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Stimulation of Type II Adenylyl Cyclase by Chemoattractant Formyl Peptide and C5a Receptors

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SUMMARY

The capacity of *N*-formylmethionyl-leucyl-phenylalanine (fMLP) and C5a receptors to regulate type II adenylyl cyclase was examined in transient transfection studies. Coexpression of either one of the chemoattractant receptors with type II adenylyl cyclase in human embryonic kidney 293 cells allowed the corresponding chemotactic factor to stimulate cAMP accumulation in a dose-dependent manner. The chemoattractant-induced stimulation of type II adenylyl cyclase was absolutely dependent on the presence of GTP-bound α subunit of G_s, as revealed by the coexpression of α_s-Q227L, a constitutively activated mutant of α_s. Stimulation of type II adenylyl cyclase by either fMLP or C5a was mediated via pertussis toxin-sensitive G_i-like proteins, because the response was abrogated by the toxin. The ability of G_z (a pertussis toxin-insensitive G protein that can couple to a number of G_i-linked receptors) to replace G_i in chemoattractant-induced stimulation of type II adenylyl cyclase was examined. The chemoattractant-induced response became insensitive to pertussis toxin upon coexpression of the α subunit of G_z. Interestingly, coexpression of α_z significantly enhanced the chemotactic factor-stimulated type II adenylyl cyclase activities. When other G protein α subunits were tested under similar experimental conditions, all three forms of α_i and α_{o1} were able to potentiate the fMLP response to various extents, whereas α_q and α_t slightly inhibited the fMLP response. The α subunit-mediated potentiation of the type II adenylyl cyclase response appears to reflect a productive coupling between α subunits and the fMLP receptor, because such enhancements were not seen with the constitutively activated α subunit mutants. Coexpression of the constitutively activated mutants of α_z, α_q, α_{o1}, and α_{i1-3} neither enhanced nor inhibited the fMLP-stimulated cAMP accumulation. These results indicated that the observed enhancement of type II adenylyl cyclase responses was dependent on the ability of the wild-type α subunits to functionally interact with the fMLP receptor and that the fMLP receptor can couple to G_{i1-3}, G_z, and G_{o1} but not to G_s, G_q, or G_t.

The chemotactic peptide fMLP and the anaphylatoxin complement fragment C5a are potent activators of PMNs. Activation of receptors for these chemoattractants has been shown to induce PMN adherence, phagocytosis, release of granule enzymes, and generation of superoxide radicals (1, 2). The chemoattractant-induced responses are known to involve regulatory as well as small molecular weight G proteins (3). Agonist binding to the fMLP or C5a receptor triggers a series of intracellular events, including activation of PI-PLC and Ca^{2+} mobilization and influx, eventually leading to the activation of PKC (4).

The cloning of fMLP (5) and C5a (6, 7) receptors has enabled researchers to dissect their signaling pathways in detail. Expression of the fMLP and C5a receptors in cultured cell lines has confirmed that these receptors can indeed stimulate PI-PLC and mobilize calcium (8). More interestingly, the fMLP receptor was shown to inhibit adenylyl cyclase (9, 10). This inhibitory effect of fMLP is in direct contrast to that observed in neutrophils, where fMLP transiently increases intracellular cAMP levels. It has been suggested that the transient increase in cAMP accumulation is largely mediated indirectly via either the release of adenosine (11) or inhibition of phosphodiesterase (12). However, a direct interaction between the fMLP receptor and adenylyl cyclase has not been formally excluded. With the existence of multiple isozymes of adenylyl cyclase (13), one possible scenario is that activation of the fMLP receptor may lead to stimulation of type II or type IV adenylyl cyclases via the $\beta\gamma$ subunits of G proteins (14). In fact, several classical inhibitory receptors, such as the α_2-adrenergic, dopamine D_2, and adenosine A_1 receptors, can stimulate the type II adenylyl cyclase in a PTX-sensitive manner (15). It is therefore likely that the fMLP receptor may have the capacity to regulate type II adenylyl cyclase in a similar fashion.

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ABBREVIATIONS: fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PI-PLC, phosphoinositide-specific phospholipase C; PMN, polymorphonuclear neutrophil; PTX, pertussis toxin; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; MEM, minimum essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

In this report, we present evidence that the chemoattractant receptors can induce the accumulation of cAMP by stimulating type II adenylyl cyclase in a PTX-sensitive manner. The experimental approach involved transient transfections of cDNAs encoding the human chemoattractant receptors, the type II adenylyl cyclase, and various G protein α subunits. The co-transfection methodology further allowed us to examine whether the fMLP receptor regulates type II adenylyl cyclase by interacting directly with the α subunit of G_s. Coexpression studies indicated that the fMLP receptor might have the ability to interact with α_z, α_{o1}, and all three forms of α_i.

Materials and Methods

Reagents. cDNAs encoding the human fMLP receptor (in the pCDM8 vector) and the human C5a receptor (in the pEE6hCMV.neo mammalian vector) were kindly provided by Dr. F. Boulay (Laboratoire de Biochemie, Grenoble, France) and Dr. Michael D. Barker (Krebs Institute, Sheffield, UK), respectively. The origin and construction of other cDNAs have been described elsewhere (16, 17). PTX was purchased from List Biological Laboratories (Campbell, CA). Human embryonic kidney 293 cells (CRL-1573) were obtained from the American Type Culture Collection. [^{3}H]Adenine and [^{3}H]fMLP were purchased from Amersham and New England Nuclear, respectively. Plasmid purification columns were obtained from Qiagen. Cell culture reagents were obtained from Life Technologies, and all other chemicals were purchased from Sigma.

