**Communication**

**Gα-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, Gα-coupled receptors, such as the α2a adrenergic receptor, were shown to mediate Ras-dependent MAPK activation via a pathway requiring G-protein βγ subunits (Gβγ) and many of the same intermediates involved in receptor tyrosine kinase signaling. In contrast, Gα-coupled receptors, such as the M1 muscarinic acetylcholine receptor (M1AChR), 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 M1AChR and platelet-activating factor receptor (PAFR) mediate MAPK activation via the α-subunit of the Gα protein. Gα-mediated MAPK activation was sensitive to treatment with pertussis toxin but insensitive to inhibition by a Gα-sequestering peptide (πARK1ct). M1AChR and PAFR catalyzed Gα subunit GTP exchange, and MAPK activation could be partially rescued by a pertussis toxin-insensitive mutant of Gαi1, but not by similar mutants of Gαi1 or Gαiα-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, M1AChR and PAFR, which have previously been shown to couple to Gαi, are also coupled to Gαi to activate a novel PKC-dependent mitogenic signaling pathway.

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Mitogen-activated protein kinase (MAPK) is 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 phosphorytrosine-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, βγ subunits derived from PTX-sensitive heterotrimeric G-proteins were also shown to mediate Ras-dependent MAPK activation (7–10). Release of Gβγ promotes the tyrosine phosphorylation of Shc and its subsequent association with Grb2-SOS. Both RTK- and Gβγ-mediated MAPK activation are completely blocked by the expression of dominant negative mutants of mSOS1 and Ras, demonstrating that RTKs and Gβγ activate MAPK via a common signaling pathway involving Shc, Grb2, SOS, and Ras (7).

MAPK activation via Gα-coupled receptors, such as α2aAR and the lysophosphatidic acid receptor, is sensitive to inhibition by the C-terminal fragment of πARK1ct, a competitive inhibitor of Gβγ-mediated signals (10). However, not all GPCRs mediate MAPK activation exclusively via receptor-catalyzed release of βγ subunits. For example, in COS-7 cells, MAPK activation via receptors coupled to members of the PTX-insensitive Gαq family, such as M1AChR and the α1 adrenergic receptor (α1AR), is sensitive to the Gαq-sequestrant πARK1ct peptide (11). Instead, MAPK activation occurs predominantly via a PKC-dependent pathway. The GTP-bound α-subunit of the Gα11 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 M1AChR- 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 Gα-protein and which is independent of Ras activation.

**EXPERIMENTAL PROCEDURES**

DNA Constructs—Hemagglutinin-tagged p44MAPK (p44HA-MAPK) cDNA was provided by J. Pavissig; the dominant negative mutant p21AR,17Ras cDNA was provided by D. Altschuler and M. Ostrowski; the PTX-insensitive Gα (GαPT) cDNA was provided by R. Taussig; the PTX-insensitive mutants of Gα13, Gα22, and Gα13 (Gα13PT, Gα22PT, and Gα13PT) cDNAs were provided by S. Senogles. All PTX-insensitive Gα-subunits were created by a mutation of the C-terminal cysteine, thereby removing the site of ADP-riboseylation by PTX. PAFR cDNA was provided by R. Snyderman; M1AChR cDNA was provided by E. Peralta (16); Gβ and Gγ cDNAs were provided by M. Simon; α2aAR cDNA and α2aAR, α2a adrenergic receptor; PTX, pertussis toxin; M1AChR, M1 muscarinic acetylcholine receptor; PAF, platelet-activating factor; PAFR, platelet-activating factor receptor; α1AR, α1 adrenergic receptor; PKC, protein kinase C; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; MBP, myelin basic protein.

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The abbreviations used are: MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; GPCR, G-protein-coupled receptor;
\( \alpha_{1AR} \) 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 \( \alpha^{44HA-MAPK} \) 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 \( G_{i} \) subunits was assayed by immunoblotting whole cell lysates using standard methods and anti-\( G_{i} \), rabbit polyclonal antibody, anti-\( G_{i} \), rabbit polyclonal antibody, or anti-\( G_{i} \), rabbit polyclonal antibody (Upstate Biotechnology Inc.).

