Differential coupling of the formyl peptide receptor to adenylate cyclase and phospholipase C by the pertussis toxin-insensitive G_z protein

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In neutrophils, activation of receptors for the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP) leads to changes in intracellular events such as phosphoinositide turnover and Ca^{2+} mobilization. Studies have shown that activation of the cloned fMLP receptor can also lead to inhibition of cyclic AMP (cAMP) accumulation [Lang, Boulay, Li and Wollheim (1993) *EMBO J.* **12**, 2671–2679; Uhing, Gettys, Tomhave, Snyderman and Didsbury (1992) *Biochem. Biophys. Res. Commun.* **183**, 1033–1039]. These responses are apparently mediated through pertussis toxin-sensitive G_i proteins. Since other chemotactic factor receptors can couple to multiple G proteins, we examined the ability of the fMLP receptor to utilize a pertussis toxin-insensitive G protein, G_z , in its signal transduction pathways. The human fMLP receptor was transiently expressed in 293 and Ltk⁻ cells, and subsequently assayed for receptor-mediated inhibition of cAMP accumulation and stimulation of

phosphoinositide-specific phospholipase C. In transfected 293 cells, fMLP inhibited choriogonadotropin-stimulated cAMP accumulation by 50% and the response could be abolished by pertussis toxin. Co-expression of the fMLP receptor with the α subunit of G_z rendered the fMLP response pertussis toxin-insensitive, indicating that the endogenous G_i proteins can be substituted efficiently by G_z . In contrast, Ltk⁻ cells expressing the fMLP receptor were able to respond to fMLP with an increase in the production of inositol phosphates, but this response was completely abolished by pertussis toxin even in cells co-expressing the α subunit of G_z . Thus, although both signalling pathways appeared to utilize G_i -like proteins, G_z can only replace G_i in mediating inhibition of cAMP accumulation, and not in the stimulation of phospholipase C. Differential interaction with G_z might represent a novel mechanism by which fMLP receptors regulate intracellular events.

INTRODUCTION

An important inflammatory response to infection is the recruitment of polymorphonuclear neutrophils (PMN) from the circulation to the site of inflammation [1]. The migratory responses of PMN are regulated by chemotactic factors. Known chemotactic factors include the peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP), platelet activating factor, leukotriene B₄, anaphylatoxin complement fragment C5a and interleukin 8. Apart from directing the migration of phagocytic cells, these chemotactic factors exhibit many pro-inflammatory properties including the ability to stimulate PMN adherence, phagocytosis of particles, release of lysosomal enzymes and superoxide $O_2^{\cdot-}$ production [2,3]. Signal transduction processes that mediate these responses are exemplified by the actions of the chemotactic peptide fMLP [4]. fMLP is produced by bacteria as the initiator sequence for prokaryotic protein synthesis and interacts with specific receptors on the cell surface of PMN. Agonist binding to the fMLP receptor triggers a series of intracellular events, including activation of phosphoinositide-specific phospholipase C (PI-PLC), Ca^{2+} mobilization and subsequent protein kinase C activation [5,6].

The cloning of several mammalian forms of the fMLP receptor [7,8] indicates that the receptor belongs to the G protein-coupled receptor family. Members of this receptor family utilize the heterotrimeric ($\alpha\beta\gamma$) G proteins to regulate intracellular effectors such as enzymes and ion channels. The involvement of G proteins in fMLP-mediated responses is well established [9]. In dimethyl

sulphoxide-differentiated HL-60 cells, fMLP stimulates chemotaxis and the respiratory burst by interacting with G proteins that are substrates for pertussis toxin-catalysed ADP-ribosylation [10,11]. Two G protein α subunits, G_{12} and G_{13} , that can be modified by pertussis toxin (PTX) are expressed in neutrophils and monocytes [12]. Biochemical studies in differentiated HL-60 cells suggest that the fMLP receptor can indeed couple to G_{12} [13–15]. Whether stimulation of PI-PLC by fMLP is mediated by G_{12} remains to be determined.

Consistent with many biochemical studies, experiments utilizing the mutationally activated α subunits of G_{12} , as well as G_{11} and G_{13} , reveal that one of the functions of G_i proteins is to inhibit cyclic AMP (cAMP) accumulation [16,17]. However, the prominent intracellular responses to fMLP in HL-60 cells are stimulation of PI-PLC and Ca^{2+} mobilization. Regulation of adenylate cyclase activities by fMLP has been limited to a transient increase in cAMP accumulation, which is largely mediated indirectly via the release of adenosine [18]. Nevertheless studies have shown that the cloned fMLP receptor can indeed inhibit adenylate cyclase when expressed in insulin-secreting RINm5F cells and human embryonic kidney 293 cells [19,20]. Receptors for other chemotactic factors, including those for C5a and interleukin 8, are known to utilize multiple G proteins in their signal transduction pathways [21–23]. Here we have tested the ability of the cloned fMLP receptor to couple to a PTX-insensitive G protein, G_z . G_z was chosen on the basis that it can substitute for G_i to mediate hormonal inhibition of cAMP accumulation [17], and it is endogenously expressed in basophilic

Abbreviations used: fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PI-PLC, phosphoinositide-specific phospholipase C; cAMP, cyclic AMP; IP, inositol phosphate, regardless of the number of phosphate groups; α_z , α subunit of the G protein G_z ; PMN, polymorphonuclear neutrophils; PTX, pertussis toxin; hCG, human choriogonadotropin; LHR, luteinizing hormone receptor; MEM, minimum essential medium; DMEM, Dulbecco's modified Eagle's medium; PVDF, poly(vinylidene difluoride).

