Signal transduction pathways that mediate C5a and fMet-Leu-Phe (fMLP)-induced pertussis toxin (PTx)-sensitive activation of phospholipase C (PLC) have been investigated using a cotransfection assay system in COS-7 cells. The abilities of the receptors for C5a and fMLP to activate PLC β2 and PLC β3 through the Gβγ subunits of endogenous G proteins in COS-7 cells were tested because both PLC β2 and PLC β3 were shown to be activated by the Gβγ subunits of G proteins in vitro reconstitution assays. Neither of the receptors can activate endogenous PLC β3 or recombinant PLC β3 in transfected COS-7 cells. However, both receptors can clearly activate PLC β2 in a PTx-sensitive manner, suggesting that the receptors may interact with endogenous PTx-sensitive G proteins and activate PLC β2 probably through the Gβγ subunits. These findings were further corroborated by the results that PLC β3 could be slightly activated by Gβ1γ1 or Gβ1γ2 in cotransfection assay, whereas the Gβγ subunits strongly activated PLC β2 under the same conditions. PLC β3 can be activated by Gγ, Gγ, and Gγ in the cotransfection assay. In addition, the Gγ and Gγ mutants with substitution of the C-terminal Cys residue by a Ser residue, which can inhibit wild type Gβγ-mediated activation of PLC β2, were able to inhibit C5a or fMLP-mediated activation of PLC β2. These Gγ mutants, however, showed little effect on m1-muscarinic receptor-mediated PLC activation, which is mediated by the Gγ class of G proteins. These results all confirm that the Gγ subunits are involved in PLC β2 activation by the two chemoattractant receptors and suggest that in COS-7 cells activation of PLC β3 by Gβγ may not be the primary pathway for the receptors.

Heterotrimeric GTP-binding protein (G protein)-mediated signal transduction pathways are involved in a variety of biological processes, ranging from neuronal activities, metabolism, hematopoietic functions, to some sensory processes (1-3). These pathways can be divided into two groups based on their sensitivities to Pertussis toxin (PTx). PTx is a bacterial toxin released from the Gi proteins. Gi proteins and transducin are PTx-resistant (3). Many G protein-coupled receptors transduce their signals through the activation of phospholipase C (PLC). Some receptors, such as the m1-adrenergic (5, 6) and m1-muscarinic cholineric receptors (7), act mainly through the Gq class of G proteins and are, thus, resistant to PTx treatment. Other receptors appear to activate PLC in a PTx-sensitive manner. Typical examples are found in leukocytes, where responses to a number of chemoattractants, including interleukin-8 (IL-8), C5a, and fMet-Leu-Phe (fMLP), are mostly PTx-sensitive (8-16). A mechanism involved in the PTx-sensitive processes has recently been proposed; ligand-bound receptors may interact with PTx-sensitive G proteins, such as the Gq proteins, and release Gβγ, which then activates PLC. This hypothesis is based on the findings that the Gβγ subunits of G proteins can activate certain isoforms of PLC β, while the Gα subunits cannot. The Gβγ subunits were shown to activate PLC β but not PLC β1 or PLC β4 in a cotransfection assay (17-19) and active PLC β3 and PLC β2 in reconstitution assays with purified proteins (20-25). Our previous report on reconstitution of PTx-sensitive, IL-8-induced activation of PLC β2 in cotransfected COS-7 cells supports the hypothesis that the chemokine receptor acts through Gβγ activation of the PLC β2 isoform (26).

In this report, we investigated the signal transduction pathways for the C5a and fMLP receptors, which play important roles in inflammation (8). C5a and fMLP receptors have previously been shown to couple selectively to PTx-insensitive Gαi subunit, Gα16 to activate PLC (27, 28). There must, however, also be a distinct pathway that mediates the PTx-sensitive responses to fMLP and C5a. The PTx-sensitive pathways may be the predominant ones in mature leukocytes, because responses to chemoattractants were found to be largely PTx-sensitive in these cells. Since C5a and fMLP, like IL-8, induce Ca2+ efflux and leukocyte chemotaxis that are sensitive to PTx treatment, the C5a and fMLP receptors may utilize the same signal transduction pathways as the IL-8 receptors (26). By using the cotransfection assay, we found that these two receptors can specifically activate PLC β2 but not PLC β3, presumably through the Gαγ subunits released from the Gq proteins. The finding that the Gγ and Gγ mutants, with substitution of the C-terminal Cys residues by Ser residues, can act as dominant negative inhibitors to block Gαγ-mediated activation of PLC β2 in cotransfected COS-7 cells supports the notion that the Gαγ subunits are involved in the signal transduction processes of these chemoattractant receptors.
MATERIALS AND METHODS

