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Coupling of the C5a Receptor to G_i in U-937 Cells and in Cells Transfected with C5a Receptor cDNA

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SUMMARY

The signaling properties of the receptor for the chemoattractant C5a (C5aR) were investigated in differentiated U-937 cells and in NIH/3T3 cells transfected with the C5aR. In both U-937 cells and transfected cells (2A3 cells), C5a induced the mobilization of intracellular calcium, phosphoinositide breakdown, and activation of mitogen-activated protein kinase. In addition, in 2A3 cells C5a induced the inhibition of forskolin-stimulated cAMP generation. Pretreatment with pertussis toxin suppressed all C5a-mediated signal transduction in both cell lines. In the presence of cholera toxin, C5a induced the ribosylation of a 39–40-kDa protein in membranes of both U-937 cells and 2A3 cells. Similar phenomena have been described in other systems, whereby $G_{i\alpha}$ subunits are substrates for cholera toxin-induced ribosylation in the presence of receptor agonists. Moreover, the C5a-induced ribosylation was eliminated in membranes of cells that had been pretreated with pertussis toxin. The G protein α subunit $G_{\alpha 16}$, which is insensitive to pertussis toxin, has been reported to couple selectively to C5aR in cells co-transfected with C5aR and $G_{\alpha 16}$ cDNAs. $G_{\alpha 16}$ expression was not detected in U-937 cells or in 2A3 cells, either by reverse transcription-polymerase chain reaction or by immunoblotting. Because pertussis toxin modifies only G_α subunits of the $G_{i/o}$ family and all signaling by C5aR was abolished by pertussis toxin pretreatment, the results strongly suggest that, in U-937 and 2A3 cells, C5a-mediated responses can be accounted for entirely through coupling with G proteins of the G_i subtype.

Chemotactic factors amplify inflammatory responses by directing the migration of phagocytic cells. One of these factors is the anaphylatoxin C5a, which is generated by cleavage of the complement component C5 by specific C5 convertases. C5a interacts with specific receptors on plasma membranes of neutrophils, macrophages, and other blood cells. The C5aR has been cloned and sequenced (1, 2) and has been shown to belong to the family of receptors that tranduce their signals through G proteins. Similarly to other chemoattractant receptors, activation of C5aR results in stimulation of PLC, with subsequent increase in $[Ca^{2+}]_i$, activation of protein kinase C, superoxide generation, and release of enzymes from neutrophils (3).

Identification of the proteins involved in the signaling mechanisms through C5aR is still unresolved. Seemingly, a PLC of the β subtype, which is the only PLC subtype sensitive to G protein activation (4), must be involved. The nature of the G protein that mediates C5a-induced PLC stimulation is unclear. PTX sensitivity has been described for C5a-mediated stimulation of neutrophils (5) and basophils (6), suggesting that the G protein involved belongs to the G_i subfamily. Furthermore, purification of C5aR from human neutrophils results in a complex of C5aR with G_i (7). On the other hand, expression of C5aR in COS cells (8) and 293 cells (9) leads to C5a-mediated PLC stimulation only if $G_{\alpha 16}$ is co-transfected with C5aR, suggesting that $G_{\alpha 16}$ is required for C5aR signaling.

In the present study, signaling mechanisms for C5aR were investigated in differentiated U-937 cells and in NIH/3T3 cells transfected with human C5aR. The data presented herein indicate that in U-937 cells C5aR is coupled with a G protein that is sensitive to PTX. Such coupling can account for all of the signaling mechanisms observed with C5aR.

Experimental Procedures

Materials

CTX and PTX were from List Laboratories (Campbell, CA). GTPγS was from Boehringer Mannheim (Indianapolis, IN). [^{3}H]Inositol was from Amersham (Arlington Heights, IL). [^{32}P]NAD was from DuPont (Boston, MA). Fura-2/AM was from Molecular Probes (Eugene, OR). Cell culture media, HBSS, and G-418 (Geneticin) were from GIBCO (Gaithersburg, MD). Streptolysin O was from Murex Diagnostics (Dartford, UK). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

U-937 cells were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI 1640 medium con-

ABBREVIATIONS: C5aR, C5a receptor(s); PLC, phospholipase C; CTX, cholera toxin; PTX, pertussis toxin; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; IP_3, inositol trisphosphate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; $[Ca^{2+}]_i$, intracellular calcium concentration; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; RT, reverse transcription; AM, acetoxymethyl ester; MAP, mitogen-activated protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

taining 10% fetal calf serum and antibiotics (penicillin and streptomycin). U-937 cells were differentiated by the addition of 1 mM dibutyryl-cAMP in the culture medium for 3–4 days. Wild-type and transfected NIH/3T3 cells were cultured in DMEM containing 10% calf serum and antibiotics. In addition, transfected cells were grown in the presence of 500 μg/ml G-418.

Amplification of C5aR cDNA, Subcloning, and Transfection in NIH/3T3 Cells

Oligonucleotide primers with sequences corresponding to the 5′ and 3′ ends of the C5aR reading frame (1) were synthesized with an automated synthesizer, by solid-phase synthesis. Total RNA from differentiated U-937 cells was obtained by using a commercial kit (Promega, Madison, WI). First-strand cDNA was synthesized utilizing a commercial kit (Pharmacia, Piscataway, NJ), using 1–5 μg of total RNA from U-937 cells as a template and oligo(dT) (provided with the kit) as a primer. An aliquot of the first-strand cDNA synthesis was used for C5aR cDNA amplification by PCR. PCR incubations contained 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.02 units/reaction of *Thermus aquaticus* polymerase, 50 mM KCl, 10 mM Tris, pH 9, 0.1% Triton X-100, 1 μM concentrations of each primer, and a 1-μl aliquot of the first-strand cDNA synthesis reaction. Temperature cycling was as follows: one cycle of 2 min at 94°, followed by 35 cycles of 30 sec at 94°, 30 sec at 55°, and 90 sec at 72°, with a final extension cycle of 7 min at 72°. The 1-kilobase fragment amplified was subcloned into the PCR II vector (Invitrogen, San Diego, CA) and sequenced using a commercial kit (*fmol*; Promega, Madison, WI) based on the dideoxynucleotide termination procedure.