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cells [24], which are involved in anaphylactic reactions. The experimental approach involves transient transfections of cDNAs encoding the human fMLP receptor [7], the rat luteinizing hormone receptor (LHR) [25] and various α subunits of G proteins [16,17]. The co-transfection methodology allowed us to examine receptor-mediated regulation of adenylate cyclase and PI-PLC, as well as the role of individual G protein α subunits in the signal transduction cascade.

EXPERIMENTAL

Materials

The human fMLP receptor cDNA (in the pCDM8 vector) was generously supplied by Dr. F. Boulay, LBIO/Laboratoire, Grenoble, France. Other cDNAs were constructed or obtained as previously described [16,17]. PTX was purchased from List Biological Laboratories (Campbell, CA, U.S.A.). Human chorionadotropin (hCG) was obtained from the National Pituitary Agency (Bethesda, MD, U.S.A.). Ltk⁻ fibroblasts and 293 cells were obtained from Dr. O. Civelli (Vollum Institute for Advanced Biomedical Research, Portland, OR, U.S.A.) and the American Type Culture Collection (A.T.C.C. CRL-1573) respectively. Radiochemicals and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Corp. Plasmid purification columns were obtained from Qiagen. Cell culture reagents were obtained from GIBCO and all other chemicals were purchased from Sigma.

Transient transfection of 293 and Ltk⁻ cells

293 cells were cultured in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μ g/ml streptomycin, in 5% CO₂. Cells were seeded on to 12-well plates at 1×10^5 cells/well 1 day before the transfection. DNAs were purified by Qiagen column chromatography and transfected into 293 cells in the presence of DEAE-dextran essentially as described previously [26]. Briefly, cells were incubated in growth medium supplemented with 400 μ g/ml DEAE-dextran, 100 μ M chloroquine and various DNAs. After 2 h the cells were shocked for 1 min at room temperature in PBS containing 10% (v/v) DMSO, rinsed with PBS and returned to growth medium for 24 h. Ltk⁻ fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum and antibiotics, in 5% CO₂. The Ltk⁻ transfection procedure was similar to that used for 293 cells with the following modifications. Ltk⁻ cells were seeded at 5×10^4 cells/well 2 days before the transfection, the cells were then incubated in growth medium containing 200 μ g/ml DEAE-dextran and cDNAs, and transfected for 4 h. All other steps were the same as with the 293 cells. Under these conditions, approx. 40–50% of the transfected 293 or Ltk⁻ cells expressed the exogenous proteins, as indicated by co-transfection studies using a plasmid encoding β -galactosidase as a reporter gene.

cAMP accumulation

At 24 h after transfection, 293 cells were labelled with [³H]adenine (1 μ Ci/ml) in MEM containing 1% (v/v) fetal calf-serum. PTX (100 ng/ml) was added where appropriate. After 18–24 h, the cells were washed with 2 ml of assay medium (20 mM Hepes-buffered MEM) and incubated at 37 °C for 30 min with 1 ml of assay medium containing 1 mM 1-methyl-3-isobutylxanthine and the indicated drugs. After incubation with drugs the assay medium was replaced with 1 ml of ice-cold 5% (w/v) trichloroacetic acid containing 1 mM ATP and 1 mM cAMP. Lysed cells

were then kept at 4 °C for 30 min. Intracellular [³H]cAMP was isolated by sequential chromatography and estimated by determining the ratios of [³H]cAMP to total [³H]ATP and [³H]ADP pools as reported previously [26,27]. Absolute values for cAMP accumulation varied between experiments, but variability within a given experiment was in general less than 10%.

Assay for inositol phosphate (IP) formation

Labelling of Ltk⁻ cells was done with 0.75 ml of DMEM containing myo-[³H]inositol (2.5 μ Ci/ml) and 5% (v/v) fetal calf serum. Where necessary, PTX was added 24 h later. At 48 h after labelling, the cells were rinsed in 2 ml of assay medium (20 mM Hepes-buffered DMEM with 20 mM LiCl) and then incubated in 1 ml of assay medium containing the indicated drugs at 37 °C for 1 h. IP production was estimated by determining the ratio of [³H]IP to [³H]inositol plus [³H]IP as described [28]. Briefly, following drug treatments the cells were lysed with 0.75 ml of ice-cold 20 mM formic acid at 4 °C for 30 min. Soluble fractions were loaded on to AG1-8X Dowex columns, immediately followed by the addition of 3 ml of 50 mM NH₄OH (pH > 9.0). The flow-throughs were collected in scintillation vials as the [³H]inositol fractions. The columns were then washed with 4 ml of 40 mM ammonium formate, and [³H]IP fractions were eluted with 5 ml of 2 M ammonium formate. For 293 cells, labelling with myo-[³H]inositol (2.5 μ Ci/ml) in MEM containing 1% (v/v) fetal calf serum was carried out for 16–20 h. The assay conditions were the same as for the Ltk⁻ cells from this point, except that the 20 mM Hepes-buffered MEM assay medium contained 5 mM LiCl.