Photoaffinity Labeling of Plasma Membrane G Proteins—[\( \alpha^{32} \)P|GTP azidoanilide] was made from [\( \alpha^{32} \)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 \( \mu \)g/ml soybean trypsin inhibitor) and then incubated with the same buffer (500 \( \mu \)l) containing [\( \alpha^{32} \)P|GTP azidoanilide (\(-3 \times 10^{6} \) Ci) and 3 \( \mu \)M 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 \( \mu \)l 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 \( \times \)g for 20 min. The supernatants were harvested and incubated for 1 h with 5 \( \mu \)l of anti-\( \alpha_{i} \) antibody (Upstate Biotechnology Inc., Lake Placid, NY) and 25 \( \mu \)l 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 AnalystTM/IPC software (Bio-Rad).

RESULTS

Effects of Pertussis Toxin Treatment and the \( G_{i} \) Sequestrant \( \beta \)ARK1ct Peptide on MAPK Activation in COS-7 and CHO Cells—Fig. 1 depicts the effects of PTX and \( \beta \)ARK1ct, an inhibitor of \( G_{i} \),-mediated signaling (10, 19), on MAPK activation by GPCRFs and overexpressed \( G_{i} \) subunits in COS-7 and CHO cells. In COS-7 cells, \( \alpha_{i} \)AR- and \( M_{i} \)AChR-mediated MAPK activation was insensitive both to PTX treatment and the expression of the \( \beta \)ARK1ct peptide (Fig. 1A). The \( \alpha_{i} \)AR-coupled \( \alpha_{2A} \)AR activated MAPK in a PTX- and \( \beta \)ARK1ct-sensitive manner, while MAPK activity mediated by transiently transfected \( G_{i} \) subunits was blocked by \( \beta \)ARK1ct. 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 \( \alpha_{i} \)AR and \( M_{i} \)AChR, is primarily mediated by the \( \alpha \)-subunits of PTX-insensitive G proteins; the other, employed by the \( \alpha_{2A} \)AR, is primarily dependent upon \( G_{i} \), subunits derived from PTX-sensitive \( G_{i} \) proteins.

In CHO cells, three patterns emerged. First, the PTX-insensitive \( \alpha_{i} \)AR-mediated signal remained PTX-insensitive, as found in COS-7 cells. Similarly, the \( \alpha_{i} \)AR-dependent signals, mediated by either \( \alpha_{2A} \)AR or by transfected \( G_{i} \), remained sensitive to \( \beta \)ARK1ct. In contrast, stimulation of PAFR-transfected CHO cells mediated a 5-fold increase in MAPK activity. Moreover, MAPK activation via \( M_{i} \)AChR and PAFR was abolished by PTX treatment but remained insensitive to \( G_{i} \), sequestration by \( \beta \)ARK1ct expression (Fig. 1B). Thus, \( M_{i} \)AChR 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 \( \beta \)-subunits. Like the \( M_{i} \)AChR, PAFR can mediate PTX-sensitive, \( \beta \)ARK1ct-insensitive MAPK activation in CHO cells. Interestingly, \( M_{i} \)AChR and PAFR mediated PTX-insensitive phosphorylation and hydrolysis in both COS-7 and CHO cells (data not shown).

\( M_{i} \)AChR and PAFR Are Coupled to \( G_{i} \), 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 \( \alpha \)-subunits, we compared \( G_{i} \), subunit expression between COS-7 and CHO cells. As shown in Fig. 2A, the levels of expression of \( G_{i} \) and \( G_{i} \) were comparable between COS-7 and CHO cells. The \( \omega \)-subunits of \( G_{i} \) and \( G_{i} \) also showed similar expression in COS-7 cells only (data not shown). In contrast, \( G_{i} \), expression was detected only in CHO cells. The expression of \( G_{i} \) in CHO, but not in COS-7 cells, suggested that \( G_{i} \) subunits might mediate PTX-sensitive MAPK activation in these cells.

PAFR has previously been shown to couple to \( G_{i} \), in NCB-20 cells (20), whereas \( M_{i} \)AChR, like \( \alpha_{i} \)AR, is known to couple only to members of the \( G_{i} \) family (21, 22). To determine whether these receptors were capable of coupling to \( G_{i} \), in CHO cells, we measured \( G_{i} \), GDP exchange in permeabilized cell preparations. As shown in Fig. 2B, agonist stimulation of either PAF or \( M_{i} \)AChR mediated a 2–3-fold increase in the incorporation of the photactivatable GTP analog into \( G_{i} \), in immunoprecipitates from CHO cell lysates, indicating that both receptors are
capable of coupling to and activating Go. The specificity of the anti-Goα subunit antibody was confirmed by its inability to detect Goα subunits in immunoblotting assays of whole cell lysates or partially purified membrane preparations (data not shown).