Expression of C5aR in NIH/3T3 Cells

Utilizing standard cloning techniques, C5aR cDNA was inserted into the *Bam*HI site of the expression vector pZip Neo SV(X) (10). The size and orientation of the insert were determined by restriction mapping. The construct was transfected into NIH/3T3 cells using the Lipofectamine system (GIBCO). Transfected cells were selected by culturing in the presence of 1.0 mg/ml G-418. Expression of C5aR was monitored in mass cultures and in individual clones by binding of ^{125}I-C5a.

Binding of ^{125}I-C5a to Transfected Cells

Cells were subcultured in 24-well plates, at a density of 5×10^5/well, and incubated overnight in regular medium. The medium was then aspirated, and the cells were rinsed twice with 1 ml of binding buffer (1× PBS containing 0.2% bovine serum albumin and 0.15% bacitracin). Cells were incubated for 2 hr at 4° with binding buffer containing 0.1 μCi/ml (70 pM) ^{125}I-C5a, with continuous rocking. Nonspecific binding was determined by the addition of 20 nM unlabeled C5a. Cells were then rinsed three times with 1 ml of binding buffer. Then 500 μl of 1 N NaOH were added to each well, and the wells were incubated for 30 min at room temperature. Lysates were transferred to vials and radioactivity was determined in a γ counter.

Determination of $[Ca^{2+}]_i$ with Fura-2

Differentiated U-937 cells were harvested by centrifugation and washed twice with PBS. Cells were then incubated for 30 min at 37° in HBSS containing no calcium, 10 mM HEPES, pH 7.4, and 0.2 μM fura-2/AM, at a density of $1–2 \times 10^6$/ml.

Confluent plates containing 2A3 cells were treated with serum-free DMEM for at least 4 hr and were then incubated for 30 min at 37° in DMEM containing 1 μM fura-2/AM. Medium was aspirated and replaced with HBSS containing 10 mM HEPES, pH 7.4, and cells were incubated for another 30 min at 37°. Cells were harvested by scraping the plates, and after centrifugation the cells were treated with 0.05% trypsin/0.53 mM EDTA (GIBCO-BRL) to disrupt cell aggregates.

Fura-2-loaded U-937 and 2A3 cells were then centrifuged and resuspended in HBSS containing 10 mM HEPES, pH 7.4. Aliquots of $0.7–1 \times 10^6$ U-937 or 2A3 cells were used for $[Ca^{2+}]_i$ determinations. A Hitachi F-2000 fluorimeter with a thermostatic chamber and stirrer was used. Excitation wavelengths were 340 nm and 380 nm, and the emission wavelength was 510 nm. Emission was monitored and ratios and concentrations of calcium were calculated by using software supplied by the instrument manufacturer, based on the procedure of Grynkiewicz *et al.* (11).

Phosphoinositide Breakdown

Differentiated U-937 cells. Cells were differentiated for 3 days (see above) and then incubated for 14 hr in minimum essential medium containing 1% fetal calf serum and [^{3}H]inositol (5 μCi/ml). Cells were centrifuged and resuspended in buffer A (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 3 mM $CaCl_2$, 10 mM LiCl, 0.5 mM EDTA, 10 mM glucose, 100 μg/ml bovine serum albumin, 20 mM HEPES, pH 7.4). Aliquots of cells ($3–4 \times 10^6$/tube) were incubated at 37° for 10 min, C5a was added, and incubations were continued for 15 min. They were stopped by addition of 2 ml of $CHCl_3$/methanol/HCl (50:50:1, by volume). Then 0.75 ml each of distilled water and $CHCl_3$ were added, the tubes were shaken and centrifuged, and [^{3}H]inositol phosphates in the upper aqueous layer were analyzed by anion exchange chromatography (12).

Permeabilized 2A3 cells. The procedure was performed as described previously (13). Cells were subcultured in 12-well plates at a density of 5×10^5 cells/well, in the presence of 10 μCi/ml [^{3}H]inositol. On the following day, the medium was aspirated and cells were washed twice with 1 ml of incubation buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.5 mM EDTA, 10 mM glucose, 20 mM HEPES, pH 7.4) containing no calcium. Then 500 μl of permeabilization buffer (incubation buffer containing 200 μM ATP, 7 μM $CaCl_2$, and 0.4 unit/ml streptolysin O; calculated concentration of free calcium, 100 nM) were added. After 10 min at 37°, agents were added at the indicated concentrations and incubations were carried out for 15 min. Incubations were terminated by the addition of 250 μl of 12% trichloroacetic acid. Cells were scraped from the bottom of the wells, transferred to microfuge tubes, and centrifuged for 2 min at $12,000 \times g$. [^{3}H]Inositol bisphosphate and [^{3}H]IP_3 in the supernatant were analyzed by anion exchange chromatography (12).

Determination of cAMP Levels in Transfected Cells

2A3 cells were seeded at a density of 4×10^5/well in a 24-well plate and were incubated for 24 hr at 37°. Medium was then aspirated and 500 μl of incubation buffer (4.7 mM KCl, 20 mM HEPES, 108 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 3.0 mM $CaCl_2$, 10 mM glucose, pH 7.4) containing 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, were added. Cells were incubated for 10 min at 37°. Agents were added at the indicated concentrations and incubations were carried out for 30 min at 37°. After aspiration of the medium, cells were lysed by addition of 500 μl of 0.2 N HCl and incubation for 30 min at room temperature. Cell lysates were cleared by centrifugation and were neutralized by the addition of 10 μl of 10 N NaOH. The levels of cAMP in each sample were determined by a binding displacement procedure, using protein kinase A as the binding protein.

Activation of MAP Kinase

2A3 cells were seeded at a density of 5×10^5/well in six-well plates, and differentiated U-937 cells were seeded at a density of 1×10^6/ml in suspension; cells were then incubated for 14 hr at 37° in serum-free medium. If PTX treatment was performed, PTX (100 ng/ml) was added to the serum-free medium and maintained for 14 hr at 37°. Treatment of 2A3 cells was performed directly in the culture wells. U-937 cells were aliquoted in glass tubes at 37°, and treatments were initiated by addition of the agents to the tubes. C5a, PDGF, or PMA was added at the indicated concentrations and cells were incubated for 5 min at 37°. In the case of 2A3 cells, medium was aspirated and 300 μl of preheated (95°) Laemmli sample buffer were added to cell monolayers in each well. In the case of U-937 cells, tubes were centrifuged at $500 \times g$ for 3 min, medium was aspirated, and 300 μl of

preheated (95°) Laemmli sample buffer were added to cell pellets. Lysates were homogenized by being forced five to 10 times through a 21-gauge syringe. Aliquots of these lysates (50 μl) were analyzed by SDS-PAGE (15% acrylamide/0.087% bisacrylamide gels). Proteins were then electrotransferred to nitrocellulose sheets as described (14). After the blots were blocked with TTBS (0.05% Tween-20, 20 mM Tris·HCl, pH 7.4, 0.9% NaCl) containing 3% nonfat dry milk, they were incubated with a monoclonal antibody (05–157; UBI, Lake Placid, NY) that specfically recognizes 42-kDa MAP kinase. Detection of immunoblots was performed with the enhanced chemiluminescence system (Amersham), after incubation with anti-mouse IgG horseradish peroxidase-coupled antibodies.

