Lipopeptides activate G_i-proteins in dibutyryl cyclic AMP-differentiated HL-60 cells

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Synthetic lipopeptides activate superoxide-anion (O_2^-) formation in human neutrophils in a pertussis-toxin (PTX)-sensitive manner, suggesting the involvement of G-proteins of the G_i family in the signal-transduction pathway. We compared G-protein activation by lipopeptides and the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP) in dibutyryl-cyclic-AMP-differentiated HL-60 cells. The lipopeptide (2*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoyl-SK_4 (Pam_3AhhSK_4) and fMLP activated high-affinity GTPase, i.e. the enzymic activity of G-protein α-subunits, in HL-60 membranes in a time- and protein-dependent manner, but they had no effect on Mg^{2+}-ATPase and Na^+/K^+-ATPase. Pam_3AhhSK_4 and fMLP increased $V_{max.}$ of GTP hydrolysis. Pam_3AhhSK_4 activated GTP hydrolysis with half-maximal and maximal effects at about 2 μM and 10 μM respectively. Other lipopeptides activated GTP hydrolysis as well. Lipopeptides were less effective than fMLP to activate GTPase. In membranes from PTX-treated cells, the stimulatory effects of lipopeptides and fMLP on GTPase were abolished. In *N*-ethylmaleimide-treated membranes, the relative stimulatory effect of Pam_3AhhSK_4 on GTP hydrolysis was enhanced, whereas that of fMLP was diminished. fMLP and Pam_3AhhSK_4 activated GTPase in an over-additive manner in *N*-ethylmaleimide-treated membranes. Unlike fMLP, Pam_3AhhSK_4 did not enhance incorporation of GTP azidoanilide into, and cholera-toxin-catalysed ADP-ribosylation of G_i-protein α-subunits in, HL-60 membranes and did not induce rises in cytosolic Ca^{2+} concentration. Pam_3AhhSK_4 and fMLP stimulated phosphatidic acid formation in a PTX-sensitive manner. Pam_3AhhSK_4 itself did not activate O_2^- formation, but potentiated the stimulatory effects of fMLP. Our data suggest that (i) lipopeptides activate the GTPase of G_i-proteins, (ii) lipopeptides and fMLP activate G_i-proteins differently, (iii) lipopeptides stimulate phospholipase D via G_i-proteins, and (iv) phosphatidic acid formation is not sufficient for activation of O_2^- formation.

INTRODUCTION

Human neutrophils play an important role in host defence against bacterial infections and are activated by the bacterial chemotactic peptide fMLP (for review see Rossi, 1986; Seifert and Schultz, 1991). fMLP, after binding to specific heptahelical membrane receptors, activates the PTX-sensitive G-proteins, G_{i2} and G_{i3} (Gierschik et al., 1989; Offermanns et al., 1990). This process leads to the activation of phospholipase C and of non-selective cation channels (resulting in an increase in $[Ca^{2+}]_i$) and of phospholipase D (resulting in PA formation) (Pai et al., 1988; Bauldry et al., 1991; Kessels et al., 1991; Seifert et al., 1992a; Krautwurst et al., 1992). Stimulation by fMLP of neutrophils and differentiated HL-60 leukaemic cells cumulates in the activation of the O_2^--forming NADPH oxidase (for review see Rossi, 1986; Seifert and Schultz, 1991).

The outer cell wall of Gram-negative bacteria contains lipoprotein (for review see Braun, 1975). Lipoprotein and synthetic lipopeptides are effective activators of B-lymphocytes (Resch and Bessler, 1981; Bessler et al., 1985). We have shown recently that lipopeptides activate O_2^- formation in human neutrophils in a PTX-sensitive manner, suggesting the involvement of G_i-proteins in the signal-transduction pathway (Seifert et al., 1990). As there is no known heptahelical receptor for lipopeptides, and as only lipopeptides bearing positive charges activate O_2^- formation, we put forward the hypothesis that lipopeptides activate G_i-proteins directly, i.e. in a manner similar to that of other cationic-amphiphilic peptides such as substance P and the wasp venom mastoparan (Higashijima et al., 1988, 1990; Mousli et al., 1990; Seifert et al., 1990; Tomita et al., 1991). However, the cationic-amphiphilic lipopeptides Pam_3CSK_4 and Pam_3AdhSK_4 do not stimulate GTP hydrolysis by G-proteins in HL-60 membranes (Seifert et al., 1992b). Additionally, we have shown that lipopeptides, i.e. Pam_3CSK_4 and Pam_3AhhSK_4, activate tyrosine phosphorylation in Bt_2cAMP-differentiated HL-60 cells (Offermanns et al., 1992). The latter finding prompted us to examine the effects of Pam_3AhhSK_4 on G-protein activation in membranes from Bt_2cAMP-differentiated HL-60 cells and to study its effects on $[Ca^{2+}]_i$, PA formation and O_2^- formation in these cells.

