide expression on basal and agonist-stimulated MAPK activity, assessed as phosphorylation of MBP by immunoprecipitated $p44^{HA\text{-}MAPK}$, was determined following a 5-min exposure to epinephrine (100 μM), carbachol (1 mM), PAF (100 nM), or UK14304 (10 μM). For $G_{\beta\gamma}$, cells were transfected with G_β and G_γ cDNAs (stimulated) either with or without βARK1ct. *B*, CHO cells were transiently co-transfected with $p44^{HA\text{-}MAPK}$ plus the indicated cDNAs with or without the βARK1ct peptide cDNA. MAPK activity was determined as described above. Data shown represent the mean ± S.D. for duplicate samples from a representative experiment, which was replicated 3 times with comparable results. *White column,* basal; *shaded column,* stimulated; *black column,* stimulated (PTX treated); *hatched column*, stimulated plus βARK1ct.

can activate MAPK via two distinct pathways, one sensitive (CHO cells) and one insensitive (COS-7 cells) to PTX, while neither pathway appears to be mediated by G-protein $\beta\gamma$ subunits. Like the M_1AChR, PAFR can mediate PTX-sensitive, βARK1ct-insensitive MAPK activation in CHO cells. Interestingly, M1AChR and PAFR mediated PTX-insensitive phosphoinositide hydrolysis in both COS-7 and CHO cells (data not shown).

M_1AChR and PAFR Are Coupled to G_o in CHO Cells—To determine whether the PTX-sensitive MAPK activation pathway in CHO cells might be due to the differential expression of G-protein α-subunits, we compared G_α subunit expression between COS-7 and CHO cells. As shown in Fig. 2*A*, the levels of expression of $G_{q\alpha}$ and $G_{i3\alpha}$ were comparable between COS-7 and CHO cells. The α-subunits of G_{i1} and G_{i2} also showed similar expression in COS-7 cells only (data not shown). In contrast, $G_{o\alpha}$ expression was detected only in CHO cells. The expression of G_o in CHO, but not in COS-7 cells, suggested that $G_{o\alpha}$ subunits might mediate PTX-sensitive MAPK activation in these cells.

PAFR has previously been shown to couple to G_o in NCB-20 cells (20), whereas M_1AChR, like α_1AR, is known to couple only to members of the $G_{q/11}$ family (21, 22). To determine whether these receptors were capable of coupling to G_o in CHO cells, we measured $G_{o\alpha}$ GTP exchange in permeabilized cell preparations. As shown in Fig. 2*B*, agonist stimulation of either PAFR or M_1AChR mediated a 2–3-fold increase in the incorporation of the photoactivatable GTP analog into $G_{o\alpha}$ in immunoprecipitates from CHO cell lysates, indicating that both receptors are

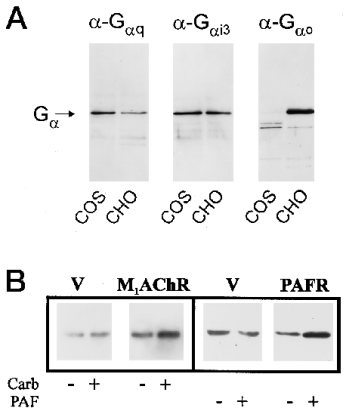

FIG. 2. **Activation of G_o-protein α-subunit by M_1AChR and PAFR in CHO cells.** *A*, CHO whole cell lysates were immunoblotted using the indicated anti-G_α subunit polyclonal antibody and visualized by enzyme-linked chemiluminescence. *B*, CHO cells were transiently transfected with M_1AChR or PAFR cDNAs. $G_{o\alpha}$ GTP exchange was measured, after a 10-min stimulation with the indicated agonist (100 nM PAF or 1 mM carbachol (*Carb*)), by GTP azidoanilide labeling and visualized by autoradiography (36). Control cells were transfected with empty pRK5 vector (*V*).

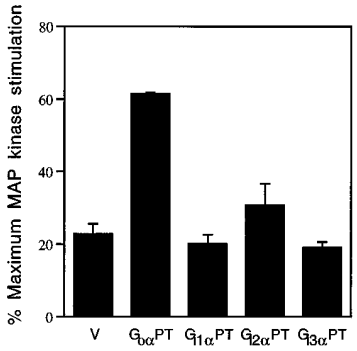

FIG. 3. **Effect of co-expression of PTX-insensitive G-protein α-subunits on M_1AChR-mediated MAPK activation.** CHO cells were transiently co-transfected with $p44^{HA\text{-}MAPK}$, M_1AChR, and the indicated PTX-insensitive mutant G-protein α-subunit. Cells were incubated overnight in serum-free medium in the presence of 100 ng/ml PTX prior to the determination of carbachol-induced MAPK activation. Data are presented as the percent of carbachol-stimulated MAPK activity measured in the absence of PTX. Data shown represent the mean ± S.D. for duplicate samples from a representative experiment, which was replicated 3 times with comparable results. Control cells were transfected with empty pRK5 vector (*V*).

