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THE HUMAN CHEMOATTRACTANT COMPLEMENT C5a RECEPTOR INHIBITS CYCLIC AMP ACCUMULATION THROUGH G_i AND G_z PROTEINS

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The human C5a receptor is known to signal through G_i proteins. The ability of the cloned C5a receptor to inhibit adenylyl cyclase or to stimulate phospholipase C through G_i proteins was examined in transfected cells. Activation of recombinant C5a receptors resulted in the stimulation of phospholipase C in Ltk$^-$ cells and inhibition of adenylyl cyclase in 293 cells. Pertussis toxin potently abolished both responses indicating the involvement of G_i proteins. Previous studies have shown that G_i-mediated inhibition of adenylyl cyclase can be similarly regulated by the pertussis toxin-insensitive G_z. In 293 cells co-transfected with the α subunit of G_z, the C5a-mediated inhibition of cAMP accumulation became pertussis toxin-resistant, signifying functional coupling between the C5a receptor and G_z. However, G_z cannot substitute for G_i in the C5a-induced stimulation of phospholipase C or inhibition of adenylyl cyclase in Ltk$^-$ cells.

C5a is a chemoattractant anaphylatoxin known to aggregate polymorphonuclear leukocytes (PMNs) and to generate superoxide radicals in these cells. The recent cloning of the C5a receptor from U937 and HL-60 cells (1,2) provides an opportunity to study the signalling pathway(s) leading to these events. The cloned C5a receptor belongs to the rhodopsin superfamily of G protein-coupled receptors. Attempts to delineate the signal transduction pathways of the cloned C5a receptor have unveiled an association between the receptor and G_{16} (3,4), a pertussis toxin (PTX) insensitive G

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The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; hCG, human choriogonadotropin; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; IBMX, 1-methyl-3-isobutylxanthine; IP, inositol phosphate, regardless of the number of phosphate groups; LHR, luteinizing hormone receptor; MEM, minimum essential medium; PI-PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin; PMNs, polymorphonuclear leukocytes.

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protein belonging to the G_q/G_{11} class which stimulates phosphoinositide-specific phospholipase C (PI-PLC). This signalling pathway is unusual because both C5a and *N*-formylmethionyl-leucyl-phenylalanine (fMLP) receptors are known to signal through G_i-like proteins in PMNs (5,6). Recent studies have established that the fMLP receptor is indeed capable of inhibiting adenylyl cyclase via G_i proteins. Expression of the human fMLP receptor in human embryonic kidney 293 cells and insulin secreting cells allowed the chemotactic factor to attenuate cAMP formation in a PTX-sensitive manner (7,8). Like the fMLP receptor, the C5a receptor utilizes G_i proteins in their signal transduction pathways (5). Although sequence homology exists between the fMLP and C5a receptors, inhibition of adenylyl cyclase has yet to be examined within the signalling cascade of the C5a receptor. To further study the relationship between the C5a receptor and G_i proteins in signal transduction, we examined the ability of the human C5a receptor to inhibit adenylyl cyclase and to stimulate PI-PLC in transient transfection studies. Our results indicate that, like the fMLP receptor, the human C5a receptor can interact with G_i proteins to inhibit adenylyl cyclase and stimulate PI-PLC. Moreover, the C5a receptor has the capacity to couple to another G protein, G_z, leading to a PTX-insensitive inhibition of cAMP accumulation in 293 cells but not in Ltk$^-$ fibroblasts.

MATERIALS AND METHODS

Materials: The human C5a receptor cDNA (in the pEE6hCMV.neo mammalian vector was subcloned into pcDNAI) (9) was a gracious gift from Dr. Michael D. Barker (Krebs Institute, UK). Human choriogonadotropin (hCG) was provided by the National Pituitary Agency. The sources of the following reagents are given in parenthesis: pcDNAI (Invitrogen), Geneclean II kit (Bio 101, Inc.), PTX (List Biological Laboratories), radiochemicals and ECL kit (Amersham Corp), cell culture media (Life Technologies, Inc.), DNA columns (Qiagen), and all other chemicals (Sigma).