Go proteins mediate M1AChR-dependent MAPK Activation in CHO Cells—PTX-insensitive α-subunits of Go have previously been shown to rescue the PTX-mediated inhibition of adenyl cyclase by D2 dopamine receptor (23), whereas a PTX-sensitive mutant of Goα rescued norepinephrine-mediated inhibition of voltage-dependent calcium current (24). To examine whether Goα could mediate PTX-sensitive activation of MAPK, we determined whether the M1AChR signal in PTX-treated CHO cells could be rescued by co-expression of a PTX-insensitive mutant of Goα.

Agonist-stimulated MAPK activity was measured in CHO cells co-transfected with M1AChR plus the PTX-insensitive mutants of Gi1α, Gi2α, Gi3α, and Goα (Go1αPT, Go2αPT, Go3αPT, and GoαPT, respectively). As shown in Fig. 3, MAPK activation by M1AChR was almost completely inhibited by PTX in control cells and in cells transfected with the PTX-insensitive mutants of Gi. In contrast, M1AChR-mediated MAPK activation was rescued by GoαPT in cells treated with PTX, suggesting that the Goα protein is able to mediate MAPK activation by M1AChR in CHO cells. GoαPT was unable to rescue M1AChR-mediated MAPK activation from PTX inhibition (data not shown).

Goα-mediated MAPK Activation is Ras-independent and PKC-dependent—MAPK activation via Goα 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 Goα-mediated MAPK activation in CHO cells, we assessed the effects of expression of a dominant negative mutant of Ras (25) (N17Ras) on M1AChR- and PAFR-mediated MAPK activation. N17Ras did not affect MAPK activation by M1AChR and PAFR or by the Gβγ1-coupled α2AR, whereas Gβγ2 and α2AR-mediated signaling was significantly inhibited (Fig. 4). These data are consistent with the observation that M1AChR- 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 M1AChR and PAFR, we pretreated cells overnight with phorbol ester to deplete endogenous PKC. As shown in Fig. 4, MAPK activation by α1AR, M1AChR, and PAFR was completely blocked by PKC depletion, whereas Gβγ and Gγ coupled α2AR were unaffected. Thus, in CHO cells, M1AChR and PAFR couple to Goα 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 Go protein. In CHO cells, activation of endogenous Goα mediates PKC-dependent MAPK activation. Although it is not clear that PAFR and M1AChR activate Goα under physiological conditions, we have shown that these receptors, when transiently expressed in CHO cells, activate MAPK via the α-subunits of Goα.

The Goα protein is the least well characterized of the known PTX-sensitive G proteins. Goα is localized primarily to the growth cones in the mammalian brain (26) and may be involved in neuronal development and differentiation. Goα is known to mediate a variety of intracellular effects, including inhibition of adenyl cyclase (27), inhibition of voltage-dependent Ca2+ channels (28, 29), and stimulation of phosphoinositide hydrolysis (30). Intracellular injection of a constitutively active mu-
A model of the known mitogenic signaling pathways mediated by GPCR (Fig. 5) shows how RTKs and Gα coupled receptors activate MAPK in a Ras-dependent manner, whereas receptors coupled to Gαq and Gα11 activate MAPK via a pathway that requires PKC. The mechanism by which Gαq activates PKC and subsequently MAPK remains unknown and is the subject of further investigation.

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**Fig. 5. Model of G-protein-mediated mitogenic signaling.** The convergent pathways of GPCRs- and RTK-mediated mitogenic signaling are shown. Signals mediated by RTKs and Gα coupled receptors converge at or before, Shc to mediate Ras-dependent MAPK activation. In contrast, receptors coupled to PTX-sensitive Go or PTX-insensitive Goα11 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.

The pathway is consistent with the known mechanism of Gαq-mediated mitogenic signaling, which requires G-protem βγ 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 Goα, sequestering jARK1ct peptide and, moreover, was specifically rescued by a PTX-insensitive mutant of Goαq, demonstrating the direct involvement of the α-subunit of Goα 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, Gαq-mediated MAPK activation was unaffected by the N17Ras dominant negative mutant and 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 Goαq cDNA did not introduce a PTX-sensitive component to the M1AChR-mediated signal, suggesting that additional downstream components, absent from COS-7 cells, may be required for Goαq to mediate a mitogenic signal.

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