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DIFFERENTIAL ACTIVATION OF DIBUTYRYL cAMP-DIFFERENTIATED HL-60 HUMAN LEUKEMIA CELLS BY CHEMOATTRACTANTS

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Abstract—Dibutyryl cAMP-differentiated HL-60 human leukemia cells possess receptors for the chemoattractants *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), C5a and leukotriene B_4 (LTB_4). We compared the effects of these chemoattractants in HL-60 membranes and in intact HL-60 cells. fMLP, C5a and LTB_4 stimulated GTP hydrolysis and guanosine 5′-*O*-[3-thio]triphosphate (GTP[γS]) binding in HL-60 membranes with similar effectiveness and in a pertussis toxin (PTX)-sensitive manner. They also stimulated photolabeling of the α-subunits of the guanine nucleotide-binding proteins (G-proteins), G_{i2} and G_{i3} with similar effectiveness. Chloride salts of monovalent cations differentially enhanced and inhibited chemoattractant-induced GTP hydrolyses. C5a was less effective than fMLP in enhancing cholera toxin-catalysed ADP-ribosylation of G_{ia2} and G_{ia3}, and LTB_4 was ineffective. fMLP was more effective than C5a and LTB_4 in stimulating Ca^{2+} influx in HL-60 cells. C5a- and LTB_4-induced rises in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were PTX-sensitive, whereas the effect of fMLP was partially PTX-insensitive. LTB_4-induced rises in $[Ca^{2+}]_i$ were more sensitive towards homologous desensitization than those induced by C5a, and the effect of fMLP was resistant in this regard. C5a was considerably less effective than fMLP in activating superoxide anion formation and azurophilic granule release, and LTB_4 was ineffective. Our data suggest that fMLP, C5a and LTB_4 effectively activate the G-proteins, G_{i2} and G_{i3}, in HL-60 cells and that fMLP may additionally activate PTX-insensitive G-proteins. fMLP, C5a and LTB_4 are full, partial and incomplete secretagogues, respectively, and these differences may be due to differences in homologous receptor desensitization and qualitative G_i-protein activation.

Key words: HL-60 cells; formyl peptides; complement C5a; leukotriene B_4; chemoattractant receptors; G_i-proteins

Human neutrophils and differentiated HL-60 human leukemia cells possess receptors for the chemoattractants fMLP,† complement C5a and LTB_4 [1, 2]. Principally, these chemoattractants activate similar signal transduction processes. Specifically, the occupation of chemoattractant receptors with agonists results in the activation of PTX-sensitive guanine nucleotide-binding proteins (G-proteins) of the G_i-family with the subsequent stimulation of phospholipase C [EC 3.1.4.10] [1, 2]. Subsequently, Ca^{2+} is mobilized from intracellular stores and Ca^{2+} also enters the cytosol through non-selective cation channels [1, 2]. Activation of the Ca^{2+} influx is required for stimulation of the superoxide anion (O_2^-)-forming NADPH oxidase [EC 1.6.99.6] and of β-glucuronidase [EC 3.2.1.31] release from azurophilic granules [3].

Although fMLP, C5a and LTB_4 all effectively activate G_i-proteins as assessed by measurement of high-affinity GTP hydrolysis and GTP[γS] binding and photolabeling of α-subunits with GTP azidoanilide [4–6], substantial differences in the effects of fMLP and LTB_4 have been observed. Specifically, LTB_4, unlike fMLP, does not enhance CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in membranes of DMSO-differentiated HL-60 cells [7, 8]. In addition, LTB_4, in marked contrast to fMLP, is only a poor activator of NADPH oxidase in neutrophils and DMSO-differentiated HL-60 cells [7, 9]. From these data it was concluded that fMLP and LTB_4 induce different activation states of G_i-proteins, resulting in different cellular responses [7, 8]. Some data indicate that there are also differences in the effects of fMLP and C5a in neutrophils and HL-60 cells. For example, C5a-induced NADPH oxidase activation in neutrophils is more rapid in onset than that induced by fMLP, but the response towards C5a is transient [10, 11]. In addition, C5a is substantially more potent than fMLP in activating rises in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in HL-60 cells and β-glucuronidase release in neutrophils [12, 13]. Moreover, cAMP-increasing substances differentially inhibit fMLP- and C5a-induced β-glucuronidase release [11, 12]. Finally, a cell-permeant analog of cGMP and NO-containing substances enhance the stimulatory effects of C5a on O_2^- formation and/or exocytosis, whereas the effects of fMLP are inhibited by these substances [11, 12].