Cell culture and transfection. 293 cells were maintained in Eagle's MEM supplemented with 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°. Twenty-four hours before transfection, 293 cells were seeded onto 12-well plates at 1×10^5 cells/well. DNAs were purified by Qiagen column chromatography and transfected into 293 cells in the presence of DEAE-dextran, essentially as described previously (18). In brief, cells were incubated in growth medium supplemented with 400 μg/ml DEAE-dextran, 100 μM chloroquine, and various cDNAs. After a 2-hr incubation at 37°, the cells were shocked for 1 min at room temperature in phosphate-buffered saline containing 10% (v/v) dimethylsulfoxide, rinsed with phosphate-buffered saline, and returned to growth medium for 24 hr. Under these conditions, approximately 40–50% of the transfected 293 cells expressed the exogenous proteins, as indicated by co-transfection studies using a vector containing β-galactosidase as a reporter gene.

cAMP accumulation. Twenty-four hours after transfection, 293 cells were labeled for 16–20 hr with [^{3}H]adenine (1 μCi/ml) in growth medium, with or without PTX (100 ng/ml). The labeled cells were rinsed once with 2 ml of assay medium (MEM containing 20 mM HEPES, pH 7.4) and incubated at 37° for 30 min with 1 ml of assay medium containing 1 mM 1-methyl-3-isobutylxanthine and the indicated drugs. Intracellular [^{3}H]cAMP was isolated by sequential chromatography as described previously (19). The level of [^{3}H]cAMP was estimated by determining the ratios of [^{3}H]cAMP to total [^{3}H]ATP and [^{3}H]ADP pools, as reported previously (17). Absolute values for cAMP levels varied between experiments, but variability within any single experiment was generally <10%.

Binding assays. Membranes were prepared from transfected 293 cells. Briefly, cells were trypsinized, resuspended in lysis buffer (50 mM Tris·HCl containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, 0.001%, w/v, soybean trypsin inhibitor, 1 mM EGTA, 5 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.5), 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. Binding assays were conducted in 20 mM Tris·HCl, pH 7.5, containing 30–50 μg of membrane protein, 1 mM benzamidine-HCl, 0.001% (w/v) soybean trypsin inhibitor, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1% (w/v) bovine serum albumin, and 10 nM [^{3}H]fMLP. Nonspecific binding was measured in the presence of excess unlabeled fMLP (1 μM). After a 1-hr incubation at 25°, the samples were filtered through Whatman GF/B filters, in a PHD cell harvester, and washed with 3 × 4 ml of ice-cold 50 mM Tris·HCl, pH 7.4. Dried filters were counted in 4 ml of OptiPhase Hi-Safe 3 scintillation fluid. Nonspecific binding was generally <15% of the total binding.

Results

The first goal of these studies was to determine whether the fMLP and C5a receptors can stimulate type II adenylyl cyclase. It has previously been demonstrated that the chemoattractant receptors, as well as type II adenylyl cyclase, can be functionally expressed in human embryonic kidney 293 cells (8, 10, 15). The 293 cells are particularly suitable for the functional analysis of interactions between various signaling components because multiple exogenous proteins can be simultaneously expressed in these cells. We co-transfected 293 cells with cDNAs encoding the type II adenylyl cyclase, α_s-Q227L, and either the fMLP receptor, the C5a receptor, or the dopamine D_2 receptor. α_s-Q227L is a constitutively active mutant of α_s harboring a point mutation at codon 227 (20). Activation of either the fMLP or C5a receptors stimulated cAMP accumulation by 80–120% over basal values (Fig. 1, A and B), which was comparable to the dopamine D_2 receptor-mediated response (Fig. 1C and Ref. 15). The chemoattractant-induced stimulatory responses were apparently mediated by G_i-like proteins, because the stimulations were blocked by PTX treatment (Fig. 1, A and B). When the cDNA encoding the type II adenylyl cyclase was omitted from the transfection cocktail, activation of the fMLP receptor resulted in 50–60% inhibition of α_s-Q227L-stimulated basal cAMP accumulation (Fig. 1D). This indicates that the chemoattractant-induced stimulatory responses were mediated via the exogenous type II adenylyl cyclase, whereas the endogenous type III adenylyl cyclase (21) was inhibited by the activated fMLP receptor. Fig. 1E shows that the addition of 10 pM to 1 μM fMLP led to a concentration-dependent stimulation of the type II adenylyl cyclase response, with an EC_{50} of approximately 0.5 nM and with the maximal response occurring at 20 nM. Similar results were obtained when α_s-Q227L was replaced by the ligand-bound rat luteinizing hormone receptor.[1]

Activation of type II adenylyl cyclase by G_i-coupled receptors is contingent upon the presence of activated α_s subunits (14). This prerequisite has been clearly demonstrated for the dopamine D_2 and adenosine A_1 receptors (15). However, promiscuous receptors such as the α_2-adrenergic receptor can stimulate type II adenylyl cyclase by interacting with G_s (15). Using slightly modified experimental conditions, we examined whether the chemoattractant receptors can couple to G_s. 293 cells were co-transfected with cDNAs encoding the type II adenylyl cyclase and a chemoattractant receptor, without α_s-Q227L. The omission of α_s-Q227L from the transfections would eliminate the ability of the chemoattractants to stimulate type II adenylyl cyclase, unless the chemoattractant receptors were able to activate endogenous α_s subunits. Un-

[1] R. C. Tsu, R. A. Allen, and Y. H. Wong, unpublished observations.