Cell Culture and Transfection—Cos-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum under 5% CO2 at 37 °C. For transfection, Cos-7 cells were seeded into 24-well plates at a density of 1 × 104 cells/well the day before transfection (32–34). The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technologies, Inc.) containing 2 μl of lipofectamine (Life Technologies, Inc.) and 0.5 μg of plasmid DNA were added to each well. Five hours later the transfection media were replaced by the culture media. Then the cells were labeled with 10 μCi/ml myo-[3H]inositol 24–48 h after the transfection. The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technologies, Inc.) and 0.5 μg of plasmid DNA were added to the plates at a density of 1 × 104 cells/well the day before transfection (32–34). The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technologies, Inc.) and 0.5 μg of plasmid DNA were added to the plates at a density of 1 × 104 cells/well the day before transfection (32–34). The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technologies, Inc.) and 0.5 μg of plasmid DNA were added to the plates at a density of 1 × 104 cells/well the day before transfection (32–34). The media were removed the next day, and 0.25 ml of Opti-MEM (Life Technologies, Inc.) and 0.5 μg of plasmid DNA were added to the plates at a density of 1 × 104 cells/well the day before transfection (32–34).

Construction of GY Mutants—The GyCS mutant was generated by polymerase chain reaction with the high fidelity DNA polymerase, pfu (Stratagene), and the mutation was confirmed by DNA sequencing. The GyCS and GY2CS mutants were kindly provided by Dr. Narasimhan Gautam from the Washington University, MO.

Ligand-binding Assays—Cos-7 cells in 24-well plates were cotransfected with CDAs encoding the receptors and various proteins including PLCs, Gβ, Gγ, or β-galactosidase. Varying amounts of 125I-labeled Csa (2200 Ci/mmol, Du Pont NEN) were incubated with the transfectants for 1 h on ice. Then the cells were washed three times with cold phosphate-buffered saline containing 0.5% bovine serum albumin and solubilized in 0.1 N sodium hydroxide. Aliquots were counted in a γ-counter. The values derived from cells transfected with the β-galactosidase DNA were taken as nonspecific binding. The maximal ligand-binding sites and affinities were determined by Scatchard analysis.

RESULTS

We used the cotransfection assay in COS-7 cells to characterize signal transduction pathways that mediate PTX-sensitive PLC activation by the receptors for C5a and fMLP. COS-7 cells transfected with the cDNA encoding the C5a receptor or fMLP receptor alone showed no ligand-dependent accumulation of IPs (Fig. 1), confirming the report that the C5a and fMLP receptors do not couple to Gα11 (27) (COS-7 cells contain endogenous Gα11, but not Gαq (26)). However, when the cells were cotransfected with the PLC β2 cDNA and the C5a or fMLP receptor, there were marked ligand-induced increases in accumulation of IPs, and these ligand-induced responses were sensitive to PTX (Fig. 1). Cells cotransfected with the cDNA encoding PLC β1 instead did not show any ligand-induced responses (Fig. 1). Knowing that Gβγ can only activate PLC β2, but not PLC β1 in the cotransfection system, and that COS-7 cells contain PLC β1, but not PLC β2 (17, 18), we interpret this result to conclude that the receptors for C5a and fMLP may interact with endogenous PTX-sensitive G proteins, presumably Gγ3 proteins, and cause release of the βγ subunits, which activate the recombinant PLC β2. We assume that the interaction is with Gγ3 because only Gγ3, but not other known PTX-sensitive G proteins including the G12, G13, or G proteins, was detected in COS-7 cells (17).

Since COS-7 cells contain endogenous PLC β3 (18), we predict that the C5a or fMLP receptor would induce IP accumulation by activating endogenous PLC β3 through Gβγ. However, this did not occur (Fig. 1), suggesting that the Gβγ subunits released from the endogenous G proteins were unable to activate the endogenous PLC β3. To investigate whether the C5a or fMLP receptor can activate the recombinant PLC β3, we cotransfected COS-7 cells with cDNAs encoding PLC β3 and the C5a or fMLP receptor. No ligand-induced accumulation of IPs was observed (Fig. 1), indicating that the receptors cannot activate the recombinant PLC β3 either. To test whether the recombinant PLC β3 can be activated by recombinant G protein subunits, we cotransfected COS-7 cells with cDNA encoding β-galactosidase (as control), Gαq, Gα11, Gα16, Gβ3γ11, or Gβ1γ5, and cDNA encoding PLC β3 as well as other PLC β isoforms as controls. As shown in Fig. 2, the recombinant PLC β3 as well as PLC β1 and β2 can all be activated by Gαq, Gα11, or Gα16 (Fig. 2) as cells cotransfected with cDNAs encoding PLC β and Gαq, Gα11, or Gα16 showed marked accumulation of IPs over those transfected with PLC β or Gαq alone. However, Gβ3γ11 or Gβ1γ5 showed only weak activation of the recombinant PLC β3, whereas the Gβγ subunits markedly activated the recombinant PLC β2 (Fig. 2). The Gβγ subunits did not activate PLC β1 or PLC β4 (Fig. 2), as we have demonstrated previously (17–19). The weak activation of PLC β3 by Gβγ may explain why the C5a or fMLP receptors could not activate PLC β3.