Transient transfection of 293 and Ltk$^-$ cells: The human embryonic kidney 293 cells (ATCC CRL-1573) were kept in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin, and in 5% CO_2. 293 cells were transfected with various cDNAs by the DEAE-Dextran method as described previously (10). Ltk$^-$ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, antibiotics (same as in 293 cells), and in 5% CO_2. Transfection of Ltk$^-$ cells was similar to that of the 293 cells (10) except the cells were transfected for 4 h in the presence of 200 μg/ml DEAE-Dextran without chloroquine.

Assay for cAMP accumulation and inositol phosphate formation: 293 cells were labelled with [^{3}H]adenine (1 μCi/ml) in MEM with 1% FCS. When needed, PTX (100 ng/ml) was added simultaneously. 18-24 h later, the cells were assayed in 20 mM Hepes-buffered MEM containing the appropriate drugs and 1 mM IBMX for 30 min at 37°C. Intracellular [^{3}H]cAMP was determined by sequential chromatography (10,11). Transfected Ltk$^-$ cells were labelled with [^{3}H]*myo*-inositol (2.5 μCi/ml) in DMEM containing 5% FCS for 48 h. PTX (100 ng/ml) was added 24 h after labelling. Cells were assayed in 20 mM Hepes-buffered DMEM containing 20 mM LiCl. The formation of [^{3}H]inositol phosphate (IP) in response to recombinant C5a peptide (1h, 37°C) was determined according to established protocols (12). The intracellular cAMP contents in transfected Ltk$^-$ cells were determined by cAMP assay kit from Amersham Corp.

Immunodetection of α_z expression: Membranes were prepared from Ltk$^-$ cells transfected with cDNAs encoding the C5a receptor with or without the α_z-pcDNAI as previously described (13). For each sample, 150 μg of membrane proteins were separated on a 12.5% polyacrylamide SDS gel and electrophorectically transferred to PVDF membranes. Immunodetection of α_z by the α_z-specific antiserum P-961 (14) was visualized by chemiluminescence.

RESULTS

The 293 cells are known for their efficient expression of exogenous G protein subunits (12,13) and cloned receptors, including the C5a and fMLP receptors (4,7,15). When 293 cells were co-transfected with cDNAs encoding the rat luteinizing hormone receptor (LHR-pCIS) and the human C5a receptor (C5aR-pcDNAI), cAMP accumulation was increased in response to the LHR agonist, hCG. The hCG response was significantly inhibited (>50%) in the presence of 100 nM of recombinant C5a (Fig. 1A). Inhibition of cAMP accumulation by C5a was dependent on the amount of C5aR-pcDNAI used in the transfections (Fig. 1B) and was not seen in cells transfected with LHR-pCIS alone (Fig. 1A). The C5a response was mediated by G_i proteins since it was abolished in cells pretreated with PTX (Fig. 1A). We next examined if the C5a-mediated inhibition of cAMP accumulation was saturable by agonist. As shown in Figure 1C, the addition of between 1 pM and 100 nM recombinant C5a led to a concentration-dependent inhibition of the hCG response, with a maximal inhibition of 50 to 60%. The EC_{50} of C5a for inhibiting cAMP accumulation was around 0.03 nM.

C5a is known to stimulate PI-PLC through G_i proteins in PMNs (6). The ability of the cloned human C5a receptor to regulate PI-PLC in a PTX-sensitive manner was examined in Ltk$^-$ cells, where receptor-mediated activation of PI-PLC via G_i proteins have been demonstrated (16,17). Ltk$^-$ cells were transfected with C5aR-pcDNAI, labelled with [^{3}H]*myo*-inositol, and assayed for IP production. 100 nM of C5a significantly increased the formation of IP over the basal and this response was not seen in mock-transfected Ltk$^-$ cells (Fig. 2A). PTX completely abolished the C5a-induced IP formation (Fig. 2A). This G_i-mediated C5a response was not as robust as that obtained with G_q-mediated stimulation of PI-PLC, but was analogous to those seen with the activation of dopamine-D_2 and 5-HT_{1A} receptors (16,17). Responses to 100 nM of recombinant C5a increased with increasing concentrations of C5aR-pcDNAI used in the transfections (Fig. 2B). In transfected Ltk$^-$ cells, the EC_{50} of C5a for stimulating PI-PLC was around 0.3 nM (Fig. 2C).