In order to learn more about the signal transduction

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† Abbreviations: Bt_2cAMP, dibutyryl cAMP; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CTX, cholera toxin; DMSO, dimethyl sulfoxide; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; G-proteins, guanine nucleotide-binding proteins; GTP[γS], guanosine 5′-*O*-[3-thio]triphosphate; LTB_4, leukotriene B_4; O_2^-, superoxide anion; PTX, pertussis toxin.

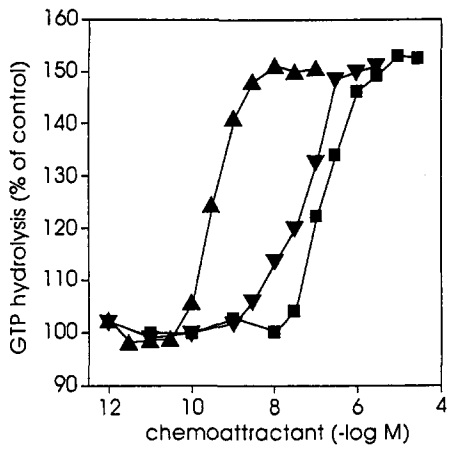

Fig. 1. Concentration–response curves for chemoattractants on high-affinity GTP hydrolysis in HL-60 membranes. High-affinity GTPase activity in HL-60 membranes was determined as described in Materials and Methods in the presence of fMLP (■), C5a (▲) and LTB_4 (▼) at the indicated concentrations. Data shown are the means of assay quadruplicates. The SD values were generally less than 5% of the means. Basal GTP hydrolysis amounted to 24.4 ± 0.5 pmol/mg/min. Similar results as those shown were obtained in three independent experiments.

pathways activated by C5a, we studied the effects of C5a in comparison to those of fMLP and LTB_4 in intact Bt_2cAMP-differentiated HL-60 cells and in membranes of these cells. Here, we show that there are not only differences in the state of G_i-protein activation induced by fMLP and LTB_4, but also between fMLP and C5a as well as between C5a and LTB_4.

MATERIALS AND METHODS

Materials. Human recombinant C5a, fMLP and LTB_4 were obtained from Sigma Chemie (Deisenhofen, Germany). PTX was obtained from List Biological Laboratories (Campbell, CA, U.S.A.). [^{35}S]GTP[γS] (1000–1500 Ci/mmol) was obtained from Dupont/New England Nuclear (Bad Homburg, Germany). Sources of other materials have been described elsewhere [12–20].

Cell culture and membrane preparation. HL-60 cells were grown in suspension culture at 37° and were differentiated towards neutrophil-like cells with Bt_2cAMP (0.2 mM) for 48 hr [15]. HL-60 membranes were prepared as described [14, 16]. PTX (100 ng/mL) or its carrier (control) were added to cell cultures 24 hr before experiments with intact cells or membrane preparation. Under these conditions, virtually all G_i-protein α-subunits were ADP-ribosylated as assessed by *in vitro* ADP-ribosylation of membranes with activated PTX and [^{32}P]NAD (data not shown).

GTPase assay. High-affinity GTP hydrolysis was determined as described [16]. In brief, reaction mixtures (100 μL) contained HL-60 membranes (3.0–7.0 μg of protein/tube), 0.5 μM [γ-^{32}P]GTP (0.1 μCi/tube), 0.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ATP, 1 mM adenosine 5′-[β,γ-imido]-triphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, 1 mM dithiothreitol, 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4, and chemoattractants at various concentrations. Reactions were conducted for 15 min at 25°.

GTP[γS] binding assay. GTP[γS] binding as described recently [17] was carried out with few modifications. Briefly, reaction mixtures (100 μL) contained HL-60 membranes (3.0–5.0 μg of protein/tube), 0.4 nM [^{35}S]GTP[γS] (50 nCi/tube), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 3 μM GDP, 0.2% (w/v) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4, and various chemoattractants. Reactions were conducted for 30 min at 25°.

Assay for photolabeling of membrane proteins and immunoprecipitation of photolabeled proteins. HL-60 membranes (200 μg of protein in a total volume of 120 μL) were incubated at 30° in a buffer consisting of 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 10 μM GDP, 10 mM NaCl and 30 mM HEPES/NaOH, pH 7.4. After exposure to various chemoattractants for 3 min, samples were incubated for another 3 min with 10 nM [α-^{32}P]GTP azidoanilide (3.5 μCi/tube). The stopping of reactions, washing and irradiation of samples were performed as described [19]. Immunoprecipitation of photolabeled G-protein α-subunits was performed with the anti-peptide antiserum, $\alpha_{i\ common}$ (AS 266), as described [20].