Immunodetection of G_z α subunit (α_z) expression

Membranes were prepared from transiently transfected Ltk⁻ cells. Briefly, transfected cells were harvested in PBS containing 1 mM EDTA. Cells were resuspended in lysis buffer (50 mM Tris/HCl containing 1 mM PMSF, 1 mM benzamidine/HCl, 1 mM EGTA and 1 mM dithiothreitol; pH 7.4) and lysed by one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle. After removal of nuclei by centrifugation, membranes were collected, washed and resuspended in lysis buffer. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. For each sample, 150 μ g of membrane proteins was separated on a 12.5% polyacrylamide gel in the presence of SDS and electrophoretically transferred to poly(vinylidene difluoride) (PVDF) membranes. Localization of protein markers on the PVDF membrane was by Ponceau S staining. Immunodetection of α_z by the α_z -specific antiserum P-961 [29] was visualized by chemiluminescence using the ECL kit from Amersham Corp.

RESULTS

Inhibition of cAMP accumulation in 293 cells

Previous studies have demonstrated that 293 cells can support functional and high-efficiency expression of cloned receptors and G protein subunits [16,17], including the fMLP receptor [20,30]. Multiple recombinant receptors, G protein subunits and effectors can be simultaneously expressed in 293 cells, thus allowing functional analysis of interactions between the various signalling components. To study hormonal regulation of adenylate cyclase, 293 cells were co-transfected with the rat LHR and the human fMLP receptor. LHR is known to activate G_s, leading to an increase in intracellular cAMP levels. When the transfected cells were incubated with the LHR agonist hCG, cAMP accumulation

Figure 1 fMLP-mediated inhibition of cAMP accumulation in transfected 293 cells

293 cells were transiently transfected (DEAE-dextran method), labelled with 1 $\mu\text{Ci}/\text{ml}$ [^3H]adenine for 18–24 h and then assayed for cAMP accumulation in the presence of 20 mM Hepes, 1 mM 1-methyl-3-isobutylxanthine and various concentrations of fMLP, as described in the text. Cells transfected with cDNAs encoding 0.15 $\mu\text{g}/\text{ml}$ LHR and 0.25 $\mu\text{g}/\text{ml}$ of the fMLP receptor (fMLP-R) were then labelled with [^3H]adenine in the absence or presence of PTX (100 ng/ml) and subsequently assayed for responses to 5 ng/ml hCG with or without 200 nM fMLP (a). Cells were transfected with cDNAs encoding 0.15 $\mu\text{g}/\text{ml}$ of the LHR and various concentrations of the fMLP receptor (up to 3 $\mu\text{g}/\text{ml}$). fMLP at 200 nM significantly inhibited the hCG-stimulated activity at all cDNA concentrations tested (paired *t* test; $P < 0.05$) (b). Cells transfected with cDNA encoding the LHR and the fMLP receptor as in (a) were then assayed for their responses to 5 ng/ml hCG in the presence of various concentrations of fMLP (up to 200 μM). The hCG-stimulated cAMP accumulation was significantly inhibited by fMLP at all concentrations tested (paired *t* test, $P < 0.05$) (c). 293 cells were co-transfected with 0.25 $\mu\text{g}/\text{ml}$ of the constitutively active α_s -Q227L mutant and 0.25 $\mu\text{g}/\text{ml}$ of the fMLP receptor, labelled in the absence or presence of PTX (100 ng/ml) and subsequently assayed for cAMP production with or without 200 nM fMLP (d). Data shown in each panel represent the means $\pm$ S.D. of triplicate determinations in a single experiment; two additional experiments yielded similar results. For panels (b) and (c) results are expressed as percentage inhibition of the hCG-stimulated activity as compared with that measured in the absence of fMLP. * fMLP significantly reduced the hCG- or α_s -Q227L-stimulated activity; paired *t* test; $P < 0.05$.

was increased by 20-fold over basal values (Figure 1a). In the presence of 200 nM fMLP, the hCG-stimulated cAMP accumulation was attenuated by about 50%. The effect of fMLP, like that of hCG, required expression of the appropriate receptor, since fMLP-mediated inhibition of the hCG response was not observed in cells which had been transfected with LHR alone (Figure 1a). As expected with receptors that couple to G_i proteins, pretreating the transfected cells with PTX (100 ng/ml) completely abolished the fMLP response (Figure 1a).

The fMLP-mediated inhibition of cAMP accumulation was dependent on the amount of fMLP receptor cDNA used in the experiments. The extent of the inhibition increased with increasing concentrations of cDNA, with maximum suppression of cAMP levels at or around 0.3 $\mu\text{g}/\text{ml}$ fMLP receptor cDNA (Figure 1b). In each case the amount of fMLP receptor cDNA was varied while the amount of LHR remained the same (0.15 $\mu\text{g}/\text{ml}$). Likewise, we titrated the amount of agonist needed to elicit the maximal inhibitory response in 293 cells co-transfected

with LHR (0.15 $\mu\text{g}/\text{ml}$) and a sub-maximal concentration of fMLP receptor (0.25 $\mu\text{g}/\text{ml}$). Various concentrations of the agonist fMLP were added to the assay medium containing 5 ng/ml hCG. Maximal inhibition of cAMP accumulation was observed with 1 nM fMLP, giving an EC₅₀ of around 0.1 nM (Figure 1c). Since these results are in good agreement with those obtained in similar studies [19,20], and since the fMLP response was absolutely dependent on the inclusion of cDNAs encoding the fMLP receptor (Figure 1a), no attempt was made to characterize the kinetics of fMLP binding to the receptor.