Agonist-Dependent Ribosylation with CTX

The procedure was essentially as described by Dell'Aqua *et al.* (15). Cells were washed twice with PBS, collected in homogenization buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$), and lysed by being forced through a 21-gauge syringe five to 10 times. The suspension was centrifuged at 500 × *g* for 5 min, and the supernatant was then centrifuged at 30,000 × *g* for 30 min at 4°. The pellet of plasma membranes was suspended in 100 mM HEPES, pH 7.4, 1 mM EDTA. Cell suspensions were kept at −20° until used.

Ribosylation reactions containing membranes (50–100 μg of protein), 10 mM thymidine, 1 mM ADP-ribose, 2.5 μM NAD, 100 μg/ml preactivated CTX, 0.5–1 μCi of [^{32}P]NAD, 100 mM HEPES, pH 7.4, 1 mM EDTA, and 400 nM C5a, where indicated, were carried out in a final volume of 50 μl for 60 min at 37°. Reactions were terminated by the addition of 50 μl of ice-cold termination buffer (5 mM NAD, 50 mM HEPES, pH 7.4, 0.5 mM EDTA). After centrifugation and an additional wash with termination buffer, membrane pellets were suspended in Laemmli sample buffer, heated at 95° for 3 min, and analyzed by SDS-PAGE in 10% acrylamide gels. Gels were dried and exposed to Hyperfilm (Amersham) film, with an intensifying screen, for 1–10 days at −70°.

RT-PCR of $G_{\alpha 16}$

Total RNA from U-937, 2A3, or 2C (NIH/3T3 cells transfected with $G_{\alpha 16}$ cDNA)[1] cells was obtained using a commercial kit (as described above). First-strand cDNA synthesis was performed using a commercial kit (as described above). MOLT-4 cell cDNA was obtained from Clontech (San Francisco, CA). $G_{\alpha 16}$ cDNA was provided by Dr. Allan Weissman (National Institutes of Health). PCR of $G_{\alpha 16}$ cDNA was performed using the following primers: sense (5′), CTGTCTAGACCGCACCATGGCCCGCTCGCTGACC; antisense (3′), GTGTCTAGAGGGTCACAGCAGGTTGATCTCGTCC. Such primers have been successfully used for $G_{\alpha 16}$ cDNA amplification (16). Incubations were as described above for C5aR cDNA amplification. Temperature cycling was as follows: one cycle of 2 min at 94°, followed by 35 cycles of 30 sec at 94°, 30 sec at 55°, and 120 sec at 72°, with a final extension cycle of 10 min at 72°. Aliquots of the amplification reactions were separated on 0.8% agarose gels and were visualized with ethidium bromide.

Immunoblots of G_α Subunits in U-937 and 2A3 Cell Membranes

Cell membranes were obtained as indicated for ribosylation studies (see above). Aliquots of membrane preparations (20–50 μg of protein) were dissolved in Laemmli sample buffer, heated at 95° for 3 min, and analyzed by 10% SDS-PAGE. Proteins were then electrotransferred to nitrocellulose sheets as described (14). After the blots were blocked with TTBS containing 3% nonfat dry milk, they were incubated, as recommended by the manufacturer, with the following commercial polyclonal antibodies: RM/1 (anti-$G_{\alpha s}$; DuPont), Go/1 (anti-$G_{\alpha o}$; DuPont), 06–236 (anti-$G_{\alpha i1-2}$; UBI), and EC/2 (anti-$G_{\alpha i3}$; DuPont). For $G_{\alpha 16}$ an antipeptide antibody (number 3345) was used that was prepared by immunization of rabbits with a 14-amino acid peptide, corresponding to the carboxyl terminus of human $G_{\alpha 16}$ (17), coupled to keyhole limpet hemocyanin. The blot was incubated with a 1/1000 dilution of the antiserum for 2 hr at room temperature. Detection of immunoblots was performed with the enhanced chemiluminescence system (Amersham), after incubation with anti-rabbit IgG horseradish peroxidase-coupled antibodies.

[1] M. Vanek, F. Gusovsky, manuscript in preparation.

Results

NIH/3T3 cells were transfected with the construct C5aR/ZipNeo and selected in G-418-containing medium. Individual clones were isolated and tested for binding of ^{125}I-C5a. Specific binding of ^{125}I-C5a was detected in several clones (Table 1).

C5a induced a dose-dependent increase in $[Ca^{2+}]_i$ in differentiated U-937 cells (Fig. 1A), with an EC_{50} value of 0.9 nM. The changes in $[Ca^{2+}]_i$ consistently showed a rapid phase (5–10 sec), followed by a long lasting plateau (Fig. 1B). In one of the transfected clones, 2A3 cells, the signaling properties of C5aR were studied further. C5a induced an increase in $[Ca^{2+}]_i$ in 2A3 cells (Fig. 1C). Overnight treatment of cells with PTX (100 ng/ml) resulted in a complete loss of the C5a-induced $[Ca^{2+}]_i$ response in both cell lines (Fig. 1, B and C). Control and PTX-treated cells responded equally to ionomycin (1 μM), a calcium ionophore (data not shown). In U-937 cells, C5a induced phosphoinositide breakdown, measured as the accumulation of [^{3}H]inositol monophosphate in intact cells, and the response was eliminated in cells that had been pretreated with PTX (Table 2). C5a induced a small but consistent stimulation of PLC in permeabilized 2A3 cells, measured as the generation of [^{3}H]inositol bisphosphate plus [^{3}H]IP_3 (Table 3). This stimulation was eliminated in cells that had been pretreated with PTX (Table 3).