Assay for CTX-catalysed ADP-ribosylation of membrane proteins. ADP-ribosylation was determined according to Klinker *et al.* [16]. In brief, reaction mixtures (50 μL) contained HL-60 membranes (50 μg of protein/tube), 3 μM [^{32}P]NAD (5 μCi/tube), 3 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 0.2% (w/v) bovine serum albumin, 2 μg of activated CTX and 0.1 M potassium phosphate, pH 7.4, in the presence of various chemoattractants. Reactions were conducted for 60 min at 30°.

Miscellaneous Protein was determined according to Lowry *et al.* [21]. [γ-^{32}P]GTP was prepared as described [22]. [α-^{32}P]GTP azidoanilide was prepared according to Offermanns *et al.* [19]. [^{32}P]-NAD was synthesized according to Cassel and Pfeuffer [23]. SDS–PAGE, immunoblotting and autoradiography were performed as described by Rosenthal *et al.* [18]. Immunoblotting studies using anti-peptide antisera, $\alpha_{i\ common}$ (AS 266), α_{i2} (AS 269) and α_{i3} (AS 105) [20], revealed that photolabeled and ADP-ribosylated 40 and 41 kDa proteins in membranes of Bt_2cAMP-differentiated HL-60 cells corresponded to $G_{i\alpha 2}$ and $G_{i\alpha 3}$, respectively (data not shown). $[Ca^{2+}]_i$ was determined using the fluorescent dye, Fura-2, as described [13]. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase [EC 1.15.1.1], using an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, Germany) [15]. The absolute amounts of O_2^- generated within 10 min were calculated. β-Glucuronidase release was determined as described by Wenzel-Seifert and Seifert [24].

Table 1. Chemoattractant-stimulated high-affinity GTP hydrolysis and GTP[γS] binding in HL-60 membranes: effect of PTX

Stimulus	GTP hydrolysis (pmol/mg/min)		GTP[γS] binding (pmol/mg)	
	Control	PTX	Control	PTX
H_2O (solvent)	26.0 ± 0.9	15.4 ± 0.8	1.09 ± 0.05	0.62 ± 0.01
fMLP	47.7 ± 2.1	14.8 ± 0.5	2.71 ± 0.13	0.63 ± 0.02
C5a	48.9 ± 1.0	14.2 ± 0.4	2.65 ± 0.07	0.62 ± 0.02
LTB_4	44.7 ± 0.7	15.5 ± 1.1	2.81 ± 0.10	0.64 ± 0.03

Treatments with carrier (control) and PTX were performed as described in Materials and Methods. High-affinity GTPase activity and GTP[γS] binding in HL-60 membranes were determined as described in "Materials and Methods". The concentrations of fMLP, C5a and LTB_4 were 10 μM, 10 nM and 1 μM, respectively. For determination of basal GTP hydrolysis, H_2O (solvent) was added instead of stimulus. Data shown are the means ± SD of assay quadruplicates. Similar results were obtained in four independent experiments.

Table 2. Effects of chloride salts of monovalent cations on chemoattractant-stimulated high-affinity GTP hydrolysis in HL-60 membranes

Stimulus	GTP hydrolysis (% stimulation)				
	Solvent (H_2O)	NaCl	KCl	LiCl	Choline chloride
fMLP	80	123	160	118	127
C5a	84	57	71	45	77
LTB_4	65	145	146	116	84

High-affinity GTPase activity in HL-60 membranes was determined in the presence of various chloride salts of monovalent cations (150 mM each) or solvent as described in Materials and Methods. The concentrations of fMLP, C5a and LTB_4 were 10 μM, 10 nM and 1 μM, respectively. Data shown are the means of assay quadruplicates. The SD values were generally less than 5% of the means. Basal GTP hydrolyses in the presence of solvent, NaCl, KCl, LiCl and choline chloride were 20.2 ± 0.6, 20.3 ± 0.5, 19.6 ± 0.3, 25.3 ± 0.4 and 19.0 ± 0.5 pmol/mg/min, respectively. The stimulatory effects of chemoattractants are referred to these basal GTP hydrolyses. Similar results as those shown were obtained in three independent experiments.

RESULTS

We first studied the effects of chemoattractants on high-affinity GTPase activity (EC 3.6.1.-) in HL-60 membranes. fMLP activated GTP hydrolysis with an EC_{50} of 190 nM and a plateau at 10–30 μM (Fig. 1). The corresponding values for C5a were 300 pM and 10–100 nM, respectively. The EC_{50} of LTB_4 for GTPase stimulation was 60 nM, and a plateau was reached at 1–3 μM. At maximally stimulatory concentrations, the chemoattractants were similarly effective in activating GTP hydrolysis. As has been shown for fMLP in HL-60 membranes [16], C5a and LTB_4 also increased V_{max} of GTP hydrolysis (data not shown).