MATERIALS AND METHODS

Materials

The lipopeptides Pam_3AhhSK_4 Pam_3CSK_4RPQASGVYMGNLTAQ and Pam_3CSK_4RPQASVYMNLTAQ were kindly provided by Dr. J. Metzger, Dr. K.-H. Wiesmüller and Dr. G. Jung, Institut für Organische Chemie der Universität Tübingen, Germany. The lipopeptide Pam_3CSK_4YGGFL was kindly provided by Dr. C. Sakarellos, Department of Chemistry, University

Abbreviations used: Bt_2cAMP, dibutyryl cAMP; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CTX, cholera toxin; G_i-proteins, family of highly similar G-proteins (G_{i1}–G_{i3}); fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; NEM, *N*-ethylmaleimide; O_2^-, superoxide anion; PA, phosphatidic acid, Pam_3, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]; Pam_3Adh, (2*S*)-palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl; Pam_3Ahh, (2*S*)-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoyl; PTX, pertussis toxin. Peptide chains linked to Pam_3, Pam_3Adh and Pam_3Ahh are given in the one-letter code.

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of Ioannina, Greece. PTX was from List Biological Laboratories (Campbell, CA, U.S.A.). 1-*O*-[^{3}H]Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (60 Ci/mmol) was from Dupont/New England Nuclear (Bad Homburg, Germany). NEM was from Sigma Chemie (Deisenhofen, Germany). Stock solutions of lipopeptides (1 mM each) and dilutions were prepared in distilled water and stored at −20 °C. Sources of other materials have been described elsewhere (Ebel et al., 1976; Rosenthal et al., 1986; Seifert and Schultz, 1987; Seifert et al., 1990, 1992a,b; Offermanns et al., 1990, 1991; Krautwurst et al., 1992; Wenzel-Seifert and Seifert, 1993).

Cell culture and membrane preparation

HL-60 cells were grown in suspension culture at 37 °C and were differentiated towards neutrophil-like cells with Bt_2cAMP (0.2 mM) for 48 h (Wenzel-Seifert and Seifert, 1993). For determination of the activities of high-affinity GTPase, Mg^{2+}-ATPase and Na^+/K^+-ATPase, and for photolabelling, HL-60 membranes were prepared as described by Seifert and Schultz (1987). PTX (100 ng/ml) or its carrier (control) was added to cell cultures 24 h before measurement of PA formation or membrane preparation. Under these conditions, more than 95% of G_i-protein α-subunits were ADP-ribosylated (results not shown).

GTPase assay

GTP hydrolysis was determined as described by Wenzel-Seifert and Seifert (1993). Reaction mixtures (100 μl) contained membranes from Bt_2cAMP-differentiated HL-60 cells (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′-[βγ-imido]triphosphate, 5 mM phosphocreatine, 40 μg of creatine kinase, 1 mM dithiothreitol and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. Reaction mixtures contained substances at various concentrations and were preincubated for 3 min at 25 °C. Reactions were initiated by addition of [γ-^{32}P]GTP and were conducted for 10–20 min, unless stated otherwise. Low-affinity GTPase activity was determined in the presence of 50 μM GTP and amounted to < 5% of total GTPase activity.

Treatment of HL-60 membranes with NEM

HL-60 membranes were treated with NEM as described by McLeish et al., (1989) with modifications. Briefly, membranes (5.0 μg of protein/tube) were incubated for 10 min in reaction mixtures containing 0.1 mM NEM or solvent (control), 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 phosphocreatine, 40 μg of creatine kinase and 0.2% BSA in 50 mM triethanolamine/HCl, pH 7.4. Thereafter, dithiothreitol (5 mM) was added, and reaction mixtures were incubated for a further 10 min. The membranes were then used immediately for the GTPase assay.

Mg^{2+}-ATPase and Na^+/K^+-ATPase assays

The activities of Mg^{2+}-ATPase and Na^+/K^+-ATPase in HL-60 membranes were determined as described by Ebel et al. (1976). Reaction mixtures (100 μl) contained 6.0 μg of protein and solvent (control), Pam_3AhhSK_4 (10 μM) or fMLP (10 μM). The amount of P_i in supernatant fluids of reaction mixtures was determined as described by Ames (1966).

Assay for photolabelling of membrane proteins

HL-60 membranes (50 μg of protein in 60 μl) were incubated at 30 °C in a buffer consisting of 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 10 μM GDP and 30 mM Hepes/NaOH, pH 7.4. After exposure to various substances, samples were incubated for another 3 min with 10 nM [α-^{32}P]GTP azidoanilide (1 μCi/tube). Stopping of reactions and irradiation of samples were performed as described by Offermanns et al. (1990).