Because PTX-sensitive G proteins are also involved in the inhibition of adenylate cyclase (18), the possibility of C5aR being linked to this inhibitory response was evaluated in 2A3 cells. C5a induced the inhibition of forskolin-stimulated cAMP generation in 2A3 cells, and the response was also eliminated after PTX treatment (Fig. 2).

TABLE 1

Binding of ^{125}I-C5a to the clones of NIH/3T3 cells transfected with C5aR

Cells were plated at a density of 2–3 × 10^5 cells/well in 24-well plates in regular medium. The following day the medium was aspirated and cells were rinsed with incubation buffer. Cells were incubated with ^{125}I-C5a, in the presence (+C5a) or absence (−C5a) of 20 nM C5a, for 2 hr at 4°. Cells were then washed and dissolved in 1 N NaOH, and radioactivity was determined in aliquots of cell lysates with a γ counter. Results correspond to single experiments performed in triplicate.

Cell line	^{125}I-C5a binding	
	cpm	*fmol/10^6 cells*
NIH/3T3		
−C5a	106 ± 4	
+C5a	110 ± 7	
E3		
−C5a	1859 ± 179	1.97
+C5a	703 ± 2	
E4		
−C5a	3986 ± 8	6.27
+C5a	309 ± 11	
F8		
−C5a	4621 ± 252	5.59
+C5a	1342 ± 34	
2A3		
−C5a	6463 ± 166	4.28
+C5a	1444 ± 86	

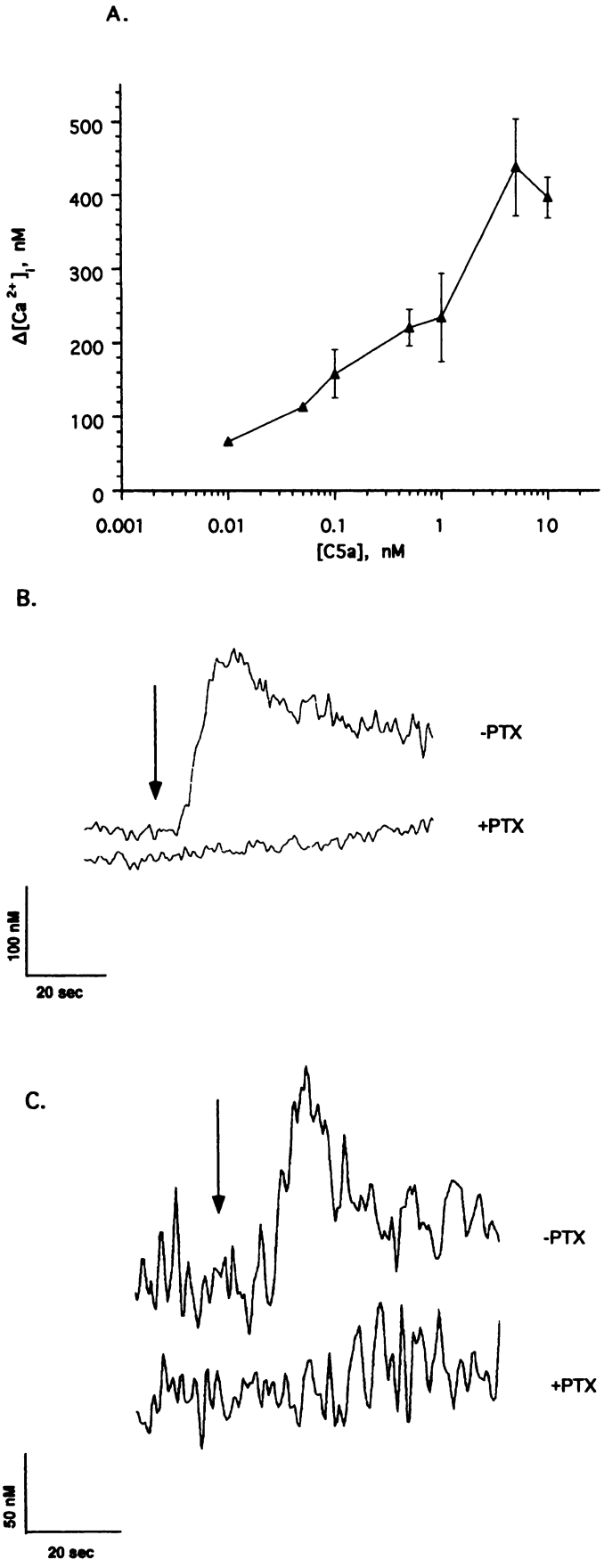

TABLE 2

PTX effects on C5a-mediated stimulation of phosphoinositide breakdown in U-937 cells

Differentiated and [^{3}H]inositol-labeled U-937 cells were treated with PTX (100 ng/ml) (+PTX) or regular medium (−PTX) for 14 hr at 37°. Cells were incubated in the presence of buffer or 100 nM C5a for 15 min at 37°, as indicated. [^{3}H]Inositol monophosphate was extracted and analyzed as described in Experimental Procedures. Results correspond to the mean ± standard error of at least three independent determinations performed in triplicate.

	[^{3}H]Inositol monophosphate	
	−PTX	+PTX
	cpm	
Control	222 ± 41	229 ± 33
C5a	610 ± 130 (275%)[a,b]	236 ± 36 (103%)

[a] Values in parentheses, percentage of control.
[b] $p < 0.05$ versus control.

TABLE 3

PTX effects on C5a-mediated stimulation of phosphoinositide breakdown in permeabilized 2A3 cells

[^{3}H]Inositol-labeled 2A3 cells were treated with PTX (100 ng/ml) (+PTX) or regular medium (−PTX) for 4 hr at 37°. Cells were then permeabilized with streptolysin O-containing buffer. After 10 min at 37°, GTPγS (10 μM) or GTPγS plus 100 nM C5a was added and incubations were carried out for 15 min. [^{3}H]Inositol bisphosphate ([^{3}H]IP_2) and [^{3}H]IP_3 were extracted and analyzed as described in Experimental Procedures. Results correspond to the mean ± standard error of at least three independent determinations performed in triplicate.

	[^{3}H]IP_2 + [^{3}H]IP_3	
	−PTX	+PTX
	cpm	
Control	399 ± 123	451 ± 144
GTPγS	2690 ± 80	2317 ± 180
GTPγS + C5a	3239 ± 129[a]	2181 ± 128
C5a	716 ± 75	554 ± 34

[a] $p < 0.025$ versus GTPγS alone.