The expression levels of PLC β3 were determined. The levels of the recombinant PLC β3 are at least 5-fold higher than those of the endogenous PLC β3, and the levels of the recombinant PLC β3 in various transfectants are rather constant (Fig. 2B). The ligand-binding sites on cells expressing the C5a receptor were also determined using 125I-labeled C5a. Cells coexpressing the C5a receptor and β-galactosidase, PLC β3, or PLC β2 showed similar numbers of ligand-binding sites (300–375 fmol per 1 × 105 cells) with affinities of 2.5–4 nM. Thus, the abilities of Gβγ or the C5a receptor to activate PLC β3 were not the result of variations in protein expression. Furthermore, we compared the expression level of the recombinant PLC β3 with that of PLC β2 in transfected COS cells. Dilutions of cell extracts from cells expressing recombinant PLC β2 or PLC β3, together with dilutions of purified PLC β2 or β3 proteins with defined protein concentrations, were analyzed by Western blotting with antibodies specific to PLC β2 or β3. Based on the signal intensities, we estimated that the expression levels of
recombinant PLC \( \beta \) and PLC \( \beta \) in transfected COS-7 cells are 0.1–0.3 \( \mu \)g/10^5 cells (Fig. 2C). Therefore, it is reasonable to conclude that G\( \beta \gamma \) is a poor activator for PLC \( \beta 3 \) when compared with PLC \( \beta 2 \) in the cotransfection assay.

In order to obtain more evidence for involvement of G\( \beta \gamma \) in C5a- or fMLP-mediated activation of PLC, we investigated whether the G\( \gamma \) mutants, with substitution of the C-terminal Cys residues by Ser residues, can act as dominant negative mutants to inhibit G\( \beta \gamma \)-mediated PLC activation. The G\( \gamma 1 \) and G\( \gamma 2 \) mutants, designated G\( \gamma CS \) and G\( \gamma SC \), were found to be able to bind to the G\( \beta \) subunits and bring them into the cytosol (30, 31). These G\( \gamma \) mutants, when paired with the G\( \beta \) subunits, failed to activate PLC \( \beta 2 \) (17, 18), suggesting that the complex of G\( \gamma CS \) and G\( \beta \) is nonfunctional. Recently, we introduced the equivalent mutation into G\( \gamma A \). The G\( \gamma 3 \) mutant G\( \gamma SC \), like G\( \gamma CS \) and G\( \gamma SC \) (17), cannot activate PLC \( \beta 2 \) (Fig. 3A). However, G\( \gamma CS \) differs from G\( \gamma SC \) and G\( \gamma SC \) in its ability to associate with the particulate fractions; G\( \gamma SC \) unlike G\( \gamma CS \) and G\( \gamma SC \) (17), can still associate with the particulate fraction (Fig. 3B).