Table 1 compares the effects of chemoattractants at maximally stimulatory concentrations on GTP hydrolysis and GTP[γS] binding in control membranes and in membranes of PTX-treated HL-60 cells. As was the case for GTP hydrolysis, fMLP, C5a and LTB_4 were similarly effective in stimulating GTP[γS] binding. PTX abolished the stimulatory effects of chemoattractants on GTPase and GTP[γS] binding.

Chloride salts of monovalent cations enhance fMLP-induced GTPase activation in HL-60 membranes, presumably through an alteration of receptor/G-protein interaction [3, 25]. NaCl, KCl, LiCl and choline chloride (150 mM each) enhanced the relative stimulatory effects of fMLP on GTPase in HL-60 membranes by 54, 100, 48 and 59%, respectively (Table 2). The corresponding values for LTB_4 were 123, 125, 78 and 29%, respectively. In contrast, NaCl, KCl, LiCl and choline chloride diminished C5a-stimulated GTP hydrolysis by 32, 15, 46 and 8%, respectively.

Next, we assessed the effects of fMLP, C5a and LTB_4 on photolabeling of G_i-protein α-subunits with GTP azidoanilide. After photolabeling, G-protein α-subunits were immunoprecipitated with the $\alpha_{i\ common}$ antiserum and then subjected to SDS–PAGE and autoradiography. fMLP, C5a and LTB_4 were similarly effective in enhancing incorporation of GTP azidoanilide into 40 kDa proteins ($G_{i\alpha 2}$) and into 41 kDa proteins ($G_{i\alpha 3}$) (Fig. 2). The abundance of $G_{i\alpha 2}$ in HL-60 cells is much higher than that of $G_{i\alpha 3}$ [6, 26], so that the intensity of labeling of the latter protein is considerably lower than that of the former.

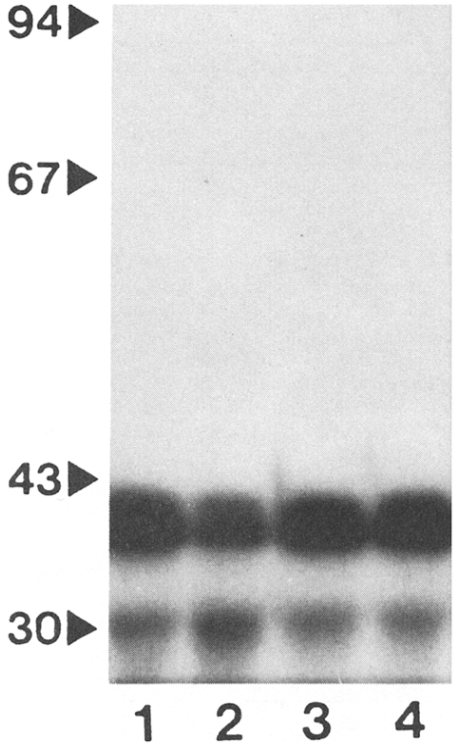

Fig. 2. Effects of chemoattractants on incorporation of GTP azidoanilide into G_i-protein α-subunits in HL-60 membranes. Photolabeling and immunoprecipitation were performed as described in Materials and Methods. The autoradiogram of an SDS gel containing 4.3 M urea and 8% (w/v) acrylamide is shown. Lane 1, fMLP (10 μM); lane 2, H_2O added instead of stimulus (control); lane 3, LTB_4 (1 μM); lane 4, C5a (1 μM). Numbers on the left, molecular masses of marker proteins (kDa). 40 kDa proteins represent $G_{i\alpha 2}$, and 41 kDa proteins represent $G_{i\alpha 3}$. The autoradiogram shown is representative of four independent experiments.