C5a induced the activation of MAP kinase in both U-937 and 2A3 cells, measured as the appearance of a phosphorylated form of lesser electrophoretic mobility in an immunoblot utilizing 42-kDa MAP kinase-specific monoclonal antibodies (Fig. 3). The phorbol ester PMA and PDGF induced similar MAP kinase activation in U-937 and 2A3 cells, respectively, but, whereas the C5a-induced response was completely eliminated by PTX pretreatment (100 ng/ml, 14 hr), PMA- and PDGF-induced responses were unaffected (Fig. 3).

The ability of receptor agonists to sustain CTX-mediated ribosylation of PTX-sensitive G protein α subunits has been utilized as a means to identify receptor/G protein coupling in the membrane environment (15, 19–21). In membranes of differentiated U-937 cells and 2A3 cells, C5a induced the ribosylation of a 39–40-kDa protein in the presence of CTX (Fig. 4). The C5a-dependent ribosylation was suppressed in membranes of cells that had been pretreated with PTX (100 ng/ml) (Fig. 4). In contrast, a 45–46-kDa protein in U-937 cell membranes and a 50–52-kDa protein in 2A3 cell membranes were ribosylated independently of the presence of C5a or PTX pretreatment (Fig. 4). These labeled proteins presumably represent two forms of $G_{\alpha i}$, which is known to be modified by CTX (18).

Fig. 1. A, Dose-dependent increase of $[Ca^{2+}]_i$ induced by C5a. Differentiated U-937 cells were loaded with 0.2 μM fura-2/AM, washed, and incubated with the indicated concentrations of C5a. Fluorescence readings correspond to maximal elevations of $[Ca^{2+}]_i$ achieved 15–25 sec after the addition of C5a. See Experimental Procedures for details. B and C, Changes in $[Ca^{2+}]_i$ in U-937 cells (B) or in 2A3 cells (C) after the addition of 10 nM C5a, in normal buffer containing 3 mM $CaCl_2$, in control cells (*−PTX*) and cells treated with PTX (100 ng/ml, 14 hr) (*+PTX*).

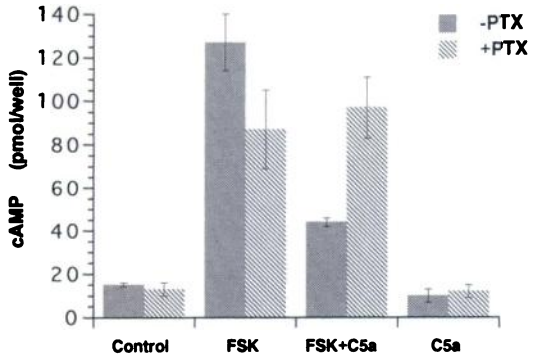

Fig. 2. Inhibition by C5a of cAMP formation in 2A3 cells. After being washed with medium, control cells (*–PTX*) or cells that had been pretreated with PTX (*+PTX*) were preincubated for 10 min with buffer containing isobutylmethylxanthine (1 mM), and then buffer (*Control*), forskolin (10 μM) (*FSK*), and/or C5a (100 nM) were added. Incubations were carried out for 15 min at 37°. Extraction and determination of cAMP levels were performed as described in Experimental Procedures. Results correspond to the mean ± standard error of at least three independent experiments performed in triplicate.

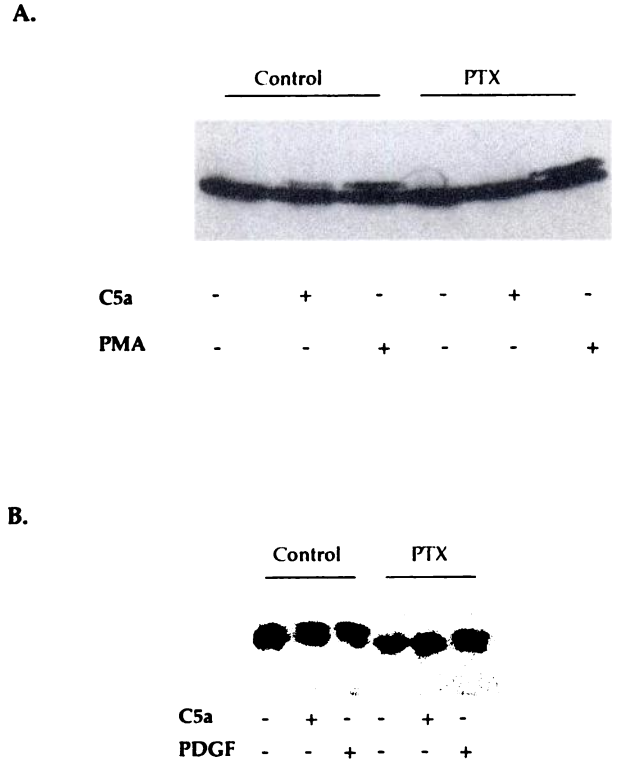

Fig. 3. Stimulation of MAP kinase in U-937 (A) and 2A3 (A) cells. Cells were incubated overnight in serum-free medium, in the absence or presence of 100 ng/ml PTX. Cells were then incubated for 5 min at 37° with 10 nM C5a, 25 ng/ml PDGF, or 1 μM PMA as indicated. Medium was then removed and cells were lysed. Aliquots were analyzed by SDS-PAGE and immunoblotting as described in Experimental Procedures.

It has been demonstrated that $G_{\alpha 16}$ is able to transduce C5aR signaling in transfected cells (8, 9). To determine whether $G_{\alpha 16}$ is involved in C5aR signaling in U-937 and 2A3 cells, RT-PCR amplification was performed, using total RNA as the initial template. Amplification products corresponding to the molecular weight expected for $G_{\alpha 16}$ cDNA were not detected in either U-937 or 2A3 cell RNA/cDNA (Fig. 5). In the same experiment, cDNA from 2C cells, which were transfected with $G_{\alpha 16}$, and from MOLT-4 cells, which express $G_{\alpha 16}$ (17), generated the expected amplification products (Fig. 5). Using the same preparation of total RNA from U-937 cells, C5aR cDNA was successfully amplified as described in Experimental Procedures (data not shown).