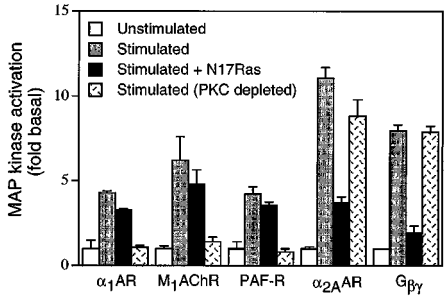

FIG. 4. **Effects of PKC depletion and dominant negative N17Ras expression on MAPK activation in CHO cells.** CHO cells were transiently co-transfected with the indicated receptor cDNA (or $G_{\beta\gamma}$) with or without the N17Ras cDNA. Where indicated, cells were incubated overnight in serum-free medium in the presence or absence of PMA (1 μM) to deplete endogenous PKC activity prior to the determination of MAPK activity. PKC depletion was confirmed by unresponsiveness to further PMA stimulation (data not shown). Data shown represent the mean ± S.D. for duplicate samples from a representative experiment, which was replicated 3 times with comparable results.

capable of coupling to and activating G_o. The specificity of the anti-$G_{o\alpha}$ antibody was confirmed by its inability to detect $G_{i\alpha}$ subunits in immunoblotting assays of whole cell lysates or partially purified membrane preparations (data not shown).

G_o-proteins Mediate M_1AChR-dependent MAPK Activation in CHO Cells—PTX-insensitive mutant α-subunits of G_i have previously been shown to rescue the PTX-mediated inhibition of adenylyl cyclase by D2 dopamine receptor (23), whereas a PTX-insensitive mutant of $G_{o\alpha}$ rescued norepinephrine-mediated inhibition of voltage-dependent calcium current (24). To examine whether $G_{o\alpha}$ could mediate PTX-sensitive activation of MAPK, we determined whether the M_1AChR signal in PTX-treated CHO cells could be rescued by co-expression of a PTX-insensitive mutant of G_o.

Agonist-stimulated MAPK activity was measured in CHO cells co-transfected with M_1AChR plus the PTX-insensitive mutants of $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$ ($G_{i1\alpha}$PT, $G_{i2\alpha}$PT, $G_{i3\alpha}$PT, and $G_{o\alpha}$PT, respectively). As shown in Fig. 3, MAPK activation by M_1AChR was almost completely inhibited by PTX in control cells and in cells transfected with the PTX-insensitive mutants of G_i. In contrast, M_1AChR-mediated MAPK activation was rescued by $G_{o\alpha}$PT in cells treated with PTX, suggesting that the G_o protein is able to mediate MAPK activation by M_1AChR in CHO cells. $G_{o\alpha}$PT was unable to rescue α_{2A}AR-mediated MAPK activation from PTX inhibition (data not shown).

$G_{o\alpha}$-mediated MAPK Activation Is Ras-independent and PKC-dependent—MAPK activation via $G_{\beta\gamma}$ and PTX-sensitive G-proteins has recently been shown to involve the activation of Ras (10) in addition to many of the same intermediates involved in RTK-mediated mitogenic signaling (7). To determine the involvement of Ras in G_o-mediated MAPK activation in CHO cells, we assessed the effects of expression of a dominant negative mutant of Ras (25) (N17Ras) on M_1AChR- and PAFR-mediated MAPK activation. N17Ras did not affect MAPK activation by M_1AChR and PAFR or by the $G_{q/11}$-coupled α_1AR, whereas $G_{\beta\gamma}$- and α_{2A}AR-mediated signaling was significantly inhibited (Fig. 4). These data are consistent with the observation that M_1AChR- and PAFR-mediated MAPK activation in CHO cells occurs in the absence of Ras activation (15).

To determine the role of PKC in the PTX-sensitive activation of MAPK by M_1AChR and PAFR, we pretreated cells overnight with phorbol ester to deplete endogenous PKC. As shown in Fig. 4, MAPK activation by α_1AR, M_1AChR, and PAFR was completely blocked by PKC depletion, whereas $G_{\beta\gamma}$ and G_i-coupled α_{2A}AR were unaffected. Thus, in CHO cells, M_1AChR and PAFR couple to G_o to activate MAPK via a signaling pathway that is independent of Ras but dependent on the activity of PKC.

DISCUSSION

We have characterized the mitogenic signaling pathways mediated by several G protein-coupled receptors in COS-7 and CHO cells. The data demonstrate the existence of a novel mitogenic signaling pathway mediated via the α-subunit of the G_o protein. In CHO cells, activation of endogenous G_o mediates PKC-dependent MAPK activation. Although it is not clear that PAFR and M_1AChR activate G_o under physiological conditions, we have shown that these receptors, when transiently expressed in CHO cells, activate MAPK via the α-subunits of G_o.