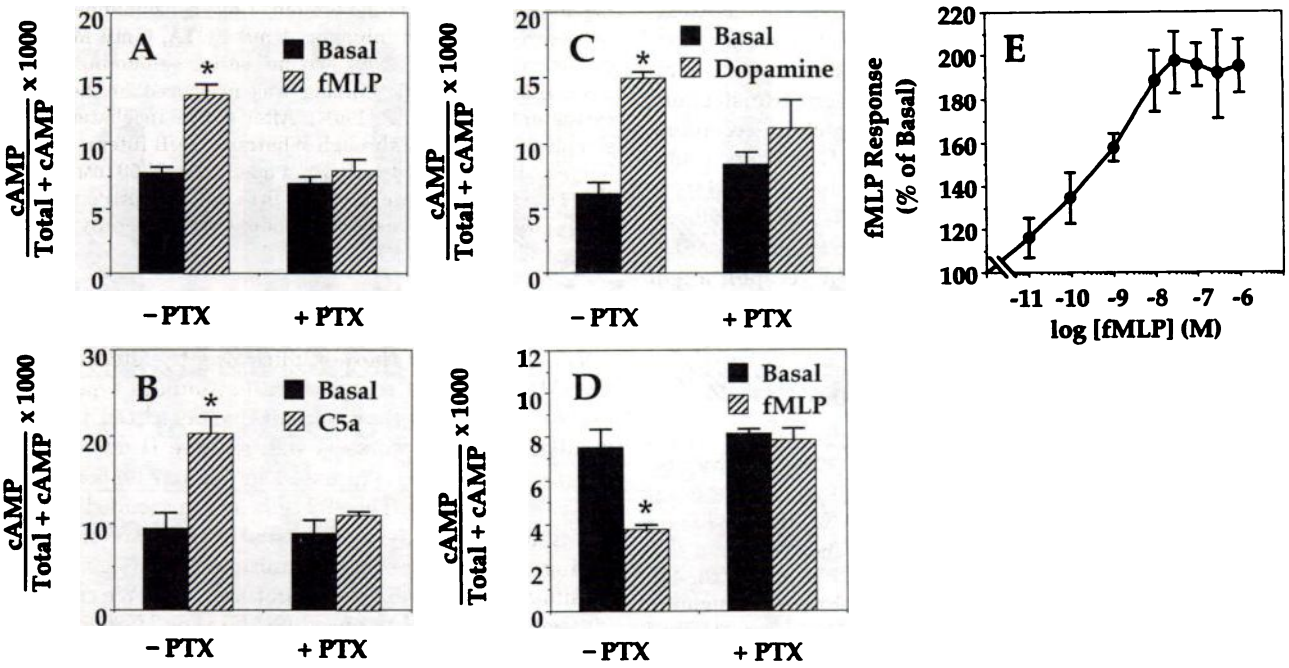

Fig. 1. Chemoattractant-induced stimulation of type II adenylyl cyclase activity in transfected 293 cells. 293 cells were transiently transfected (by the DEAE-dextran method), labeled with [^{3}H]adenine (1 μCi/ml) for 18–24 hr, and then assayed for cAMP accumulation in the presence of 20 mM HEPES, 1 mM 1-methyl-3-isobutylxanthine, and various concentrations of drugs as described in the text. A–D, 293 cells were transfected with cDNAs encoding type II adenylyl cyclase (in pcDNAI; 0.25 μg/ml, except D), α_s-Q227L (in pcDNAI; 0.025 μg/ml), and one of the following three receptor cDNAs: fMLP receptor (in pCDM8; 0.25 μg/ml) (A and D), C5a receptor (in pcDNAI; 0.25 μg/ml) (B), or dopamine D_2 receptor (in pcDNAI; 0.25 μg/ml) (C). Cells were then labeled with [^{3}H]adenine in the absence or presence of PTX (100 ng/ml) and assayed for responses to either 200 nM fMLP, 100 nM C5a, or 1 μM dopamine. *, Agonists significantly stimulated (A–C) or inhibited (D) cAMP accumulation over basal values (paired *t* test, $p < 0.05$). E, Cells were transfected as in A and the transfected cells were then assayed for cAMP accumulation in the presence of various concentrations of fMLP (up to 1 μM). Results are expressed as percentage of cAMP formation, compared with basal values. fMLP significantly increased cAMP accumulation at all of the concentrations tested (paired *t* test, $p < 0.05$). Data shown represent the mean ± standard deviation of triplicate determinations in a single experiment; two additional experiments yielded similar results.

der these conditions, no significant stimulation of cAMP accumulation was observed in the presence of 200 nM fMLP (Fig. 2). These results implied that the fMLP receptor could not couple to G_s and that the stimulation of type II adenylyl cyclase was mediated via the $\beta\gamma$ subunits released through the activation of G_i. Similar results were obtained with the C5a receptor, whereas activation of the α_2-adrenergic receptor resulted in significantly elevated cAMP levels (Fig. 2).