Immunoblotting of G protein α subunits was performed in lysates of U-937 and 2A3 cell membranes (Fig. 6). Analysis of $G_{\alpha s}$ with antibody RM/1 revealed that in U-937 cells a short form is predominant, whereas in 2A3 cells a long form is more abundant. The same pattern of $G_{\alpha s}$ expression could be detected with CTX labeling in the absence of C5a (Fig. 4). U-937 cells lack $G_{\alpha o}$ but express $G_{\alpha i3}$ and $G_{\alpha i2}$. (Although antibody 06–236 recognizes $G_{\alpha i1}$ and $G_{\alpha i2}$, $G_{\alpha i1}$ is found only in brain tissues.) In 2A3 cell membranes $G_{\alpha o}$, $G_{\alpha i3}$, and $G_{\alpha i2}$ are present. In agreement with the RT-PCR data (Fig. 5), neither U-937 cells nor 2A3 cells showed immunoreactivity with an antibody that reacts with $G_{\alpha 16}$, whereas membranes of 2C cells (see above) showed a clear band at ~40 kDa (Fig. 6).

Discussion

The mobilization of intracellular Ca^{2+} induced by C5a initiates a cascade of events leading to the activation of neutrophils (and probably other cell types). Such activation, which results in the release of enzymes and cytokines, seems to play a critical role in the development of the inflammatory process (3). C5a, a product of the complement cascade, interacts with specific receptors present in the plasma membrane of neutrophils and other cells (1–3). A receptor for C5a has recently been cloned and sequenced (1, 2) and was shown to belong to the family of G protein-coupled receptors. Similarly to other members of this family, C5aR induces PLC activation via a G protein (4, 22), which results in the generation of the second messengers IP_3 and diacylglycerol. Increases in IP_3 result in elevation of $[Ca^{2+}]_i$, whereas diacylglycerol activates protein kinase C. Both mechanisms seem to be involved in the activation of neutrophils (3).

U-937 cells, a human monocyte-like cell line, express high levels of C5aR upon differentiation (1), and signaling mechanisms through C5aR in these cells are similar to those in neutrophils and other cell types. C5a induces elevation of $[Ca^{2+}]_i$ in these cells in a dose-dependent manner (Fig. 1A), and stimulation of phosphoinositide breakdown (Table 2). Because pretreatment with PTX resulted in complete inhibition of C5a-mediated responses (Fig. 1B; Table 2), it seems that signaling through C5aR in U-937 cells proceeds through G_i-like proteins, i.e., PTX-sensitive G proteins.

The signaling properties of C5aR were tested in transfected NIH/3T3 fibroblasts. Introduction of the construct C5aR/ZipNeo resulted in stable transfectants expressing high levels of C5aR (Table 1). In 2A3 cells, one of the C5aR-transfected clones, C5a induced elevation of $[Ca^{2+}]_i$ (Fig. 1C) and a small but consistent activation of PLC in permeabilized cells (Table 3). The PLC stimulation was G protein mediated, as evidenced by the guanine nucleotide requirement (Table 3). Both C5a-induced responses, i.e., elevation of $[Ca^{2+}]_i$ and PLC stimulation, were suppressed after PTX treatment, indicating that both responses are mediated by the same or similar G proteins and suggesting that the elevation of $[Ca^{2+}]_i$ is a consequence of the generation of intracellular IP_3 by PLC. PLC-β isozymes are responsible for G protein-mediated phosphoinositide breakdown initiated by receptor agonists (4). Members of the $G_{\alpha q}$ subfamily (α_q, α_{11}, and α_{16}) can stimulate PLC-β activity (4).

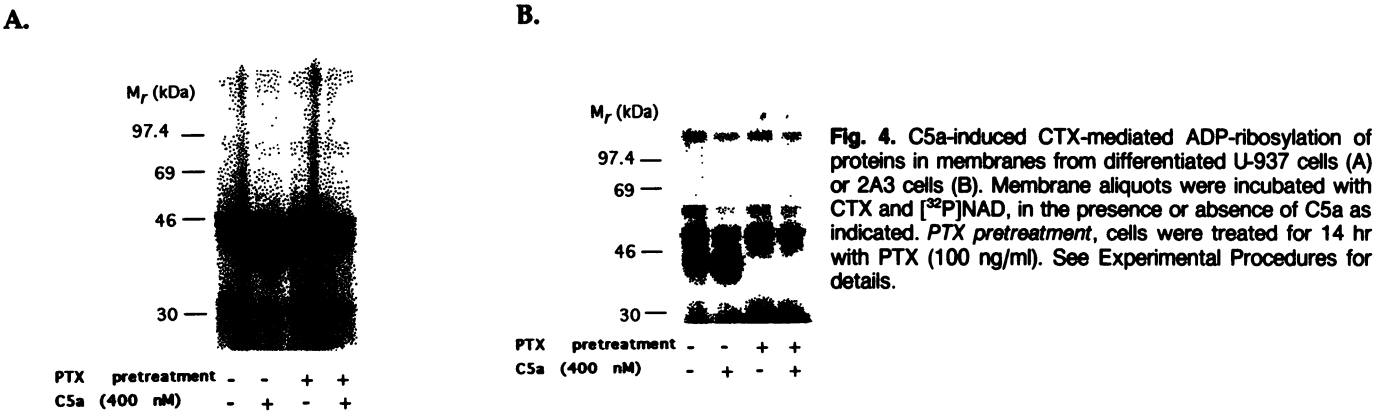

Fig. 4. C5a-induced CTX-mediated ADP-ribosylation of proteins in membranes from differentiated U-937 cells (A) or 2A3 cells (B). Membrane aliquots were incubated with CTX and [^{32}P]NAD, in the presence or absence of C5a as indicated. *PTX pretreatment*, cells were treated for 14 hr with PTX (100 ng/ml). See Experimental Procedures for details.