The cotransfection assay was used to test whether these G\( \gamma \) mutants are capable of acting as dominant negative mutants to inhibit G\( \beta \gamma \)-mediated effects. We found that cells cotransfected with cDNAs encoding PLC \( \beta 2 \), G\( \beta 1 \), G\( \gamma 1 \) and G\( \gamma 3 \) showed little accumulation of IPs compared with those cotransfected with PLC \( \beta 2 \), G\( \beta 1 \), and G\( \gamma 3 \) (Fig. 3A), suggesting that G\( \gamma 3 \) inhibited G\( \beta 1 \gamma 3 \)-mediated activation of PLC \( \beta 2 \). To test whether the G\( \gamma \) mutants can block ligand-mediated responses, we cotransfected COS-7 cells with cDNAs encoding PLC \( \beta 2 \), the C5a receptor, and one of the G\( \gamma \) mutants. As shown in Fig. 4A, G\( \gamma SC \) and G\( \gamma SC \) were capable of blocking C5a-mediated accumulation of IPs, whereas the wild types and G\( \gamma SC \) could not. Knowing that COS-7 cells contain G\( \gamma 1 \), G\( \gamma 2 \) (17) and that G\( \gamma 3 \) cannot interact with G\( \gamma 2 \) (30, 31), we interpreted the G\( \gamma SC \) results to suggest that G\( \gamma 3 \) and G\( \gamma SC \) may be able to scavenge most of the G\( \beta \) subunits in the cells to prevent them from activating PLC, whereas G\( \gamma SC \) is unable to scavenge G\( \beta 2 \), thus failing to inhibit C5a-mediated effects. We also determined the expression levels of PLC \( \beta 2 \) and the numbers of C5a-binding sites on cells expressing the C5a receptors. Coexpression of various G\( \gamma \) subunits did not significantly affect C5a-binding sites on various transfectants (350 fmol/1 \( \times 10^5 \) cells) neither did it affect the expression levels of PLC \( \beta 2 \) (Fig. 4C). Therefore, inhibition of G\( \beta \gamma \) (Fig. 3A) and C5a (Fig. 4A)-mediated PLC activation by the G\( \gamma 2,3 \) mutants is unlikely to result from changes in the expression levels of the proteins involved in activation of PLC \( \beta 2 \). The same result was also observed for the fMLP receptor, i.e. the G\( \gamma 2,3 \) mutants can inhibit fMLP-mediated activation of PLC\( \beta 2 \) in transfected COS-7 cells (data not shown). In addition, it is interesting to note that the G\( \gamma \) mutants did not appear to affect Ga-mediated effector activation. In cotransfected COS-7 cells, G\( \gamma CS \) (Fig. 4B) and G\( \gamma SC \) (data not shown) did not inhibit \( m_1 \)-muscarnic receptor-mediated activation of PLC, which is mediated by the Ga subunits of the G\( a \) class (7). Thus, the G\( \gamma \) mutants, G\( \gamma CS \) and G\( \gamma SC \), appear to only affect G\( \beta \gamma \)-mediated responses but not Ga-mediated responses. In summary, these results support...
receptors, like the IL-8 (18) and m2-muscarinic receptors (17),

may occur in vivo in cells where the expression levels of PLCβ3 are higher than those of the recombinant in COS-7 cells or where the subcellular localization of PLCβ3 or production of accessory proteins is differently regulated.

As we have demonstrated, the Gγ mutants, Gγ2CS and Gγ4CS in particular, can serve as dominant negative mutants to inhibit Gβγ-mediated, but not Gαi-mediated, activation of PLC. This is presumably due to their abilities to form complexes with all known Gβ subunits. Although the Gγ2CS and Gγ4CS mutants were demonstrated to be capable of binding to Gβ (30, 31), we do not know whether the complexes of Gγ2CS and Gβ can still interact with PLCβ2. In other words, it is not clear whether the changes in the Gγ mutants impair the abilities of the Gγ2 complexes to interact with PLCβ2 or their abilities to activate PLCβ2. However, the ability of the Gγ2CS mutant to associate with the particulate fractions indicates that the lipid modification on the Cys residue may not serve only as an anchor for Gβγ, but it may also have other functions. The lipid modification at the C-terminal Cys residue may either participate in effector activation or in orientation of the Gβγ complexes to allow better access of effectors to their substrates on the membranes. It appears paradoxical that the Gγ mutants had no effect on m1-muscarinic receptor-mediated activation of PLC because Gβγ was shown to be essential for reconstitution of m1-receptor-mediated activation of PLC in an in vitro system using purified proteins (7, 38). We suggest two possible interpretations. 1) There may still be interactions between receptors and Gαi subunits in the absence of Gβγ depending on the nature of receptors and G proteins, but Gβγ may greatly facilitate the interactions. In the COS-7 overexpression system, the requirement for Gβγ may be overcome by the high expression levels of α subunits and receptors. 2) The Gγ mutants did not scavenge all of the Gαi subunits; hence, there may be enough normal Gβγ complexes present so that Gαi-mediated activation of effectors is largely unaffected. The result that more Gβγ is required for activation of PLC than Gαi supports the second possibility (24, 39).

The Gγ2CS mutants may not only inhibit Gγ2CS-mediated activation of PLC, but they should also be able to block other Gβγ-mediated regulation of effectors, including adenylcyclases (23, 40), phosphatidylinositol 3-kinase (41), ion channels (42, 43), mitogen-activated kinase (44), and β-adrenergic receptor kinase (45, 46), etc. Therefore, the Gγ2CS mutants, joined with the other Gγ antagonists, including phosphocin (47) and the Gγ-binding region of β-adrenergic receptor kinase (48), provide useful tools to test whether a specific G protein-coupled signal pathway is mediated by Gβγ in a variety of systems.

REFERENCES

Signal Transduction Pathways for C5a and fMLP