Activation of fMLP receptors can also inhibit the activities of adenylate cyclase which have been stimulated by other signalling components. For example, in 293 cells co-expressing fMLP receptor and α_s -Q227L, a decrease of 45% in cAMP accumulation was observed upon receptor stimulation with fMLP (Figure 1d). α_s -Q227L is a constitutively activated mutant form of α_s which causes persistent activation of adenylate cyclase, resulting in an elevated basal cAMP level [31]. Moreover,

Figure 2 fMLP receptor coupling to the PTX-insensitive α_z in 293 cells

The 293 cells were co-transfected with DNA encoding 0.15 $\mu\text{g}/\text{ml}$ of the LHR and 0.25 $\mu\text{g}/\text{ml}$ of the fMLP receptor with or without 0.125 $\mu\text{g}/\text{ml}$ α_z . Transfected cells were assayed for cAMP accumulation as described in the legend to Figure 1, in the presence of 1 mM 1-methyl-3-isobutylxanthine and 5 ng/ml hCG with or without 200 nM fMLP. Results are expressed as percentage inhibition of the hCG-stimulated activity in the presence of fMLP, compared with that measured in the presence of hCG alone. * PTX significantly reduced the ability of fMLP to inhibit hCG-stimulated activity; paired Bonferroni *t* test; $P < 0.05$ (a). The 293 cells were co-transfected with 0.15 $\mu\text{g}/\text{ml}$ of the LHR cDNA and either 0.125 $\mu\text{g}/\text{ml}$ of the mammalian expression vector pcDNA I or 0.125 $\mu\text{g}/\text{ml}$ α_z cDNA. Transfected cells were assayed for cAMP accumulation in the absence or presence of 5 ng/ml hCG (b). The data in each panel represent means $\pm$ S.D. of triplicate determinations in a single experiment; two independent experiments yielded similar results.

incubation with PTX almost completely blocked this response of the fMLP receptor (Figure 1d).

fMLP receptor coupling to G_z

Previous studies have shown that receptors which normally couple to G_i proteins can also couple to G_z [17]. The α subunit of G_z does not serve as a substrate for PTX, and activation of G_z leads to inhibition of cAMP accumulation in a PTX-insensitive manner. To test whether the fMLP receptor can couple to G_z , we co-transfected 293 cells with cDNAs encoding the LHR and the fMLP receptor in the absence or presence of α_z . By treating the transfected 293 cells with PTX to inactivate endogenous α_i , we examined the ability of α_z to respond to stimulation by the fMLP receptor. In the absence of α_z , PTX abolished the inhibitory response of fMLP (Figure 2a). Co-expression of α_z with the fMLP receptor and the LHR, however, produced cells in which PTX treatment had little effect on the inhibition of cAMP accumulation by fMLP (Figure 2a). These results suggest that,

Figure 3 [^3H]IP formation in transfected 293 cells

IP formation was measured as described in the text for 1 h in the presence or absence of receptor agonists as indicated. 293 cells were transfected with either 0.25 $\mu\text{g}/\text{ml}$ fMLP receptor (fMLP-R) in pCDM8 or 0.25 $\mu\text{g}/\text{ml}$ muscarinic m1 receptor in pCDM8. Closed bars represent basal activity, whereas hatched bars indicate the activity obtained in the presence of 200 nM fMLP or 200 μM carbachol (a). 293 cells were transfected with 0.25 $\mu\text{g}/\text{ml}$ of the mammalian expression vector pcDNA I containing various wild-type α subunits (SS) or their constitutively activated mutants (ZZ), including α_{i1} -Q204L, α_{i2} -Q205L, α_{i3} -Q204L, α_{o1} -Q205L, α_z -Q205L and α_q -R183C (b). Results represent the means $\pm$ S.D. of triplicate determinations in a single experiment; two additional experiments gave similar results. * Significantly different from the basal activity; paired *t* test; $P < 0.001$.

like many receptors which couple to G_i , the fMLP receptor can functionally interact with G_z . In the absence of the fMLP receptors, expression of α_z did not affect the hCG-stimulated response (Figure 2b), indicating the activation of α_z requires ligand-bound receptors. It should be noted that expression of α_z at high levels will constitutively inhibit the hCG response [17] due to the relatively slow rate of GTP hydrolysis by α_z [29].