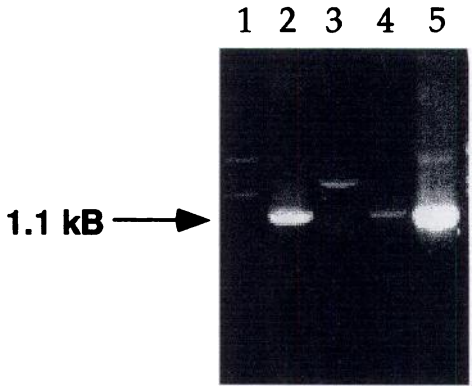

Fig. 5. RT-PCR for amplification of $G_{\alpha 16}$ cDNA. *Lanes 1-4*, cDNA corresponding to 2A3 cells (*lane 1*), 2C cells (*lane 2*), U-937 cells (*lane 3*), and MOLT-4 cells (*lane 4*) was obtained and amplified with $G_{\alpha 16}$-specific primers as described in Experimental Procedures. *Lane 5*, a sample corresponding to $G_{\alpha 16}$ cDNA was amplified as described. Aliquots of PCRs were analyzed in 0.8% agarose gels, with ethidium bromide staining.

However, PLC-β can also be stimulated by $G_{\beta\gamma}$ subunits, with PLC-β_2 being more sensitive to $\beta\gamma$ stimulation than PLC-β_1 (4). Because α_q, α_{11}, and α_{16} are insensitive to PTX action, it has been proposed that a $G_{\beta\gamma}$/PLC-β_2 pathway is involved in cells that show PTX inhibition of receptor-mediated phosphoinositide breakdown (4). It seems likely that such a pathway is activated by C5aR in U-937 and 2A3 cells (Tables 2 and 3). Alternatively, it could be conceived that C5aR, through G_i, activates the mobilization of calcium by means other than PLC. In this regard, in GH_3 pituitary cells activation of G_{i2} is required for the opening of voltage-dependent calcium channels (23), which results in elevation of $[Ca^{2+}]_i$. Whether such a mechanism pertains to U-937 or 2A3 cells is not known.

C5a inhibited forskolin-mediated cAMP accumulation in 2A3 cells by about 75% (Fig. 2). PTX pretreatment induced a slight reduction in the accumulation of cAMP induced by forskolin and completely eliminated C5a inhibition (Fig. 2). Therefore, similarly to many receptors in other cell types, C5aR, through coupling with G_i, is able to inhibit adenylate cyclase activity in 2A3 cells. It is unlikely that this mechanism is relevant in activating differentiated U-937 cells, which are grown in the presence of 1 mM dibutyryl-cAMP. However, it may pertain to C5aR signaling in other cell types.

MAP kinases are serine/threonine protein kinases that are activated by extracellular stimuli such as ligands for growth factor tyrosine kinase receptors and G protein-coupled receptors (24). The stimulation induced by growth factors, such as epidermal growth factor and PDGF, involves tyrosine phosphorylation of proteins, which leads to activation of *ras* and the protein kinases Raf, MAP kinase kinase, and MAP kinase (25). Activation of certain receptors coupled to G proteins, such as the thrombin (26), lysophosphatidic acid (26), m2 muscarinic (27), α_2-adrenergic (28), and formyl-methionyl-leucyl-phenylalanine (29) receptors, induces the activation of MAP kinase.

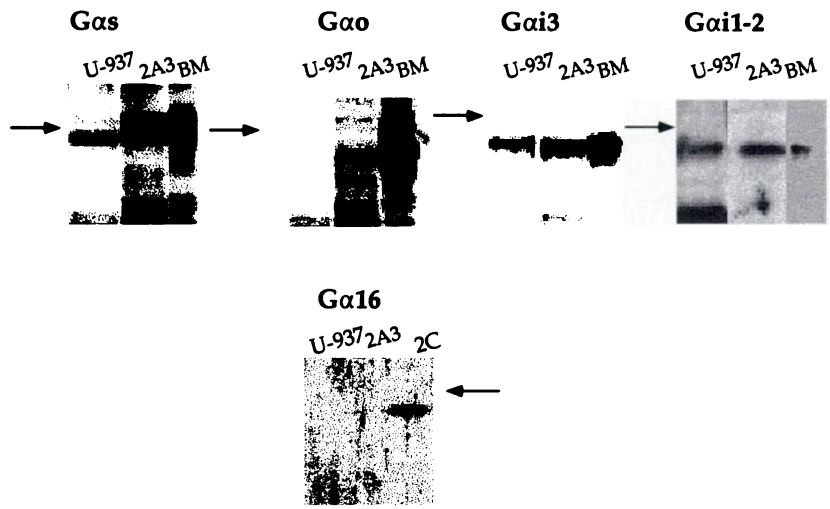

Fig. 6. Immunoblots of G protein α subunits of U-937 and 2A3 cell membranes. Aliquots of U-937 and 2A3 cell membranes and a positive control [rat brain membranes (*BM*) for $G_{\alpha s}$, $G_{\alpha o}$, $G_{\alpha i3}$, and $G_{\alpha i1-2}$ or 2C cell membranes for $G_{\alpha 16}$] were analyzed by 10% PAGE, electrotransferred, and immunoblotted with antibodies directed to the indicated G_α subunits, as follows: $G_{\alpha s}$, RM/1; $G_{\alpha o}$, Go/1; $G_{\alpha i3}$, EC/2; $G_{\alpha i1-2}$, 06–236; $G_{\alpha 16}$, 3345. See Experimental Procedures for details.

Stimulation by these agonists also seems to involve *ras*, and it is eliminated in cells that have been pretreated with PTX (26–28). Protein kinase C stimulates MAP kinase in a *ras*-independent manner (25). In U-937 and 2A3 cells, C5a induced the activation of MAP kinase (Fig. 3). After treatment with PTX, C5a-induced activation was eliminated, indicating that a "G_i-like" protein is involved in this response (Fig. 3). Stimulation of MAP kinase in U-937 cells by PMA, which activates protein kinase C, and in 2A3 cells by PDGF, which activates a tyrosine kinase receptor, was unaffected by PTX pretreatment. Whether *ras* is involved in C5a-induced MAP kinase activation is not known, but based on the results reported for other G_i-coupled receptors (see above) involvement of *ras* would be expected for C5aR as well. Similarly to the G_i-mediated activation of PLC, the activation of MAP kinase by certain G protein-coupled receptors seems to proceed through a $G_{\beta\gamma}$/effector pathway (30).