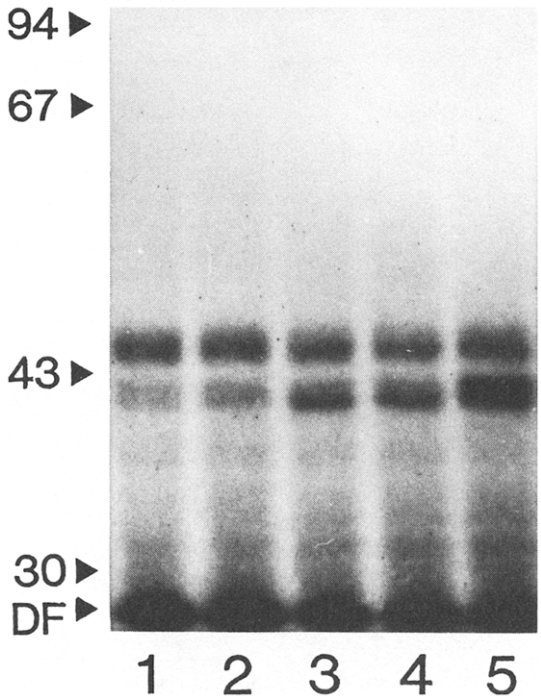

Fig. 3. Effects of fMLP and C5a on CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in HL-60 membranes. CTX-catalysed ADP-ribosylation was performed as described in Materials and Methods. The autoradiogram of an SDS gel containing 4.3 M urea and 9% (w/v) acrylamide is shown. Lane 1, H_2O added instead of stimulus (control); lane 2, C5a (10 nM); lane 3, C5a (100 nM); lane 4, C5a (1 μM); lane 5, fMLP (1 μM). Numbers on the left, molecular masses of marker proteins (kDa). DF, dye front. 40 kDa proteins represent $G_{i\alpha 2}$, and 41 kDa proteins represent $G_{i\alpha 3}$. The autoradiogram shown is representative of three independent experiments.

Labeling of a 31 kDa protein with GTP azidoanilide was observed as well (see Fig. 2). Unlike 40 and 41 kDa proteins, chemoattractants did not enhance labeling of the 31 kDa protein. Thus, the 31 kDa protein may represent a proteolytic fragment of $G_{i\alpha 2}$ and/or $G_{i\alpha 3}$ which still binds GTP azidoanilide but no longer interacts with chemoattractant receptors.

The effects of chemoattractants on CTX-catalysed ADP-ribosylation of G_i-protein α-subunits were studied as well. As has been shown for membranes of DMSO-differentiated HL-60 cells [7], LTB_4 (1 μM) did not enhance ADP-ribosylation of G_i-protein α-subunits in membranes of Bt_2cAMP-differentiated HL-60 cells either (data not shown). In contrast, fMLP (1 μM) effectively stimulated CTX-catalysed ADP-ribosylation of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (Fig. 3). By comparison to fMLP, C5a (10 nM–1 μM) was considerably less effective in enhancing ADP-ribosylation of $G_{i\alpha 2}$ and, specifically, of $G_{i\alpha 3}$.

Time courses of the rises in $[Ca^{2+}]_i$ caused by chemoattractants at maximally stimulatory concentrations [13] are shown in Fig. 4. fMLP, C5a and LTB_4 induced rapid rises in $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ values stimulated by fMLP and C5a were higher than those induced by LTB_4. In the absence of extracellular Ca^{2+}, chemoattractant-induced rises in $[Ca^{2+}]_i$ were shorter than in its presence. These data indicate that fMLP, C5a and LTB_4 induced both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx from the extracellular space. The imidazole, 1-{β-[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl-1*H*-imidazole hydrochloride (SK&F 96365), reduces fMLP-induced Ca^{2+} influx through non-selective cation channels in Bt_2cAMP-differentiated HL-60 cells [3]. SK&F 96365 (30 μM) inhibited C5a- and LTB_4-induced Ca^{2+} influxes to the same extent as that induced by fMLP (data not shown), indicating that they were mediated through non-selective cation channels as well. C5a- and LTB_4-induced Ca^{2+} influxes declined more rapidly than fMLP-induced Ca^{2+} influx. Chemoattractant-induced Ca^{2+} mobilizations were blocked by PTX. With respect to Ca^{2+} influx, approx 20% of the effect of fMLP was PTX-insensitive. In comparison, PTX inhibited C5a- and LTB_4-induced Ca^{2+} influxes almost completely.