Several G_i-coupled receptors can interact with the PTX-insensitive G_z to inhibit adenylyl cyclase (17). The fMLP and C5a receptors may also have the ability to activate G_z. To test whether the chemoattractant receptors can utilize G_z to stimulate type II adenylyl cyclase, we co-transfected 293 cells with cDNAs encoding the type II adenylyl cyclase, α_s-Q227L, and either the fMLP or C5a receptor, in the absence or presence of α_z. In the absence of α_z, PTX-mediated inactivation of endogenous α_i in the transfected 293 cells abolished the response to the chemotactic factors (Fig. 3). However, coexpression of α_z with the chemoattractant receptors produced cells in which the chemoattractant-mediated stimulation of cAMP accumulation was only partially inhibited by PTX treatment (Fig. 3), thus suggesting that, like G_i, G_z can also couple to the chemoattractant receptors and stimulate type II adenylyl cyclase, presumably by releasing $\beta\gamma$ subunits.

Interestingly, the chemoattractant-induced stimulation of cAMP accumulation was significantly enhanced in cells co-expressing α_z (Fig. 3). The α_z-dependent enhancement of cAMP accumulation and the partial blockade by PTX tend to support the idea that the chemoattractant receptors can simultaneously utilize endogenous α_i and exogenous α_z to stimulate type II adenylyl cyclase. It is noteworthy that the

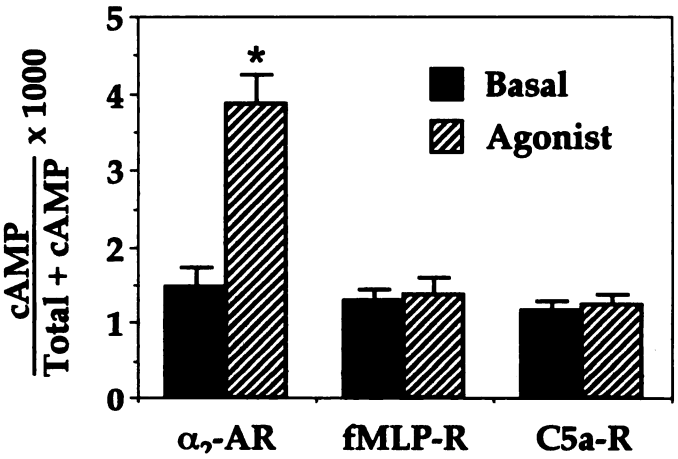

Fig. 2. Lack of chemoattractant-induced stimulation of type II adenylyl cyclase in the absence of α_s-Q227L. 293 cells were transfected with cDNAs encoding type II adenylyl cyclase (0.25 μg/ml) and one of the following three receptor cDNAs: α_2-adrenergic receptor (*α_2-AR*) (0.25 μg/ml), fMLP receptor (*fMLP-R*) (0.25 μg/ml), or C5a receptor (*C5a-R*) (0.25 μg/ml). Cells were then labeled with [^{3}H]adenine and assayed for responses to either 1 μM clonidine, 200 nM fMLP, or 100 nM C5a, as described in the text. Results represent the mean ± standard deviation of triplicate determinations in a single experiment; two additional experiments gave similar results. *, Significantly different from the basal activity (paired *t* test, $p < 0.05$).

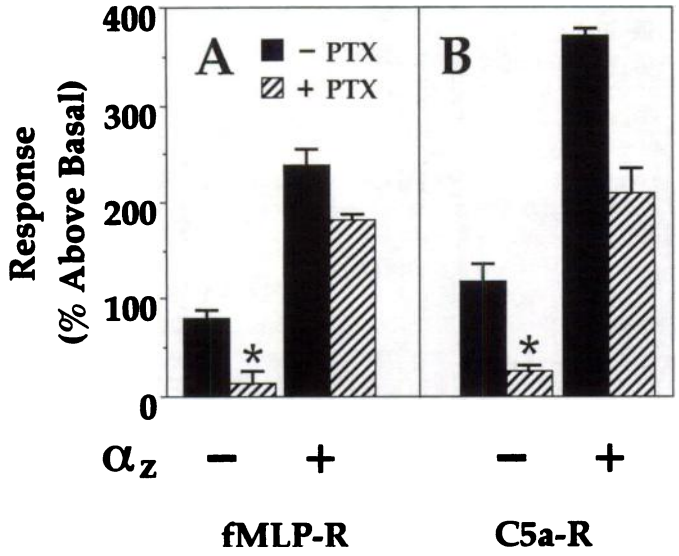

Fig. 3. Chemoattractant receptor coupling to the PTX-insensitive α_z. The 293 cells were co-transfected with DNAs encoding type II adenylyl cyclase (0.25 μg/ml), α_s-Q227L (0.025 μg/ml), and one of two receptor DNAs, i.e., fMLP receptor (*fMLP-R*) (in pcDNAI; 0.25 μg/ml) (A) or C5a receptor (*C5a-R*) (in pcDNAI; 0.25 μg/ml) (B). Each set of receptor DNAs was co-transfected with or without 0.15 μg/ml α_z in pcDNAI. Transfected cells were assayed for cAMP formation as described in the legend to Fig. 1, in the presence of 1 mM 1-methyl-3-isobutylxanthine, with or without inhibitory agonists (200 nM fMLP and 100 nM C5a). Results are expressed as percentage stimulation of cAMP production over basal activities. The basal values, expressed as the ratio ($\times 10^3$) of cAMP to total adenine nucleotides, ranged from 6.87 ± 0.55 to 8.13 ± 0.89 (A) or from 6.62 ± 0.33 to 9.39 ± 1.87 (B). For A and B, two or more separate experiments yielded results similar to those shown. *, After PTX treatment, the agonist-induced stimulation of cAMP production was not significantly different from the basal value (paired Bonferroni *t* test, $p > 0.05$).

