Pertussis Toxin-sensitive Activation of Phospholipase C by the C5a and fMet-Leu-Phe Receptors*

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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 Gq proteins in COS-7 cells were tested because both PLC β2 and PLC β3 were shown to be activated by the βγ 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 only be slightly activated by Gα1γ or Gα1βγ in the cotransfection assay, whereas the Gβγ subunits strongly activated PLC β2 under the same conditions. PLC β3 can be activated by Gαqα, Gα1γ, and Gαqβ in the cotransfection assay. In addition, the Gγ2 and Gγ2 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 Gαi class of G proteins. These results all confirm that the Gβγ subunits are involved in PLC β2 activation by the two chemotactant 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)1-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 that catalyzes ribosylation of C-terminal cysteine residues of some Gα subunits, including the Gαq subunits, Gαo subunits, and transducin α subunits. Modification by PTx prevents the interaction between Gα subunits and receptors, thus blocking ligand-mediated signal transduction (1, 4). The Gαq subunits of the Gq and Gα class of G proteins lack the C-terminal cysteine residues; hence, signal transduction pathways mediated by these Gαq subunits 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 m3-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 Gαi subunits cannot. The Gβγ subunits were shown to activate PLC β2 but not PLC β1 or PLC β4 in a cotransfection assay (17–19) and activate 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 Gαq subunit, Ga18, 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 Gγ2 subunits released from the Gγ proteins. The finding that the Gγ2 and Gγ2 mutants, with substitution of the C-terminal Cys residues by Ser residues, can act as dominant-negative inhibitors to block Gβγ-mediated activation of PLC β2 in cotransfected COS-7 cells supports the notion that the Gβγ subunits are involved in the signal transduction processes of these chemoattractant receptors.

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1The abbreviations used are: G protein, GTP-binding protein; C5a, complement factor 5a; fMLP, formyl-Met-Leu-Phe; GγCS, Gγ mutants with substitution of C-terminal Cys residues by Ser residues; PLC, phospholipase C; PTx, pertussis toxin; IP, inositol phosphate; IL-8, interleukin-8.
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 × 10⁵ cells/well the day before transfection (32–34). The media were removed the next day, and 0.25 ml of OptiMEM (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-[2-3H]inositol the following day, and the levels of inositol phosphates were determined 25 min after addition of ligand. 100 nM of C5a or fMLP receptor could not activate the recombinant PLC β3 either. To test whether the PLC β3 could be activated by recombinant G protein subunits, we cotransfected COS-7 cells with cDNA encoding β-galactosidase as control, Gₐ₅₁, Gₐ₃₁₆, Gₐ₃₁₀, Gₐ₁₅, and Gₐ₁₈ 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 all β1 and β2 can all be activated by Gₐ₅₁, Gₐ₃₁₆ (Fig. 2a) or Gₐ₁₅, Gₐ₃₁₀ (Fig. 2b) as cells cotransfected with cDNAs encoding PLC β and Gₐ₅₁, Gₐ₃₁₆, or Gₐ₁₅ showed marked accumulation of IPs over those transfected with PLC β or Gₐ₅₁ alone. However, Gₐ₃₁₀ showed only weak activation of the recombinant PLC β3, whereas the Gₐ₅₁ subunits markedly activated the recombinant PLC β3 (Fig. 2). The Gₐ₅₁ subunits did not activate PLC β3 (Fig. 2). As we have demonstrated previously (17–19). The weak activation of PLC β3 by Gₐ₅₁ may explain why the C5a or fMLP receptor could not activate the recombinant 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 inabilities of Gₐ₅₁ 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

![Fig. 1. Specific activation of PLC β2 by C5a and fMLP receptors](http://example.com/f1.png)