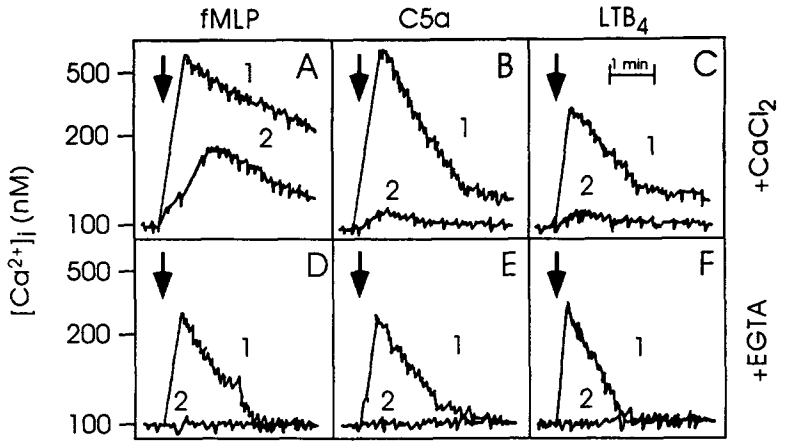

Fig. 4. Chemoattractant-induced rises in $[Ca^{2+}]_i$ in HL-60 cells: effect of PTX. Determinations of $[Ca^{2+}]_i$ were performed as described in Materials and Methods. The increases in $[Ca^{2+}]_i$ induced by fMLP (1 μM), C5a (10 nM) and LTB_4 (10 nM) in the presence of 1 mM extracellular $CaCl_2$ (A–C) or 1 mM extracellular EGTA (D–F) were assessed. Treatments with carrier (control) (traces 1) and PTX (traces 2) were performed as described in Materials and Methods. Arrows indicate the addition of chemoattractants. Superimposed original fluorescence tracings are shown. Similar results were obtained in four experiments with different preparations of HL-60 cells.

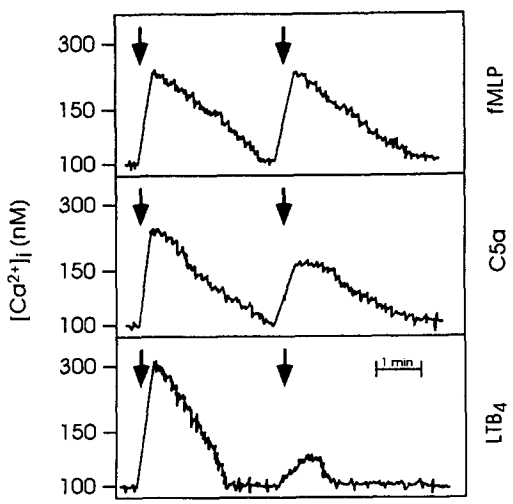

Fig. 5. Homologous desensitization of chemoattractant-induced rises in $[Ca^{2+}]_i$ in HL-60 cells. Determinations of $[Ca^{2+}]_i$ were performed as described in Materials and Methods. The increases in $[Ca^{2+}]_i$ induced by chemoattractants in the presence of 1 mM extracellular $CaCl_2$ were assessed. The arrows on the left indicate the additions of fMLP (2 nM), C5a (0.3 nM) and LTB_4 (3 nM), respectively. The arrows on the right indicate the repeated additions of chemoattractants at the same concentrations. Superimposed original fluorescence tracings are shown. Similar results were obtained in three experiments with different preparations of HL-60 cells.

Homologous desensitization of chemoattractant-induced rises in $[Ca^{2+}]_i$ was determined. HL-60 cells were stimulated with fMLP, C5a and LTB_4 at submaximally effective concentrations and were re-challenged with the chemoattractants at the same concentrations after 3 min, i.e. after $[Ca^{2+}]_i$ had again reached basal values (Fig. 5). Under these conditions, the second response to fMLP was not diminished, whereas those to C5a and LTB_4 were reduced by 50 and 85%, respectively.

Table 3. Effects of chemoattractants on O_2^- formation and β-glucuronidase release in HL-60 cells

Stimulus	O_2^- formation (nmol/10^6 cells)	β-glucuronidase release (% of cellular content)
fMLP	2.5 ± 0.3	34.1 ± 3.3
C5a	1.3 ± 0.2	21.3 ± 2.0
LTB_4	0	0

O_2^- formation and β-glucuronidase release in HL-60 cells were determined as described in Materials and Methods. The concentrations of fMLP, C5a and LTB_4 were 1 μM, 100 nM and 1 μM, respectively. In the absence of stimuli, HL-60 cells did not generate O_2^-. Basal β-glucuronidase release in the absence of stimuli was 5.5 ± 0.6% of the cellular content. Data shown are the means ± SD of assay quadruplicates. Similar results were obtained in three independent experiments.

Finally, we compared the effects of chemoattractants on O_2^- formation and β-glucuronidase release in HL-60 cells. At maximally stimulatory concentrations, C5a was approx. 50% less effective than fMLP in activating O_2^- formation (Table 3). LTB_4 did not activate O_2^- formation. PTX abolished the stimulatory effects of fMLP and C5a on O_2^- formation (data not shown). With respect to exocytosis, C5a was approx. 40% less effective than fMLP in stimulating the release of β-glucuronidase (see Table 3).