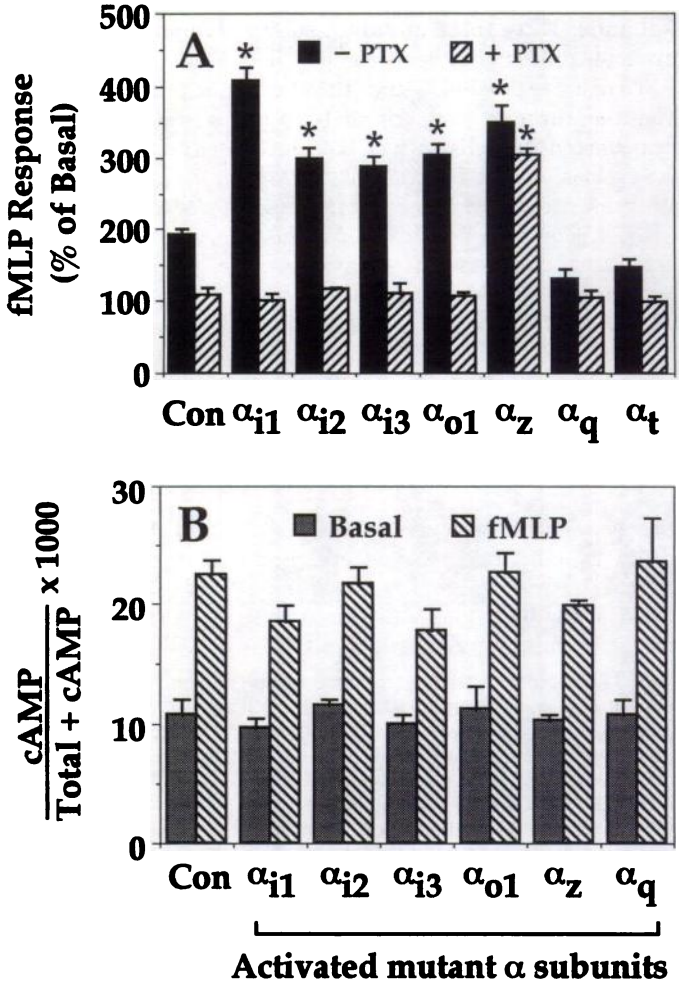

Fig. 4. Potentiation of fMLP-induced stimulation of type II adenylyl cyclase by different G protein α subunits. A, The 293 cells were co-transfected with type II adenylyl cyclase cDNA (in pcDNAI; 0.25 μg/ml), α_s-Q227L (0.025 μg/ml), and fMLP-pCDM8 (0.25 μg/ml), without (*Con*) or with 0.15 μg of cDNA encoding one of the following α subunits: α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_z, α_q, or α_t. Transfected cells were assayed for cAMP accumulation as described in the legend to Fig. 1, in the presence of 1 mM 1-methyl-3-isobutylxanthine, with or without 200 nM fMLP. Results are expressed as percentage stimulation of cAMP formation in the presence of fMLP, compared with that measured in the absence of fMLP. The basal values, expressed as the ratio ($\times 10^3$) of cAMP to total adenine nucleotides, ranged from 4.33 ± 0.32 to 7.82 ± 0.46. *, fMLP-stimulated cAMP accumulation was significantly different from that observed in the control group (paired Bonferroni *t* test, $p < 0.05$). B, Cells were transfected with various cDNAs as described for A, except that the wild-type α subunits were replaced by their respective activated mutants, i.e., α_{i1}-Q204L, α_{i2}-Q205L, α_{i3}-Q204L, α_{o1}-Q205L, α_z-Q205L, or α_q-R183C. The data represent triplicate determinations in a single experiment; two independent experiments yielded similar results.

magnitudes of inhibition by PTX were very similar to the extent of stimulation mediated solely by chemoattractant-induced activation of endogenous G_i proteins in the absence of G_z (Fig. 3). Because it is known that the chemoattractant receptors are coupled to G_i proteins, increasing the pool of G_i proteins by expression might similarly potentiate the chemoattractant-stimulated type II adenylyl cyclase response. The ability of various G proteins to enhance the chemoattractant-induced type II adenylyl cyclase response was examined by co-transfecting 293 cells with cDNAs encoding the type II adenylyl cyclase, α_s-Q227L, and the fMLP receptor, in the absence or presence of different α subunit cDNAs. The fMLP-induced stimulation of type II adenylyl cyclase was significantly increased in cells coexpressing either α_{i1}, α_{i2}, α_{i3}, α_{o1}, or α_z (Fig. 4A). Enhancement of the fMLP response was specific and was not observed in cells coexpressing α_q or α_t (Fig. 4A). In contrast, expression of α_q and α_t inhibited the fMLP-mediated type II adenylyl cyclase response. Except for cells coexpressing α_z, the enhanced fMLP responses due to coexpression of α_{i1}, α_{i2}, α_{i3}, and α_{o1} were completely abolished by PTX (Fig. 4A).