As the C5a receptor has the capacity to couple to PTX-insensitive G proteins such as G_{16} (4), we sought to test whether G_z represents yet another potential partner for the receptor. PTX-insensitive G_z has been shown to inhibit cAMP accumulation in 293 cells by coupling to the α_2-adrenergic, dopamine-D_2, and adenosine-A_1 receptors (18). Hence, 293 cells were co-transfected with LHR-pCIS and C5aR-pcDNAI in the absence or presence of α_z-pcDNAI. In 293 cells co-expressing α_z, the hCG response was significantly inhibited by 100 nM of C5a even after the endogenous α_i subunits

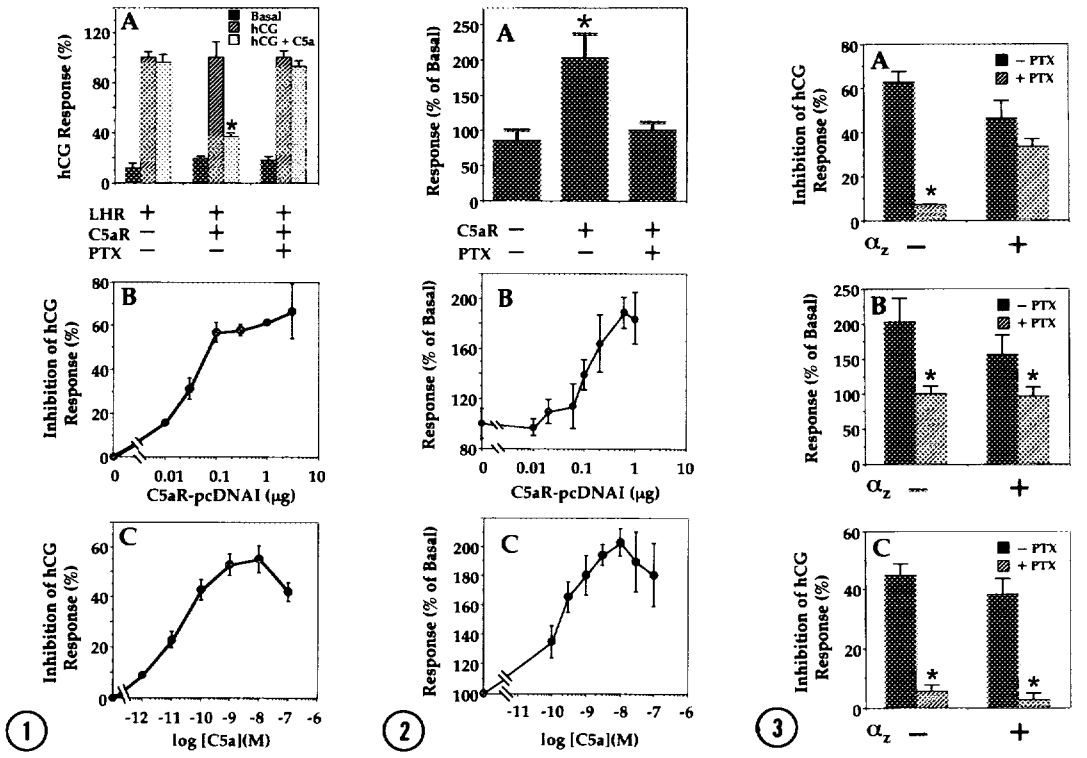

Fig. 1. C5a-mediated inhibition of cAMP accumulation in transfected 293 cells. *(A)* 293 cells were transfected with LHR-pCIS (0.15 μg/ml) and C5aR-pcDNAI (0.25 μg/ml) and treated with PTX as indicated. Transfectants were assayed for cAMP responses to hCG (5 ng/ml) with or without C5a (100 nM). Results are expressed as a percentage of the hCG response. *(B)* Cells were transfected with LHR-pCIS (0.15 μg/ml) and up to 3 μg/ml of C5aR-pcDNAI and then assayed as in *A*. *(C)* Cells were transfected and assayed as in *A* except with varying concentrations of C5a. For *B* and *C*, results are expressed as % inhibition of the hCG response. Data shown represent the mean ± S.D. of triplicate determinations in a representative experiment (n=3). * C5a significantly reduced the hCG-stimulated activity; paired Bonferroni t-test, P<0.05.