Fig. 1. Specific activation of PLC β2 by C5a and fMLP receptors. COS-7 cells were cotransfected with cDNA encoding PLC β1 (P1), PLC β2 (P2), PLC β3 (P3), or β-galactosidase (LacZ) and C5a encoding the C5a (A) or fMLP (B) receptor. Ligand-induced accumulation of inositol phosphates was determined 25 min after addition of ligand in the presence or absence of PTx (500 ng/ml). PTx was added 4 h before addition of ligand. 100 nM of C5a (A) and fMLP (B) were used in the assay. The basal inositol phosphate levels in cells transfected with the LacZ gene and with the PLC β1, β2, and β3 cDNAs are 70 ± 27, 750 ± 65, 1030 ± 78, and 770 ± 43 dpm, respectively.
COS-7 cells were cotransfected with cDNAs encoding PLC-β2 and PLC-β3, G protein subunits, and control plasmid β-galactosidase (LacZ) as indicated in the figure. The levels of inositol phosphates were determined as described under “Materials and Methods.” B, Western blot of G protein subunits and Gq/105 cells (Fig. 2). Two days after transfection the cells were separated into the cytosolic and particulate fractions, which were then subjected to Western analyses with an antibody specific to Gγ3.

However, Gγ3CS differs from Gγ1CS and Gγ2CS in its ability to associate with the particulate fractions; Gγ1CS, unlike Gγ1CS and Gγ2CS (17), can still associate with the particulate fraction (Fig. 3B).

The cotransfection assay was used to test whether these Gγ mutants are capable of acting as dominant negative mutants to inhibit Gβγ-mediated effects. We found that cells cotransfected with cDNAs encoding PLC-β2, Gβ1, Gγ3, and Gγ3CS showed little accumulation of IP3s compared with those cotransfected with PLC-β2, Gβ1, and Gγ3 (Fig. 3A), suggesting that Gγ3CS inhibited Gβ1γ3-mediated activation of PLC-β2. To test whether the Gγ mutants can block ligand-mediated responses, we cotransfected COS-7 cells with cDNAs encoding PLC-β2, the C5a receptor, and one of the Gγ mutants. As shown in Fig. 4A, Gγ2CS and Gγ3CS were capable of blocking C5a-mediated accumulation of IP3s, whereas the wild types and Gγ1CS could not. Knowing that COS-7 cells contain Gγ1 and Gγ2 (17) and that Gγ1CS cannot interact with Gβ2 (30, 31), we interpreted the Gγ1CS results to suggest that Gγ2CS and Gγ3CS may be able to scavenge most of the Gβ subunits in the cells to prevent them from activating PLC, whereas Gγ1CS is unable to scavenge Gβ2, thus failing to inhibit C5a-mediated effects. We also determined the expression levels of PLC-β2 and the numbers of C5a-binding sites on cells expressing the C5a receptors. Coexpression of various Gγ subunits did not significantly affect C5a-binding sites on various transfectants (350 fmol/105 cells) neither did it affect the expression levels of PLC-β2 (Fig. 4C). Therefore, inhibition of Gβγ- (Fig. 3A) and C5a (Fig. 4A)-mediated PLC activation by the Gγ2CS, mutants is unlikely to result from changes in the expression levels of the proteins involved in activation of PLC-β2.

The same result was also observed for the fMLP receptor, i.e. the Gγ2CS mutants can inhibit fMLP-mediated activation of PLC-β2 in transfected COS-7 cells (data not shown). In addition, it is interesting to note that the Gγ mutants did not appear to affect Gα-mediated effector activation. In cotransfected COS-7 cells, Gγ2CS (Fig. 4B) and Gγ3CS (data not shown) did not inhibit the m1-muscarinic receptor-mediated activation of PLC, which is mediated by the Gα subunits of the Gq class (7). Thus, the Gγ mutants, Gγ1CS and Gγ3CS, appear to only affect Gβγ-mediated responses but not Gα-mediated responses. In summary, these results support
FIG. 4. Effects of G YCS expression on receptor-induced activation of PLC. A, COS-7 cells were cotransfected with cDNA encoding G Y, G YCS, or the control β-galactosidase (LacZ) and cDNAs encoding the C5a receptor and PLC β2. B, COS-7 cells were cotransfected with the cDNA encoding G YCS, or the control β-galactosidase (LacZ), and the m 1-muscarinic receptor cDNA. Ligand-induced accumulation of inositol phosphates was determined 25 min after addition of ligand. 100 nM of C5a (A) or 10 μM carbachol (B) were added. The basal levels of inositol phosphates are about 1500 dpm. C, COS-7 cells were transfected or cotransfected with cDNAs encoding the C5a receptor (CSAR), PLC β2, Lz, G Y, and its mutant G YCS, as indicated in the figure. The cell extracts were subjected to Western analysis 2 days after transfection with an antibody specific to PLC β2.