Because the fMLP-induced stimulation of type II adenylyl cyclase required the presence of α_s-Q227L, decreased expression of α_s-Q227L, due to competition by other α subunits for protein synthesis, would result in an apparent enhancement of the fMLP response through the lowering of basal values. Although a decrease in basal activities was observed for some cells coexpressing α_i subunits, it was not a general observation. Because it is difficult to assess by immunodetection the level of expression of α_s-Q227L over a massive background level of endogenous α_s, we attempted to address this issue by indirect means. It has previously been demonstrated that, under the control of the cytomegalovirus promoter, both wild-type and constitutively activated, mutant α subunits could be expressed to comparable levels in 293 cells (16, 17). Because the activated mutant α subunits have impaired GTPase activity, they are maintained in the GTP-bound state and constitutively regulate their corresponding effectors but presum-

ably cannot interact productively with receptors and $\beta\gamma$ subunits. Thus, these mutant α subunits could serve as controls for protein synthesis competition.

To test the possibility that the expression of α_s-Q227L was reduced through competition by other α subunits, we co-transfected 293 cells with cDNAs encoding the type II adenylyl cyclase, α_s-Q227L, and the fMLP receptor, in the absence or presence of various mutationally activated α subunit cDNAs. Compared with the control cells, neither basal nor fMLP-stimulated type II adenylyl cyclase activities were significantly affected in cells coexpressing either α_{i1}-Q204L, α_{i2}-Q205L, α_{i3}-Q204L, α_{o1}-Q205L, α_q-R183C, or α_z-Q205L (Fig. 4B). The lack of inhibition of the basal activities suggested that the expression of α_s-Q227L was not significantly impaired by the coexpression of other α subunits. Moreover, loss of enhancement of the fMLP response reaffirmed the inability of the mutant α subunits to be regulated by receptors.

A change in the level of expression of the fMLP receptor could explain the observed enhancement of the fMLP response by the different α subunits. The abundance of the fMLP receptor in the transfected cells was therefore determined by binding assays using [^{3}H]fMLP as the ligand. Because the coexpression of type II adenylyl cyclase and α subunits may affect the affinity as well as the B_{max} of fMLP receptors, the binding studies were performed with a ligand concentration near saturation (10 nM), at which the specific binding activity is sufficiently high to reflect the changes. As shown in Table 1, no specific binding was observed in membranes prepared from cells transfected with the vector (pcDNAI) alone, indicating that the 293 cells do not express the fMLP receptor endogenously. Membranes derived from cells expressing the fMLP receptor specifically bound [^{3}H]fMLP at a level comparable to those reported in similar studies (Table 1). Coexpression of type II adenylyl cyclase and α_s-Q227L with the fMLP receptor did not significantly alter the amount of [^{3}H]fMLP bound to the membranes. Furthermore, inclusion of cDNAs encoding the various α subunits did not significantly affect the specific binding of [^{3}H]fMLP (Table 1).

TABLE 1

Specific binding of [^{3}H]fMLP to membranes prepared from various transfected cells

Membranes prepared from 293 cells that had been transfected with the indicated constructs were assayed for specific binding of [^{3}H]fMLP (10 nM) as described in Materials and Methods. Values represent the mean ± standard error of triplicate determinations in three or more independent experiments. No significant difference was observed in the specific binding of [^{3}H]fMLP to membranes from cells expressing the fMLP receptor alone or those expressing additional constructs (Students t test, $p > 0.1$).

Transfection[a]	[^{3}H]fMLP bound
	pmol/mg
pcDNAI	NS[b]
fMLP-R	4.54 ± 0.83
fMLP-R + AC II + α_s-Q227L	5.58 ± 0.55
fMLP-R + AC II + α_s-Q227L + α_{i1}	4.90 ± 0.58
fMLP-R + AC II + α_s-Q227L + α_{i2}	6.47 ± 1.27
fMLP-R + AC II + α_s-Q227L + α_{i3}	4.17 ± 0.38
fMLP-R + AC II + α_s-Q227L + α_{o1}	9.29 ± 3.77
fMLP-R + AC II + α_s-Q227L + α_z	4.70 ± 1.28
fMLP-R + AC II + α_s-Q227L + α_q	5.73 ± 3.45
fMLP-R + AC II + α_s-Q227L + α_t	3.75 ± 1.26

[a] fMLP-R, cDNA encoding the fMLP receptor; AC II, cDNA encoding type II adenylyl cyclase.
[b] NS, no specific binding was detected.

These results indicate that the expression of fMLP receptors was unaffected by the coexpression of type II adenylyl cyclase and α subunits. Although the absolute values for the [^{3}H]fMLP binding varied among the different membranes preparations (ranging from 3.75 ± 1.26 to 9.29 ± 3.77 pmol/mg and probably reflecting variations in the efficiency of transfection, which was approximately 40–50%), no correlation could be found between the apparent α subunit-mediated potentiation of fMLP responses (Fig. 4A) and the abundance of fMLP receptors (Table 1).

It has been reported that G_q-coupled receptors can stimulate type II adenylyl cyclase (22) via the actions of PKC. Although the fMLP receptor can potently activate the PI-PLC system in PMNs, it cannot stimulate the formation of inositol phosphates when expressed in 293 cells.[2] Similar findings have also been reported by others (23). The lack of coupling to PI-PLC in 293 cells suggests that the fMLP receptor does not stimulate type II adenylyl cyclase via PKC. We therefore examined whether the fMLP response can synergize with PKC in activating type II adenylyl cyclase. 293 cells were co-transfected with cDNAs encoding the fMLP receptor, type II adenylyl cyclase, and α_s-Q227L and then assayed for cAMP accumulation in the absence or presence of 100 nM PMA. As shown in Fig. 5, a 15-min incubation with PMA markedly increased the basal cAMP accumulation but did not abolish the ability of fMLP to stimulate type II adenylyl cyclase. It appears that the pathway utilized by the

[2] R. C. Tsu, R. A. Allen, and Y. H. Wong, unpublished observations.