The G_o protein is the least well characterized of the known PTX-sensitive G proteins. G_o is localized primarily to the growth cones in the mammalian brain (26) and may be involved in neuronal development and differentiation. G_o is known to mediate a variety of intracellular effects, including inhibition of adenylyl cyclase (27), inhibition of voltage-dependent Ca^{2+} channels (28, 29), and stimulation of phosphoinositide hydrolysis (30). Intracellular injection of a constitutively active mu-

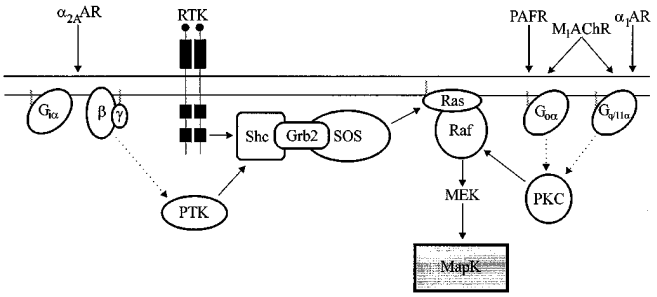

FIG. 5. **Model of G-protein-mediated mitogenic signaling.** The convergent pathways of GPCR- and RTK-mediated mitogenic signaling are shown. Signals mediated by RTKs and G_i-coupled receptors converge at, or before, Shc to mediate Ras-dependent MAPK activation. In contrast, receptors coupled to PTX-sensitive G_o or PTX-insensitive $G_{q/11}$ activate PKC which, in turn, can mediate Ras-independent MAPK activation. *Dotted arrows* indicate multiple or uncharacterized steps in the pathway. *Jagged lines* indicate lipid modifications of proteins. *MEK*, MAPK/extracellular regulated kinase kinase.

tant of $G_{o\alpha}$ (Q205LG_o-α) mediates a PKC-dependent resumption of the *Xenopus* oocyte cell cycle (31). Expression of Q205LG_o-α stimulates mitogenesis in NIH 3T3 cells, and prolonged expression leads to cellular transformation (32).

In addition to G_o, the PAF receptor has been reported to couple to the PTX-insensitive G_s and the PTX-sensitive G_{i1} and G_{i2} (20). PAF mediates a variety of physiological effects, including increased expression of the c-*fos* and c-*jun* protooncogenes (33), increased neurite outgrowth in PC12 pheochromocytoma cells (34), elevation of intracellular Ca^{2+} (35), and increased phosphoinositide hydrolysis (15). M_1AChR has previously been shown to couple primarily to $G_{q/11}$ (21, 22), whereas our data demonstrate a coupling with G_o to mediate mitogenic signaling. In contrast, α_1AR, which is also coupled to $G_{q/11}$ in COS-7 cells, is unable to couple to G_o in CHO cells, demonstrating that the interaction between M_1AChR and G_o is specific.

The activation of MAPK via a PTX-sensitive, but Ras-independent, pathway is inconsistent with the known mechanism of G_i-mediated mitogenic signaling, which requires G-protein $\beta\gamma$ subunits and the activation of Shc, Grb2, SOS, and Ras (7, 10) (Fig. 5). Our data show that the PTX-sensitive activation of MAPK was insensitive to the $G_{\beta\gamma}$ sequestering βARK1ct peptide and, moreover, was specifically rescued by a PTX-insensitive mutant of $G_{o\alpha}$, demonstrating the direct involvement of the α-subunit of G_o in mitogenic signaling.

It has been suggested that PKC stimulation is capable of mediating MAPK activation via direct phosphorylation of Raf (13). Consistent with this observation, G_o-mediated MAPK activation was unaffected by the N17Ras dominant negative mutant and required the activity of PKC. These data corroborate the observation that PAFR, when stably expressed in CHO cells, is unable to mediate an increase in the GTP-bound form of Ras (15). Interestingly, transfection of COS-7 cells with wild-type $G_{o\alpha}$ cDNA did not introduce a PTX-sensitive component to the M_1AChR-mediated signal,[2] suggesting that additional downstream components, absent from COS-7 cells, may be required for $G_{o\alpha}$ to mediate a mitogenic signal.

[2] T. van Biesen, B. E. Hawes, J. R. Raymond, L. M. Luttrell, W. J. Koch, and R. J. Lefkowitz, unpublished observations.

A model of the known mitogenic signaling pathways mediated by GPCRs (Fig. 5) shows how RTKs and G_i-coupled receptors activate MAPK in a Ras-dependent manner, whereas receptors coupled to G_o and $G_{q/11}$ activate MAPK via a pathway that requires PKC. The mechanism by which G_o activates PKC and subsequently MAPK remains unknown and is the subject of further investigation.

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