Fig. 2. C5a-mediated stimulation of IP formation in transfected Ltk⁻ cells. *(A)* Ltk⁻ cells were transfected with or without C5aR-pcDNAI (0.25 μg/ml), treated with PTX and assayed for IP formation in response to 100 nM C5a as indicated. *(B)* Cells were transfected with up to 3.0 μg/ml of C5aR-pcDNAI and assayed as in *A*. *(C)* Cells were transfected and assayed as in *A* except with varying concentrations of C5a. Results are expressed as a percentage of the basal response. Data represent the mean ± S.D. of triplicate determinations in a representative experiment (n=3). * C5a significantly stimulated the production of IP; paired Bonferroni t-test, P<0.05.

Fig. 3. C5a receptor coupling to the PTX-insensitive α_z. *(A)* 293 cells were co-transfected with 0.15 μg/ml of LHR-pCIS and 0.25 μg/ml of C5aR-pcDNAI with or without 0.125 μg/ml of α_z-pcDNAI and treated with PTX as shown. Transfectants were then assayed for cAMP accumulation as in Fig. 1. Results are expressed as % inhibition of the hCG-stimulated activity in the presence of C5a, compared to that measured in the presence of hCG alone. *(B)* Ltk⁻ cells were transfected as in *A* but without the LHR-pCIS. Transfectants were assayed for IP formation as in Fig. 2. Results are expressed as % stimulation of IP production as compared to basal activity. *(C)* Ltk⁻ cells were transfected as in *A* and the intracellular cAMP levels were determined with a cAMP assay kit. Results are expressed as a percentage of the hCG response. Data represent the mean ± S.D. of triplicate determinations in a representative experiment (n=3). * PTX significantly reduced the ability of C5a to inhibit hCG-stimulated activity *(panels A* and *C)* or stimulate the production of IP *(panel B)*; paired Bonferroni t-test, P<0.05.

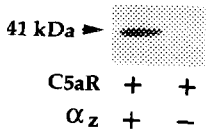

Fig. 4. **Immunodetection of α_z in transefected Ltk$^-$ cells.** Ltk$^-$ cells were transfected with C5aR-pcDNAI in the absence or presence of α_z-pcDNAI as in Fig. 3B. Membranes prepared from the transfected cells were resolved by SDS-PAGE on a 12.5% polyacrylamide gel and subsequently analysed by immunoblotting with the α_z-specific antiserum P-961.

were incapacitated by PTX (Fig. 3A). In contrast, PTX effectively abolished the C5a-mediated inhibition of the hCG response in cells that did not co-express α_z (Fig. 3A). These results suggest that in addition to G_i and G_{16}, G_z can also couple to the C5a receptor. We next addressed whether G_z can similarly replace G_i in the C5a-mediated stimulation of PI-PLC. Ltk$^-$ cells were transfected with C5aR-pcDNAI in the absence or presence of α_z-pcDNAI. Unlike the results obtained with inhibition of cAMP accumulation, co-expression of α_z did not confer upon the Ltk$^-$ cells the ability to respond to C5a in a PTX-resistant manner (Fig. 3B). In Ltk$^-$ cells co-expressing α_z, PTX effectively abolished the C5a-induced formation of IP, indicating that G_z is unable to replace G_i in the regulation of PI-PLC. Moreover, in contrast to the results observed in 293 cells, co-expression of α_z did not confer upon the transfected Ltk$^-$ cells the ability to mediate C5a-induced inhibition of cAMP accumulation in a PTX-insensitive manner (Fig. 3C). Nonetheless, expression of α_z in the transfected cells was confirmed by immunodetection with the α_z-specific antiserum P-961 (Fig. 4).