fMLP-mediated stimulation of PLC

In previous studies it has been shown that fMLP can utilize G_i proteins to stimulate PI-PLC in HL-60 cells [4] and induce Ca^{2+} mobilization in 293 cells [20]. Despite the fact that the C5a receptor was unable to exploit endogenous G proteins to stimulate PI-PLC in 293 cells [22], we examined whether the cloned fMLP receptor can regulate PI-PLC when expressed in 293 cells and, if so, whether G_z can similarly replace G_i in this signal transduction pathway. In cells transfected with cDNA encoding the human fMLP receptor (0.25 $\mu\text{g}/\text{ml}$), fMLP (up to 200 nM) did not stimulate the production of IP (Figure 3a). However, under similar conditions, 293 cells transiently expressing the muscarinic m1 receptor responded to 200 μM carbachol with a significant increase in IP formation (Figure 3a). Unlike the fMLP receptor, the muscarinic m1 receptor utilizes PTX-insensitive G_q/G_{11} proteins to stimulate PI-PLC. The inability of fMLP to regulate PI-PLC in 293 cells may be a reflection of its lack of coupling to G_q . However, a more plausible explanation is that the fMLP-induced stimulation of PI-PLC in HL-60 cells is mediated by $\beta\gamma$ subunits rather than α subunits of G proteins. Nevertheless, mutationally activated α subunits of different G proteins were transiently expressed in 293 cells to see if any of them could stimulate PI-PLC. Wild-type or constitutively activated mutant forms of α_i , α_o , α_q and α_z were transfected into 293 cells and then assayed for the formation of IP. Amongst the α subunits tested, only α_q -R183C constitutively stimulated PI-PLC (Figure 3b). None of the PTX-sensitive α subunits of G proteins showed any degree of PI-PLC activation.

The lack of PTX-sensitive PI-PLC activity in 293 cells prompted us to search for another cell line to examine the possible involvement of G_z in the regulation of PI-PLC by

Figure 4 fMLP-mediated stimulation of IP formation in transfected Ltk⁻ cells

Transfected Ltk⁻ cells were assayed for IP formation in the presence of 20 mM LiCl as described in the text. Cells were transfected with 0.25 μg/ml of fMLP receptor (fMLP-R) DNA, labelled with *myp*-[³H]inositol in the absence or presence of PTX (100 ng/ml) and subsequently assayed for responses to 200 nM fMLP. * fMLP significantly increased the formation of IP; paired *t* test; *P* < 0.05 (a). Cells were transfected with various concentrations of cDNA encoding the fMLP receptor (up to 3 μg/ml) and subsequently assayed for IP formation in response to 200 nM fMLP. fMLP at 200 nM significantly stimulated the production of IP at cDNA concentration of ≥ 0.1 μg; paired *t* test; *P* < 0.05 (b). Cells were transfected with the fMLP receptor as in (a). Transfected cells were then assayed for their responses to different concentrations of fMLP (up to 200 μM). IP formation was significantly increased by fMLP at concentrations of ≥ 1 nM; paired *t* test; *P* < 0.05 (c). For each panel, two or more separate experiments yielded results similar to those shown.

fMLP. Ltk⁻ fibroblasts have been used to demonstrate a PTX-sensitive stimulation of PI-PLC by recombinant dopamine D₂ [32] and 5-HT_{1A} [33] receptors. In Ltk⁻ cells transfected with the human fMLP receptor (0.25 μg/ml), incubations in the presence of 200 nM fMLP for 60 min stimulated the formation of IP by 50–120% over basal values (Figure 4a), with an average percentage increase of 87 ± 8.3% (*n* = 9). The fMLP-mediated stimulation of PI-PLC required the expression of fMLP receptors and was not observed in untransfected cells. The response was completely blocked by treating with cells with PTX (100 ng/ml) for 20–24 h before the addition of fMLP (Figure 4a). Though relatively small, the magnitude of the fMLP-mediated IP formation was similar to that obtained through the activation of endogenous lysophosphatidic acid receptors (about 100% over basal), and was comparable to that obtained in similar studies on the dopamine D₂ and 5-HT_{1A} receptors [32,33].

Like the fMLP-mediated inhibition of cAMP in transfected 293 cells, stimulation of IP formation in Ltk⁻ cells was dependent upon the amount of fMLP receptor cDNA used in the transfections; maximum stimulation of IP formation was observed with 0.3 μg/ml cDNA encoding the fMLP receptor (Figure 4b). Titration of the agonist-stimulated PI-PLC activities in transfected Ltk⁻ cells (0.25 μg/ml fMLP receptor cDNA) gave a maximum response at 10 nM and an EC₅₀ of 3 nM (Figure 4c). The EC₅₀ for the formation of IP was slightly higher than that required for inhibition of cAMP accumulation (0.1 nM), and was comparable with the EC₅₀ (~6 nM) for fMLP-induced production of superoxide in neutrophils [34]. Indeed, maximal stimulation of PI-PLC by fMLP occurred at 1 μM in HL-60 cells [35], and fMLP stimulated chemotaxis at 0.1 μM [10].

G_i does not couple the fMLP receptor to the stimulation of PLC in Ltk⁻ cells

Since stimulation of PI-PLC in Ltk⁻ cells was sensitive to PTX, we attempted to identify the α subunits involved by expressing the constitutively activated mutants of α subunits in Ltk⁻ cells. Similar to the results obtained with the 293 cells, none of the α_i nor α_o mutants were able to stimulate the formation of IP, whereas the α_q mutant, α_q-R183C, constitutively activated PI-PLC (results not show, but similar to Figure 3b). These results indicated that the PI-PLC in Ltk⁻ cells, like that in HL-60 cells, is likely to be regulated by βγ subunits of G proteins. Indeed, when cDNAs encoding the β₂ and γ₂ subunits were co-transfected into the Ltk⁻ cells, the formation of IP increased significantly above the basal values (Figure 5a). Expression of either β₂ or γ₂ subunit alone in Ltk⁻ cells did not stimulate PI-PLC (R. C. Tsu and Y. H. Wong, unpublished work), while co-expression of β₂γ₂ subunits resulted in IP formation. The magnitude of β₂γ₂-stimulated IP formation was 2-fold higher than that obtained via stimulation of the fMLP receptor (Figure 5a), and no further stimulation by fMLP was observed in cells co-expressing the fMLP receptor (R. C. Tsu and Y. H. Wong, unpublished work). These results are in agreement with similar studies in COS-7 cells using β₂γ₂ subunits [36].