DISCUSSION

The aim of our study was to characterize C5a-mediated signal transduction pathways in Bt_2cAMP-differentiated HL-60 cells. Specifically, we were interested in determining whether there are differences between fMLP and C5a and between C5a and LTB_4. The three chemoattractants were similarly effective in activating GTP hydrolysis, GTP[γS] binding and Ca^{2+} mobilization, the latter parameter reflecting phospholipase C activation, and their effects were completely PTX-sensitive (see Table 1 and Fig. 4). Thus, fMLP, C5a and LTB_4 activate G_i-proteins in Bt_2cAMP-differentiated HL-60 cells. Similar data have been obtained for human neutrophils and DMSO-differentiated HL-60 cells [4, 5, 7].

In order to ascertain which G_i-protein subtypes are activated by chemoattractants, we photolabeled G_i-protein α-subunits with GTP azidoanilide. In agreement with the data obtained for membranes of DMSO-differentiated HL-60 cells, we found that chemoattractants were similarly effective in activating $G_{i\alpha 2}$ in membranes of Bt_2cAMP-differentiated HL-60 membranes (see Fig. 2) [6]. In our previous study [6], we were unable to determine whether fMLP, C5a and LTB_4 also activate $G_{i\alpha 3}$ in HL-60 membranes. This could have been due to the lack of resolution of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ on SDS–PAGE [6]. Another reason could have been that the concentration ratio $G_{i\alpha 2}$/$G_{i\alpha 3}$ in HL-60 membranes is high [6, 25], so that the strong labeling of a $G_{i\alpha 2}$ masked that of $G_{i\alpha 3}$. Therefore, we increased the sensitivity of the method by immunoprecipitating G_i-protein α-subunits with the anti-peptide antiserum, $\alpha_{i\ common}$, before performing SDS–PAGE and by including urea into the gel. Under these conditions, labeling of $G_{i\alpha 3}$ was, in fact, evident. As was the case for $G_{i\alpha 2}$, chemoattractants were similarly effective in increasing photolabeling of $G_{i\alpha 3}$ (see Fig. 2).

However, when CTX-catalysed ADP-ribosylation is considered, substantial differences between fMLP, C5a and LTB_4 become apparent. Specifically, C5a activated ADP-ribosylation of $G_{i\alpha 2}$ and, particularly, of $G_{i\alpha 3}$ much less effectively than fMLP, and LTB_4 was ineffective (see Fig. 3). Intriguingly, there was no difference in the extent of ADP-ribosylation of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in the presence of fMLP, whereas with respect to incorporation of GTP azidoanilide, labeling of $G_{i\alpha 2}$ was much more prominent than that of $G_{i\alpha 3}$ (compare lane 1 in Fig. 2 with lane 5 in Fig. 3). These differences between CTX-catalysed ADP-ribosylation and photolabeling and the data concerning GTP hydrolysis and GTP[γS] binding (see Table 1) indicate that the former parameter is not suitable to assess quantitatively G_i-protein activation by chemoattractants. Rather, CTX-catalysed ADP-ribosylation is useful in revealing qualitative differences in the G_i-protein activation state caused by fMLP, C5a and LTB_4.

Differences in the effects of chemoattractants on G_i-protein activation were not only apparent with respect to CTX-catalysed ADP-ribosylation but also with regard to the effects of chloride salts of monovalent cations on GTP hydrolysis. Specifically, cations enhanced the effectiveness of fMLP and LTB_4 in activating GTP hydrolysis, whereas they decreased that of C5a (see Table 2). It is noteworthy that NaCl and KCl were similarly effective with respect to LTB_4, but with respect to fMLP, KCl was more effective than NaCl (see Table 2). When C5a is considered, LiCl was the most effective inhibitor (see Table 2). Thus, the differential effects of salts on chemoattractant GTP hydrolyses support our assumption that there are not only qualitative differences in the G_i-protein activation state induced by fMLP and LTB_4, but also between fMLP and C5a and between C5a and LTB_4.

On the level of intact cells, we observed differences between the three chemoattractants as well. Specifically, the order of effectiveness of agonists in inducing Ca^{2+} influx was fMLP > C5a > LTB_4 (see Fig. 4). In a recent study, we showed that Ca^{2+} influx is required for activation of O_2^- formation and β-glucuronidase release [3]. In accordance with the aforementioned data, the order of effectiveness of chemoattractants in activating O_2^- formation and exocytosis was found to be fMLP > C5a > LTB_4 (ineffective) (see Table 3). In agreement with our results, Stutchfield and Cockcroft [27] reported that C5a is less effective than fMLP in activating phosphatidic acid formation in Bt_2cAMP-differentiated HL-60 cells. Thus, fMLP is a full secretagogue, C5a is a partial secretagogue and LTB_4 is an incomplete secretagogue (as it induces rises in $[Ca^{2+}]_i$ but not O_2^- formation or exocytosis). Histamine, acting via H_1-receptors, is also an incomplete secretagogue in Bt_2cAMP-differentiated HL-60 cells [28]. However, unlike the effects of LTB_4 on $[Ca^{2+}]_i$, those of histamine show substantial PTX-insensitivity (see Fig. 4) [28].