Agonist-dependent ribosylation of $G_{\alpha i}$ subunits with CTX has been reported for formyl-methionyl-leucyl-phenylalanine (21), m2 muscarinic (15), and α_2-adrenergic (20) receptors and provides a method to identify the nature of the G_α subunit that is coupled to a particular receptor in intact plasma membranes. Agonist-induced CTX-mediated ribosylation experiments confirmed that C5aR is coupled to a $G_{\alpha i}$-like protein in both U-937 and 2A3 cells. Thus, in the presence of C5a (Fig. 3), CTX induced the ribosylation of 39–40-kDa protein(s) in both cell lines. The response was dependent on the presence of C5aR, because in untransfected NIH/3T3 cells the response was absent (data not shown), and, furthermore, C5a induced similar CTX-sustained ribosylation in several independent clones of transfected cells (data not shown). Because the agonist-induced ribosylation is absent in membranes of cells that have been pretreated with PTX (Fig. 4), it appears that the 39–40-kDa labeled protein belongs to the $G_{\alpha i}$ subfamily, whose members are sensitive to PTX (18). The electrophoretic mobilities of the 39–40-kDa protein(s) labeled in U-937 and 2A3 cells were very similar (Fig. 3). In addition, a member of the $G_{\alpha s}$ subfamily (45–46 kDa in U-937 cells and 50–52 kDa in 2A3 cells) seems to undergo ribosylation by CTX independently of the presence of C5a (Fig. 3).

Because the stimulation of PLC (Tables 2 and 3), the mobilization of intracellular Ca^{2+} (Fig. 1), the inhibition of cAMP generation (Fig. 2), the activation of MAP kinase (Fig. 3), and the CTX-mediated labeling of the 39–40-kDa protein (Fig. 4) induced by C5a are prevented by PTX treatment, it is likely that all phenomena involve the activation by C5aR of the same or very similar G protein(s). Immunoblot analysis of G_α subunits in U-937 and 2A3 cells was performed in an attempt to identify the G proteins involved in coupling to C5aR. $G_{\alpha s}$ immunoreactivity matched the agonist-independent labeling obtained with CTX (Fig. 4), which ribosylates $G_{\alpha s}$ subunits (18). Thus, in U-937 cells a short form is predominant, whereas in 2A3 cells a longer form is more abundant (Fig. 6). Both forms of $G_{\alpha s}$ originate from the same gene, by alternative splicing (18). In U-937 cells the PTX substrates expressed are $G_{\alpha i2}$ and $G_{\alpha i3}$, and therefore at least one of these G_α subunits is responsible for coupling to C5aR. In addition to $G_{\alpha i2}$ and $G_{\alpha i3}$, 2A3 cells contain $G_{\alpha o}$, which is also a substrate for PTX-induced ribosylation and thus could be involved in coupling to C5aR as well.

Two recent studies demonstrated that C5aR transfected into COS cells (8) or 293 cells (9) could mediate PLC activation only in the presence of $G_{\alpha 16}$. This PTX-insensitive G_α was originally cloned from HL-60 cells (26). The results of these studies suggest that $G_{\alpha 16}$ may indeed mediate signaling of C5aR (8, 9). Some evidence exists, however, against $G_{\alpha 16}$ being the transducer of C5aR in all tissues. (i) When isolated from human neutrophils, C5aR co-purifies with a G protein heterotrimer, and the G_α was identified as a $G_{\alpha i}$ (7). (ii) In HL-60 cells, $G_{\alpha 16}$ is highly expressed when cells are undifferentiated; after differentiation, $G_{\alpha 16}$ essentially disappears (17). C5aR is expressed only when HL-60 cells are differentiated (1). (iii) C5a is able to induce calcium mobilization in C5aR-transfected 293 cells (31), which do not express $G_{\alpha 16}$ (9). Interestingly, in 293 cells, C5a can stimulate PLC only if $G_{\alpha 16}$ is co-transfected with C5aR (9). C5a induced a small but consistent PTX-sensitive stimulation of PLC in permeabilized 2A3 cells and in whole U-937 cells (Tables 2 and 3), and neither of these cell lines expresses $G_{\alpha 16}$ (Figs. 5 and 6). No PLC activation could be detected in whole 2A3 cells (data not shown). It is possible that C5aR-transfected 293 cells could respond to small amounts of IP_3 to increase $[Ca^{2+}]_i$ and yet IP_3 could be undetected in whole-cell assays (9). Alternatively, C5aR could mediate intracellular Ca^{2+} mobilization through other means not involving PLC in 293 cells and 2A3 cells. Nevertheless, it should be noted that the intracellular Ca^{2+} mobilization does not involve $G_{\alpha 16}$, and at least in 2A3 cells and U-937 cells it is sensitive to PTX (Fig. 1, B and C). Thus, although the coupling of C5aR with $G_{\alpha 16}$ occurs in transfected cells, whether such coupling takes place in untransfected systems remains to be determined. In a cell line with neutrophil phenotype (differentiated HL-60 cells), $G_{\alpha 16}$ is not present (17). Because C5a proinflammatory activity involves primarily neutrophil activation (3), the C5a/$G_{\alpha 16}$ coupling may not affect such action. However, C5a activity in other cells may require the presence of $G_{\alpha 16}$. The receptors for another chemoattractant, interleukin-8, which also mediates neutrophil activation, are able to couple to $G_{\alpha 16}$ or to PTX-sensitive $G_{\alpha i}$ to induce phosphoinositide breakdown when transfected into COS-7 cells (32). However, stimulation via $G_{\alpha i}$ required the presence of PLC-β_2 (32). Thus, if PLC-β_2 is indeed expressed in U-937 cells, then conceivably C5aR-mediated stimulation of phosphoinositide breakdown could occur exclusively through $G_{\alpha i}$. The presence of PLC-β_2 may explain the difference between U-937 and 2A3 cells in the magnitude of the C5a-induced response of phosphoinositide breakdown, although other factors may play a role (rate of [^{3}H]inositol uptake, inositol phosphate metabolism, etc.). On the other hand, in the presence of $G_{\alpha 16}$, stimulation of all PLC-β subtypes by C5aR would be possible.

In summary, the results presented indicate that in differentiated U-937 cells C5aR is coupled to a $G_{\alpha i}$-like protein to mediate intracellular Ca^{2+} mobilization, phosphoinositide breakdown, and MAP kinase activation. When transfected into a murine fibroblast cell line, C5aR is able to stimulate phosphoinositide breakdown via coupling to a very similar G_α subunit, as determined by PTX sensitivity and CTX labeling in the presence of agonist. Two novel signaling systems are presented for C5aR, i.e., the inhibition of adenylate cyclase and the stimulation of MAP kinase. These mechanisms may contribute to the activation of cells involved in the inflammatory process.

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