As the fMLP stimulation of PI-PLC in transfected Ltk⁻ cells was also PTX-sensitive, it seemed possible that G_i might similarly substitute for G_i in this signalling pathway. Ltk⁻ cells were thus transfected with cDNA encoding the fMLP receptor with or without the α_z cDNA. In the absence of α_z expression, inactivation of endogenous α_i by PTX resulted in the abolition of fMLP-induced IP formation (Figure 5b). Even in cells co-expressing α_z a similar result was obtained, suggesting that α_z cannot replace α_i in coupling the fMLP receptor to PI-PLC. To confirm that the transfected cells were indeed expressing α_z,

Figure 5 Role of $\beta\gamma$ and α_z subunits in the stimulation of IP formation in Ltk⁻ cells

The Ltk⁻ cells were co-transfected with 0.125 μg of pcDNA I containing the γ_2 subunit with or without different concentrations of the β_2 subunit as indicated. Transfected cells were assayed for IP formation as described in the legend to Figure 4, in the presence of 20 mM LiCl. Results are expressed as percentages of IP formation in the basal state of control (vector-transfected) cells. IP formation in cells which had been co-transfected with 0.125 μg of γ_2 subunit and ≥ 0.125 μg of β_2 subunit was significantly higher than the control; paired *t* test; $P < 0.05$ (a). The Ltk⁻ cells were transfected with 0.25 μg of fMLP receptor DNA with or without 0.125 μg of α_z . Transfected cells were assayed for IP formation in the absence or presence of 200 nM fMLP. Results are expressed as percentage stimulation of IP formation as compared with basal activity. * PTX significantly reduced the ability of fMLP to stimulate the formation of IP; paired Bonferroni *t* test; $P < 0.05$ (b). For each panel, two or more independent experiments yielded results similar to those shown.

Figure 6 Immunodetection of α_z expression

Plasma membranes were prepared from Ltk⁻ cells transiently expressing the fMLP receptor (fMLP-R) with or without α_z . A 150 μg sample of membrane protein was separated on a 12.5% polyacrylamide/SDS gel and electrophoretically transferred to PVDF membranes. Protein markers were localized by Ponceau S staining and the presence of α_z was shown by immunodetection with the α_z -specific antiserum P-961.

membranes prepared from the transfectants were analysed by immunoblotting with an α_z -specific antiserum. The α_z -specific antiserum P-961 recognized a 41 kDa substrate in membranes prepared from Ltk⁻ cells which had been co-transfected with the fMLP receptor and α_z (Figure 6). No substrate was detected by

Figure 7 Inhibition of $\beta\gamma$ -mediated stimulation of IP formation by α subunits

The Ltk⁻ cells were co-transfected with different combinations of cDNAs consisting of 0.125 μg of α_z , 0.125 μg of γ_2 subunit and 0.25 μg of β_2 subunit, as indicated. Transfected cells were assayed for IP formation in the presence of 20 mM LiCl. Results are expressed as percentages of IP formation above the basal state of control (vector-transfected) cells (a). The Ltk⁻ cells were transfected and assayed as in (a) but with α_z replaced by α_{11} . * Co-expression of α_{11} significantly reduced the ability of $\beta_2\gamma_2$ to stimulate the formation of IP; paired Bonferroni *t* test; $P < 0.05$ (b). For each panel, two more experiments yielded results similar to those shown.

antiserum P-961 in membranes prepared from cells transfected with the fMLP receptor alone (Figure 6).

Since $\beta\gamma$ subunits form an integral part of the receptor-G-protein complex, the lack of coupling between α_z and the fMLP receptor in Ltk⁻ cells may reflect an incompatibility of α_z with the endogenous $\beta\gamma$ subunits, or the possibility that the specific $\beta\gamma$ subunits released through the activation of G_x do not stimulate PI-PLC. To gain some insight into the coupling of α_z to specific species of $\beta\gamma$ subunits, we examined whether α_z can inhibit the $\beta_2\gamma_2$ -induced stimulation of PI-PLC. As shown in Figure 7(a), co-expression of $\beta_2\gamma_2$ in Ltk⁻ cells increased IP formation by about 4-fold. The $\beta_2\gamma_2$ -induced formation of IP was not affected by the co-expression of α_z (Figure 7a). In contrast, co-expression of α_{11} significantly inhibited the ability of $\beta_2\gamma_2$ to stimulate PI-PLC (Figure 7b); α_{11} is known to exhibit a high affinity for $\beta\gamma$ subunits and has been used to block the $\beta\gamma$ -induced stimulation of type II adenylate cyclase in 293 cells [37]. Expression of either α_z or α_{11} alone did not stimulate PI-PLC (Figures 7a and 7b). These results indicate that α_z does not bind to the $\beta_2\gamma_2$ complex, whereas α_{11} can apparently interact with $\beta_2\gamma_2$.