The idea that the G Y subunits mediate FMLP- and C5a-induced activation of PLC β2.

DISCUSSION

In this report, we have demonstrated that the C5a and FMLP receptors, like the IL-8 (18) and m 2-muscarinic receptors (17), can interact with PTX-sensitive G proteins (presumably G Y) causing the release of the G Y subunits, which then specifically activate PLC β2 in a PTX-sensitive manner. Although these two receptors can also couple to G α , we believe that the G Y-mediated pathway may be the predominant one that occurs in leukocytes, because the responses to these chemoattractants in mature leukocytes are mostly PTX-sensitive. Our results are also consistent with previous results demonstrating that the C5a and FMLP receptors can interact with the G Y proteins (35–37).

Regulation of PLC β1, β2, and β4 by G Y has been tested before in the cotransfection assay (17–19), but here PLC β3 was tested in this assay system for the first time. To our surprise, PLC β3 can only be weakly activated by G Y in the cotransfection assay system. G Y was previously shown to activate PLC β3 effectively in vitro reconstitution assays with purified proteins (23–25). In fact, there are other examples of apparent specificity in the cellular system that are not seen in vitro. For instance, Schultz and his co-workers (49, 50) have demonstrated receptor-mediated specificity for G protein heterotrimeric forms that is not evident in the reconstitution system (23). There are probably other factors, such as substrate compartmentalization, modification, and membrane interaction and the involvement of accessory proteins, which may mediate specificity in the cellular system. Nevertheless, the weak activation of PLC β3 by G Y explains the inability of the chemoattractant receptors to activate endogenous or recombinant PLC β3 in COS-7 cells and may explain the lack of significant PLC activation by non-G α -coupling receptors in many systems where PLC β3 is expressed. However, activation of PLC β3 by G Y may occur in vivo in cells where the expression levels of PLC β3 are higher than those of the recombinant in COS-7 cells or where the subcellular localization of PLC β3 or production of accessory proteins is differently regulated.

As we have demonstrated, the G Y mutants, G YCS and G YCS in particular, can serve as dominant negative mutants to inhibit G Y-mediated, but not G α-mediated, activation of PLC. This is presumably due to their abilities to form complexes with all known G β subunits. Although the G YCS and G YCS mutants were demonstrated to be capable of binding to G β (30, 31), we do not know whether the complexes of G YCS and G β can still interact with PLC β2. In other words, it is not clear whether the changes in the G Y mutants impair the abilities of the G Y complexes to interact with PLC β2 or their abilities to activate PLC β2. However, the ability of the G YCS mutant to associate with the particulate fractions indicates that the lipid modification on the Cys residue may not serve only as an anchor for G Y, 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 βγ complexes to allow better access of effectors to their substrates on the membranes. It appears paradoxical that the G Y mutants had no effect on m 1-muscarinic receptor-mediated activation of PLC because G Y was shown to be essential for reconstitution of m 1-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 G α subunits in the absence of G Y depending on the nature of receptors and G proteins, but G Y may greatly facilitate the interactions. In the COS-7 overexpression system, the requirement for G Y may be overcome by the high expression levels of α subunits and receptors. 2) The G Y mutants did not scavenge all of the G β subunits; hence, there may be enough normal G Y complexes present so that G α-mediated activation of effectors is largely unaffected. The result that more G Y is required for activation of PLC than G α supports the second possibility (24, 39).

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

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