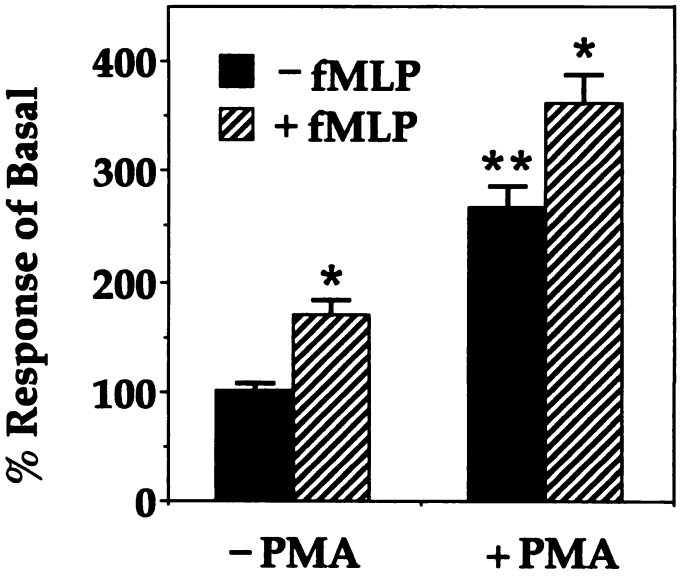

Fig. 5. Effects of PMA on basal and fMLP-stimulated activities of type II adenylyl cyclase. The 293 cells were co-transfected with type II adenylyl cyclase cDNA (in pcDNAI; 0.25 μg/ml), α_s-Q227L (0.025 μg/ml), and fMLP-pCDM8 (0.25 μg/ml). Transfected cells were pretreated with or without 100 nM PMA for 15 min at 37° before cAMP accumulation was assayed as described in the legend to Fig. 1. Results are expressed as a percentage of basal cAMP formation (the ratio of cAMP to total adenine nucleotides was 4.72×10^{-3}) in the absence of fMLP or PMA. *, fMLP-stimulated cAMP accumulation was significantly different from that observed in its absence (paired Bonferroni t test, $p < 0.05$). **, PMA significantly stimulated the cAMP formation (paired Bonferroni t test, $p < 0.05$). The data represent triplicate determinations in a single experiment; two independent experiments yielded similar results.

fMLP receptor to stimulate type II adenylyl cyclase does not involve PKC.

Discussion

The chemoattractant receptors belong to a family of G protein-coupled multifunctional receptors. Among the diverse signaling pathways linked to the chemoattractant receptors, regulation of intracellular cAMP levels is the most obscure and perhaps the least significant pathway. In PMNs, where the chemoattractant receptors are found, activation of these receptors produces a modest and transient increase in intracellular cAMP levels (24). This response has been postulated to be an indirect effect due to the release of adenosine (11) or inhibition of phosphodiesterase (12). It was discovered only recently that the chemoattractant receptors may play a more direct role in the control of intracellular cAMP levels. Both fMLP (9, 10) and C5a[3] receptors have been shown to mediate inhibition of adenylyl cyclase, which is a hallmark of G_i-coupled receptors. Because the HL-60 cells are known to express both G_{i2} and G_{i3} (25) and both of these G_i proteins can inhibit adenylyl cyclase (16, 26), one might expect the cAMP level to be lowered in response to activation of chemoattractant receptors. The observed variance may be accounted for by the remarkably complex regulatory schemes for the various isoforms of adenylyl cyclase that have been discovered to date.

The transient increase in cAMP accumulation in response to chemoattractants might be explained if type II or type IV adenylyl cyclase is expressed in PMNs. Type II adenylyl cyclase is stimulated by activated α_s, and $\beta\gamma$ subunits can produce a synergistic stimulation (14). Although type II adenylyl cyclase can also be stimulated by the activation of PKC, the observed responses to fMLP are unlikely to be mediated via such a pathway, because chemotactic factors cannot induce the formation of inositol phosphates in transfected 293 cells (23).[4] Activation of type II adenylyl cyclase by G protein $\beta\gamma$ subunits represents a mechanism by which classical inhibitory receptors (e.g., α_2-adrenoceptor) stimulate the formation of cAMP (15). In the present study we did not address the issue of which isoform of adenylyl cyclase is expressed in PMNs. Instead, we examined the ability of fMLP and C5a receptors to stimulate type II adenylyl cyclase via the $\beta\gamma$ subunits of G_i. Although it is not totally unexpected that the fMLP and C5a receptors can stimulate type II adenylyl cyclase via the activation of G_i proteins, it confirms that, depending on the intracellular environment, the activation of chemoattractant receptors can lead not only to a decrease but also to an increase in cAMP levels. This finding also suggests the possibility that the chemoattractant-induced stimulation of cAMP production in PMNs is due to the presence of type II or type IV adenylyl cyclase. Of the two isoforms, type II adenylyl cyclase is primarily expressed in the brain (27), whereas type IV adenylyl cyclase appears to be widely distributed (28). It is tantalizing to imagine that the chemotactic factors can collaborate with other hormonal signals acting on G_s-coupled receptors to regulate the levels of intracellular cAMP.