DISCUSSION

The objective of this study was to assess the signalling capacity of the cloned fMLP receptor in terms of both the specificity of coupling to G proteins and the diversity of regulation of effectors. An initial goal was to determine whether the fMLP receptor can utilize G proteins other than the PTX-sensitive G_i proteins in its signal transduction pathways. As the cloned fMLP receptor has been shown to inhibit cAMP accumulation in transfected 293 cells [20], we explored this experimental set-up to examine the possible coupling between the fMLP receptor and G_z, a PTX-insensitive G protein known to inhibit adenylate cyclase [17]. Our results demonstrate that the activated human fMLP receptor is indeed able to inhibit cAMP accumulation by interacting with either G_i or G_z proteins.

It has been previously established that other chemoattractant receptors such as the C5a and interleukin 8 receptors can activate PI-PLC by interacting specifically with the PTX-insensitive G₁₆ and G₁₄ [21–23]. Thus far, there is no evidence that the fMLP receptor can couple to any PTX-insensitive G protein. Our studies with α_z demonstrate the capacity of this subunit to act as a transducer for the fMLP receptor. This had not been assumed previously since, in neutrophils, fMLP stimulates PI-PLC exclusively via G proteins that serve as substrates for PTX-catalysed ADP-ribosylation [10,11]. In the light of the finding that the fMLP receptor can also inhibit adenylate cyclase [19,20], it appears likely that the fMLP receptor may share some mechanistic properties with other multifunctional receptors such as the dopamine-D₂ and α_2 -adrenergic receptors. Indeed, the PTX-insensitive G_z protein has been shown to inhibit cAMP accumulation in response to the activation of a variety of inhibitory receptors, including the dopamine-D₂, adenosine-A₁, α_2 -adrenergic and lysophosphatidic acid receptors [17]. The list of inhibitory receptors that can utilize G_z instead of G_i has now been extended to include the fMLP receptor. The significance of the coupling between fMLP receptors and G_z in relation to biological activities remains to be explored. Using the α_z -specific antiserum P-961 on immunoblots, we were unable to detect the presence of α_z in undifferentiated or differentiated HL-60 cells (R. C. Tsu and Y. H. Wong, unpublished work). Nevertheless, it is interesting to note that G_z is present in basophilic cells [24] and that several chemotactic factors, including fMLP and C5a, can induce histamine release from basophils [38]. However, it has also been shown that the fMLP-induced histamine release in human basophils is sensitive to PTX [39].

In the present study, the coupling between fMLP receptors and G_i proteins is highly efficient, since the maximal response can be achieved at nanomolar concentrations of fMLP. However, in HL-60 cells, fMLP-induced activation of G_i proteins results only in stimulation of PI-PLC and Ca²⁺ mobilization, with no inhibition of cAMP accumulation. The reason behind a lack of fMLP-mediated inhibition of adenylate cyclase in HL-60 cells is unclear. One possibility is that the fMLP receptor might only couple to one form of G_i to inhibit adenylate cyclase, and the differential response of fMLP in 293 and HL-60 cells may arise from differences in the expression of various forms of G_i proteins in these cell lines. G₁₂ and G₁₃ are expressed in both HL-60 and 293 cells, whereas G₁₁ is present in 293 cells but is undetectable in HL-60 cells [12,16,40]. It is unlikely that fMLP-induced inhibition of cAMP accumulation is mediated solely through G₁₁, since all three forms of G_i proteins are known to inhibit adenylate cyclase upon their activation [16,17]. Alternatively, over expression of the fMLP receptor in 293 cells may result in forced coupling to the inhibition of adenylate cyclase. Although this possibility has not been definitely ruled out, in general the

expression of fMLP receptors in 293 and other cell lines has yielded K_d and B_{max} values that are comparable with those obtained with HL-60 cells [19,30].

With the cloning and characterization of multiple forms of adenylate cyclase [41], it seems more plausible that HL-60 and 293 cells may express different forms of the effector, thus dictating the final response to G_i activation. Isoenzymes of mammalian adenylate cyclase are known to differ in tissue distribution and mode of regulation by G protein subunits. Type II and type IV adenylate cyclases are stimulated by $\beta\gamma$ subunits, contingent upon the presence of activated α_s . Although 293 cells express a type III-like adenylate cyclase [42], the isoenzymes of adenylate cyclase expressed in HL-60 cells are as yet unclassified. Nevertheless, it has been demonstrated that the cellular complement of adenylate cyclase can indeed affect the final response to activation of inhibitory receptors such as the α_2 -adrenoceptor [37]. Expression of type II adenylate cyclase in 293 cells can convert signals which normally inhibit cAMP accumulation to ones that synergize or stimulate the formation of cAMP through the actions of G protein $\beta\gamma$ subunits. If type II-like adenylate cyclase is the predominant isoform found in HL-60 cells, activation of G_i proteins would not suppress the formation of cAMP; depending on whether activated α_s subunits are present or not, it may even lead to stimulation of cAMP accumulation. Based on our current understanding of how isoenzymes of adenylate cyclases are regulated, and on the observation that the cloned fMLP receptor can functionally couple to G_i proteins when expressed in 293 cells, we predict that the HL-60 cells will contain type II-like adenylate cyclase. Indeed, in 293 cells co-expressing type II adenylate cyclase, α_s -Q227L and the fMLP receptor, enhancement of cAMP accumulation is observed in the presence of fMLP [43]. The relevance of having an adenylate cyclase which is tuned to positive regulation in neutrophils is not obvious. Paradoxically, elevated levels of cAMP inhibit phospholipase D activation [44] and possibly other neutrophil responses [45]. Nevertheless, a small transient increase in cAMP through the stimulation of the fMLP receptor has been observed to precede the respiratory burst and lysosomal secretion in PMN [45].