Differences in receptor desensitization may account, at least in part, for the differential effectiveness of chemoattractants in activating Ca^{2+} influx, O_2^- formation and β-glucuronidase release. Indeed, the order of sensitivity of chemoattractants towards homologous desensitization was LTB_4 > C5a > fMLP (resistant) (see Fig. 5). This order correlates inversely with that concerning activation of Ca^{2+} influx, O_2^- formation and exocytosis (compare Fig. 5 with Fig. 4 and Table 3). Like LTB_4-induced rises in $[Ca^{2+}]_i$, those induced by histamine in Bt_2cAMP-differentiated HL-60 cells are very sensitive to homologous desensitization [28]. Differences in homologous desensitization of receptors could also provide an explanation for the findings that C5a-induced rises in $[Ca^{2+}]_i$ and NADPH oxidase activation are shorter than those induced by fMLP (see Fig. 4) [10, 11]. Interestingly, C5a receptors are also more sensitive to desensitization via protein kinase C than formyl peptide receptors [29]. In contrast, cAMP-increasing substances inhibit fMLP-induced exocytosis, whereas C5a-induced β-glucuronidase release is unaffected [12].

Quantitative differences in activation of G_i-protein α-subunits do not contribute to the differential effects of fMLP, C5a and LTB_4 on Ca^{2+} influx, O_2^- formation and exocytosis as they showed very similar effects on GTP hydrolysis, GTP[γS] binding and photolabeling (see Table 1 and Fig. 2). However, the differences between the chemoattractants

concerning CTX-catalysed ADP-ribosylation (see Fig. 3) and regulation by chloride salts of monovalent cations (see Table 2) of GTP hydrolysis suggest that qualitative differences in G_i-protein activation states finally result in differential cellular effects of fMLP, C5a and LTB_4. The molecular basis for this differential G_i-protein activation remains to be determined.

Recently, Amatruda *et al.* [30] reported that in transfected COS-7 cells, C5a receptors mediate activation of phospholipase C through the PTX-insensitive G-protein, $G_{\alpha 16}$, a member of the G_q family. Moreover, C5a activates phospholipase C through a partially PTX-insensitive mechanism in the monocytic cell line, THP-1, which expresses $G_{\alpha 16}$ at high concentrations [30]. Furthermore, C5a mediates Ca^{2+} influx in Bt_2cAMP-differentiated U937 cells through a PTX-insensitive mechanism [31]. Evidently, activation by C5a of phospholipase C via $G_{\alpha 16}$ or another PTX-insensitive G-protein of the G_q-family is not of relevance in Bt_2cAMP-differentiated HL-60 cells, as C5a-induced Ca^{2+} mobilization was abolished by PTX (see Fig. 4). Additionally, PTX-insensitive G-proteins are also not of particular importance for mediation of C5a-induced Ca^{2+} influx in Bt_2cAMP-differentiated HL-60 cells (see Fig. 4). In human neutrophils, C5a-induced Ca^{2+}-influx is PTX-sensitive as well [31]. Taken together, these findings show that the coupling of C5a receptors to G_i-proteins and PTX-insensitive G-proteins in myeloid cells shows substantial cell type specificity.

Compared to C5a, fMLP-induced Ca^{2+} influx in Bt_2cAMP-differentiated HL-60 cells shows substantial PTX-insensitivity (see Fig. 4). However, fMLP did not show any stimulatory effect on GTPase and GTP[γS] binding in membranes of PTX-treated cells (see Table 1). An explanation for this apparent discrepancy could be the fact that the guanine nucleotide exchange rates of PTX-insensitive G-proteins of the G_q-family are low [32] so that their activation in membranes is masked by the rapidly exchanging G_i-proteins.

In conclusion, fMLP, C5a and LTB_4 activate the G-proteins, $G_{i\alpha 2}$ and $G_{i\alpha 3}$, in Bt_2cAMP-differentiated HL-60 cells, but they do so in different manners. Our data suggest that G_i-proteins are not static signal amplifiers, but that G_i-proteins transduce and enhance signals differentially, depending on the type of chemoattractant receptor being activated. Unlike fMLP, C5a is only a partial secretagogue in Bt_2cAMP-differentiated HL-60 cells and this may be due to differences in homologous receptor desensitization and qualitative G_i-protein activation.

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