The chemoattractant-induced stimulation of type II adenylyl cyclase is apparently mediated by the PTX-sensitive G_i/G_o proteins. Both fMLP and C5a receptors can couple to multiple G proteins. Besides coupling to G_i proteins, both receptors are able to utilize G_z to inhibit adenylyl cyclase.[5] Additionally, the C5a receptor can interact with G_{16} to stimulate PI-PLC (23). However, neither receptor was able to activate G_s, as demonstrated in the present study. The chemoattractant receptors thus appear to resemble the dopamine D_2 receptor more than the promiscuous α_2-adrenoceptor.

The capacity of G_z to mediate the chemoattractant-induced stimulation of type II adenylyl cyclase supports earlier findings of G_z coupling to G_i-linked receptors (17). It is interesting to note that, upon coexpression of α_z, the chemoattractant-stimulated type II adenylyl cyclase activity was significantly enhanced. The potentiation is probably due to the recruitment of α_z-associated $\beta\gamma$ subunits and not a change in the expression of the fMLP receptor. Similarly, the enhancement of fMLP-induced stimulation of type II adenylyl cyclase activity by the coexpression of α_i and α_o subunits implies a functional coupling between the receptor and the α subunits. Such an interpretation is supported by the fact that coexpression of either α_q or α_t produced an inhibitory effect instead. The inhibitions seen with α_q and α_t are presumably due to their ability to complex with free $\beta\gamma$ subunits that have been released by activated fMLP receptors, thereby abrogating the stimulatory actions of $\beta\gamma$ subunits on type II adenylyl cyclase. There is substantial evidence, including the present study, that suggests a functional relationship between the fMLP receptor and G_{i2} and G_{i3} (25). Thus far, no differences in the capacity of G_{i1}, G_{i2}, and G_{i3} to regulate effectors such as adenylyl cyclases and potassium channels have been observed (26, 29). It is therefore extremely likely that the fMLP receptor can functionally interact with G_{i1}. Possible association between the fMLP receptor and G_{o1} is also implicated in the present study. G_{o1} couples a variety of G_i-linked receptors (e.g., the muscarinic m2 receptor) to the inhibition of Ca^{2+} influx (30). Although there is little or no evidence in support of fMLP-induced inhibition of Ca^{2+} channels, it remains possible that G_{o1} can mediate other responses to fMLP. Of obvious interest is the likely involvement of G_{o1} in the fMLP-induced stimulation of PI-PLC. Indeed, G_{o1} has been shown to stimulate PI-PLC in *Xenopus* oocytes (31). A functional interaction between G_{o1} and the fMLP receptor is a tantalizing idea that remains to be proven.

The coexpression studies utilizing mutationally activated α subunits provide supportive evidence for the specificity of G_i-mediated inhibition of adenylyl cyclase. It has been previously shown that the mutant α subunits of G_{i1}, G_{i2}, G_{i3}, and G_z constitutively inhibit cAMP accumulation in 293 cells (17). The 293 cells express a type III-like isoform of adenylyl cyclase (21). Type III adenylyl cyclase is similar to type I in that it is activated by Ca^{2+}/calmodulin and inhibited by all three forms of G_i proteins (32). In contrast, type II adenylyl cyclase is practically unresponsive to inhibition by G_i proteins (32). Hence, in the present study the coexpression of mutationally activated α subunits of G_{i1}, G_{i2}, and G_{i3} did not attenuate the fMLP-induced stimulation of type II adenylyl cyclase activities. Likewise, coexpression of α_z-Q205L did not

[3] J. K. S. Shum, R. A. Allen, and Y. H. Wong, unpublished observations.
[4] R. C. Tsu, R. A. Allen, and Y. H. Wong, unpublished observations.
[5] R. C. Tsu, J. K. S. Shum, R. A. Allen, and Y. H. Wong, unpublished observations.

affect the type II adenylyl cyclase responses to fMLP. These results indicate that α_z and α_i subunits are very similar in terms of their lack of inhibitory control of type II adenylyl cyclase. In contrast, it has been shown that activation of a G_q-regulated signaling pathway can enhance hormone-stimulated type II adenylyl cyclase responses (22), presumably by activating PKC. Such cooperative actions were similarly reproduced in the present study, because 100 nM PMA, an activator of PKC, significantly enhanced basal and fMLP-mediated cAMP accumulation. Although no significant enhancement of the fMLP-induced type II adenylyl cyclase response was observed in cells co-transfected with α_q-R183C, our results are not incompatible with previous findings. The constitutive activation of PI-PLC by α_q-R183C would lead to the prolonged activation and subsequent down-regulation of PKC. Indeed, down-regulation of PKC by phorbol esters can abolish the G_q-mediated enhancement of type II adenylyl cyclase responses (22).

In summary, the present study has demonstrated that both fMLP and C5a receptors are capable of stimulating type II adenylyl cyclase in a PTX-sensitive manner. With respect to the fMLP receptor, possible interactions with G_{i1}, G_{i2}, G_{i3}, G_{o1}, and G_z have been implicated. On the other hand, it seems unlikely that the fMLP receptor can interact with either G_s, G_q, or G_t. The significance of having the ability to activate multiple G proteins is unclear but may account for the diverse effects of the chemotactic factors.

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