DISCUSSION

Although it is generally accepted that the C5a receptor is coupled to G_i proteins, little is known with regard to its regulation of the second messenger cAMP. Activation of the C5a receptor has been reported to mildly stimulate the formation of cAMP and this action may be mediated indirectly through inhibition of phosphodiesterase (19) or release of endogenous adenosine (20). Recent demonstrations of fMLP-induced inhibition of cAMP accumulation in two different cell lines (7,8) suggest that the same may hold true for the C5a receptor. In this report we have demonstrated that the C5a receptor can indeed inhibit adenylyl cyclase via G_i proteins.

The efficacy at which C5a inhibits cAMP accumulation in 293 cells is comparable to that seen with C5a-induced calcium mobilization (15). The EC_{50} values for both types of reponses are in the sub-nanomolar range. Inasmuch as C5a can efficiently inhibit adenylyl cyclase, it is hard to comprehend the lack of such a response in PMNs. The answer may lie in the cell-specific expression of the different isoforms of adenylyl

cyclase known to date (21). Activation of members of three major classes of G proteins (G_s, G_i, and G_q) may directly or indirectly lead to stimulation or inhibition of the different isoforms of adenylyl cyclase. 293 cells express a type III-like adenylyl cyclase (22) which can be inhibited by activated α_i subunits. It remains possible that PMNs lack α_i-sensitive isoforms (e.g. type II or IV) of adenylyl cyclase.

Apart from inhibition of adenylyl cyclase, activation of G_i proteins by the human C5a receptor can lead to stimulation of PI-PLC as illustrated by the studies in Ltk$^-$ cells. Stimulation of PI-PLC by the C5a receptor can apparently proceed along two distinct pathways. Firstly, in cells co-expressing G_{16}, activated C5a receptors can stimulate PI-PLC via α_{16}. Alternatively, agonist-bound C5a receptors can activate their corresponding G proteins to release $\beta\gamma$ subunits which can then stimulate specific isoforms of PI-PLC. The latter mechanism may form the basis of C5a-induced IP formation in Ltk$^-$ cells. Indeed, it has been shown that the PLC β_2 isoform present in HL-60 cells can be activated by $\beta\gamma$ subunits (23). Although our results did not directly address the issue of whether the C5a-induced IP formation was mediated via G protein $\beta\gamma$ subunits, supportive indications can be noted. Consistent with the notion that $\beta\gamma$-regulated effector pathways require much higher concentrations of agonists, the EC_{50} value for C5a-mediated stimulation of PI-PLC is 10-fold higher than that obtained with inhibition of adenylyl cyclase. Moreover, co-expression of $\beta_2\gamma_2$ subunits in Ltk$^-$ cells significantly increased the formation of IP[1].

As the IL-8 receptor is known to interact with G_{14}, G_{15}, and G_{16} to activate PI-PLC in COS-7 cells (24), it is not surprising that the C5a receptor can couple to G_z in addition to G_i and G_{16}. Many inhibitory receptors which utilize G_i to inhibit adenylyl cyclase can similarly inhibit the enzyme via G_z (18). We predict that G_z can functionally interact with most receptors which display a strong inhibitory influence on adenylyl cyclase. The significance of C5a receptor coupling to G_z is not immediately apparent. It is noteworthy that both C5a receptor and G_z are expressed in basophilic cells (25,26). Whether G_z plays a role in C5a-induced anaphylatoxic reactions remains to be elucidated. The inability of G_z to substitute for G_i in the C5a-mediated stimulation of PI-PLC and inhibition of cAMP accumulation is rather intriguing. The lack of C5a receptor coupling to α_z was not due to a lack of α_z expression (Fig. 4). The reasons behind the exclusion of G_z from C5a-mediated responses in Ltk$^-$ cells are not clear. It appears that the coupling of the C5a receptor to G_z is cell type-dependent. One plausible explanation relates to the specificity in forming the G protein heterotrimers. If the C5a receptor can only bind to a specific combination of $\alpha_z\beta\gamma$, and the specific $\beta\gamma$ complex is absent in Ltk$^-$ cells, then no productive coupling can be achieved with G_z. There is precedence in specific interactions between G protein heterotrimers and their receptors where the exact permutation of $\beta\gamma$ complex is important (27).

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[1]Tsu, R., Allen, R. A. and Wong, Y.H. (Unpublished results).

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