Recent studies on the cloned isoenzymes of PLC- β reveal complex mechanistic models for their regulation. Purified α subunits of G_q/G₁₁ can activate PI-PLC- β_1 , $-\beta_2$ and $-\beta_3$ with different efficacies [46]. It has been shown that $\beta\gamma$ subunits of G proteins can also stimulate PI-PLC- β isoenzymes [47,48]. Although indirect, results obtained in the present study appear to reinforce the notion that $\beta\gamma$ subunits of G proteins can indeed stimulate PI-PLC. Despite the fact that the production of IP in response to fMLP is mediated via PTX-sensitive G proteins in HL-60 cells, presumably through PI-PLC- β_2 , experiments designed to elucidate the role of α subunits in the regulation of PI-PLC lend little or no support to the involvement of α_i subunits. Expression of the mutationally activated α subunits of PTX-sensitive G proteins in transfected cells did not result in the constitutive activation of PI-PLC, as shown in this paper. Like the 293 cells, Ltk⁻ fibroblasts express all three forms of G_i protein [49]. The lack of fMLP stimulation of PI-PLC in 293 cells is therefore unlikely to be due to the differential expression of G_i proteins. Instead, it is more likely that the two cell lines express different isoenzymes of PI-PLC, with those present in Ltk⁻ cells being responsive to $\beta\gamma$ subunits, as demonstrated by an increased IP production in Ltk⁻ cells that were co-transfected with the G protein β_2 and γ_2 subunits. The exact compositions of the complement of PI-PLC isoenzymes in Ltk⁻ and 293 cells are not known, but differences do exist. The β_1 isoform of PI-PLC is expressed in Ltk⁻ but not in 293 cells (R. C. Tsu and Y. H. Wong, unpublished work). The mechanism by which $\beta\gamma$ subunits

stimulate the activity of PI-PLC is not clear. Regulation of PI-PLC by $\beta\gamma$ subunits is rather complex, since a large number of permutations exist between known β and γ subunits [50]. The composition of the $\beta\gamma$ subunits may play a role in signalling specificity, as it has been demonstrated that $\beta_1\gamma_1$, but not $\beta_2\gamma_1$, can stimulate PI-PLC- β_2 [36]. Nevertheless, the recent cloning of a new form of PI-PLC termed β_4 , which appears to be refractory to α_q and $\beta\gamma$ subunits [51], renews the possibility that α_i subunits can directly regulate the generation of IP.

Although it is not totally unexpected that α_z can substitute for α_i in mediating the fMLP-induced inhibition of adenylate cyclase, it is noteworthy that α_z apparently cannot replace α_i in transducing the fMLP signal which leads to the formation of IP. The difference may reflect functional specificity between α_i and α_z . As the fMLP-induced stimulation of PI-PLC may be mediated via $\beta\gamma$ subunits, the inability of α_z to replace α_i in the regulation of PI-PLC could be interpreted as a difference in the binding affinities of α subunit for various $\beta\gamma$ subunits. With regard to the $\beta_2\gamma_2$ complex, specific interactions with different α subunits appear to exist. The lack of interaction between α_z and $\beta_2\gamma_2$ is in direct contrast to the binding of α_{i1} to $\beta_2\gamma_2$. It is therefore plausible that the endogenous $\beta\gamma$ subunits in Ltk⁻ may not interact with α_z efficiently. Furthermore, the molecular composition of $\beta\gamma$ subunits has been shown to determine the specificity of coupling between receptors and G proteins. In a series of elegant experiments in which selective antisense oligonucleotides to various G protein subunits were microinjected into the nuclei of rat GH₃ cells, the muscarinic m4 and somatostatin receptors appeared to inhibit voltage-dependent Ca²⁺ channels through distinct G proteins composed of $\alpha_{o1}\beta_3\gamma_4$ and $\alpha_{o2}\beta_1\gamma_3$ respectively [52–54]. It is highly likely that α_z and α_i can bind to different β and γ subunits and thus confer upon G_z and G_i the ability to regulate diverse signalling pathways. Future experiments will resolve this and other questions regarding the specific interactions between the fMLP receptor, G_z and PI-PLC.

In summary, the present study shows that, like the C5a and interleukin 8 receptors, the fMLP receptor can couple to PTX-insensitive G proteins. The biological significance of this putative coupling between the fMLP receptor and G_z is as yet unclear. It is conceivable that the multitude of fMLP-induced pro-inflammatory responses could be mediated in part by G_z. Presumably, if G_z plays a role in mediating any response to fMLP, the uniquely slow rate of GTP hydrolysis by α_z [29] might dictate that the response be long-lasting. Whether G_z is involved in the fMLP-induced sustained Ca²⁺ influx in neutrophils remains the subject of further investigation.

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