Assay for CTX-catalysed ADP-ribosylation of membrane proteins

HL-60 cells were homogenized by 20 passes through a 26-gauge needle in a buffer consisting of 150 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 20 mM Hepes/NaOH, pH 7.4. After centrifugation at 1000 *g* for 10 min at 4 °C, the supernatant suspension was incubated for 60 min at 30 °C in the above buffer supplemented with 1 mM guanosine 5′-[βγ-imido]triphosphate. Thereafter, the suspension was centrifuged at 30000 *g* for 15 min at 4 °C. The pellet, referred to as membranes, was dissolved in a buffer containing 1 mM EDTA and 10 mM Tris/HCl, pH 7.4. CTX was activated by mixing toxin stock solution (2 mg/ml) with an equal volume of 40 mM dithiothreitol and subsequent incubation for 10 min at 30 °C. Reaction mixtures (50 μl) contained 3 μM [^{32}P]NAD^+ (5 μCi/tube), 3 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, 0.2% BSA, 2 μg of activated CTX and 0.1 M potassium phosphate, pH 7.4, and various substances. After preincubation for 3 min, reactions were initiated by addition of membranes from about 1×10^7 HL-60 cells. After incubation for 60 min at 30 °C, reactions were terminated by addition of 20 mM Hepes/NaOH, pH 7.4 (4 °C), and centrifugation at 12000 *g* for 10 min at 4 °C.

Assay for PA formation

PA formation was determined as described by Pai et al. (1988) and Bourgoin and Grinstein (1992), with modifications. In brief, HL-60 cells were centrifuged at 250 *g* for 10 min at 20 °C and were suspended at 1×10^6 cells/ml in a buffer consisting of 125 mM NaCl, 0.7 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, 0.1% fatty-acid-free BSA and 25 mM Hepes/NaOH, pH 7.2. Centrifugation was repeated. HL-60 cells were suspended at 2×10^7 cells/ml in the above buffer and incubated for 90 min at 37 °C in the presence of 10–15 μCi/ml 1-*O*-[^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine. Thereafter, cells were diluted to 1×10^6 cells/ml in the above buffer and were centrifuged at 250 *g* for 10 min at 20 °C. HL-60 cells [$(0.5–1.0) \times 10^6$ cells in 100 μl] were incubated for 6 min at 37 °C in the presence of 1 mM $CaCl_2$ and 1 μg/ml cytochalasin B. Cells were exposed to solvent (control), Pam_3AhhSK_4 (10 μM) or fMLP (1 μM) for 15 min. Reactions were terminated by the addition of 500 μl of $CHCl_3/CH_3OH$/conc. HCl (400:200:1, by vol.). Phase separation was achieved by addition of 250 μl of water and 150 μl of $CHCl_3$. The samples were centrifuged at 12000 *g* for 10 min at 4 °C. The upper phase was removed, and 400 μl of the organic phase was dried under nitrogen at 30 °C. Lipids were dissolved in 50 μl of $CHCl_3$ and spotted on t.l.c. plates (Whatman LK 6 D). Non-radioactive lipid standards were added, and the plates were developed in a solvent system consisting of $CHCl_3/CH_3OH$/acetic acid (13:3:3, by vol). Lipid standards were detected by exposure to iodine vapour, and the areas

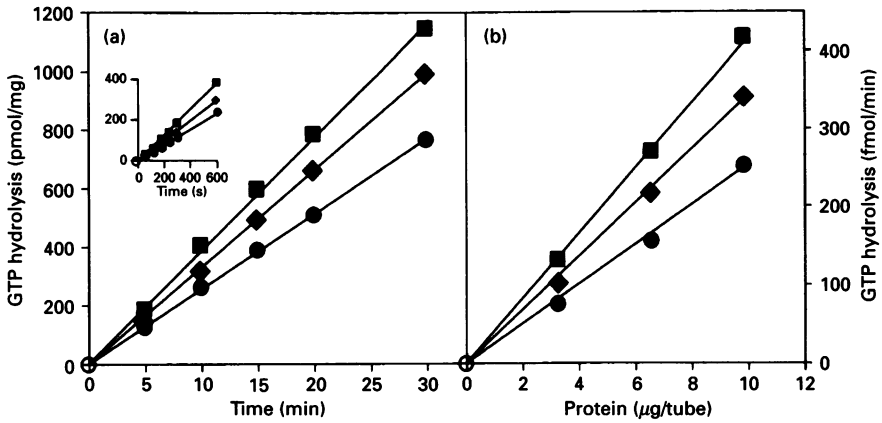

Figure 1 Time- and protein-dependence of high-affinity GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells

High-affinity GTP hydrolysis was determined as described in the Materials and methods section in the presence of Pam_3AhhSK_4 (10 μM) (◆), fMLP (10 μM) (■), or water added instead of stimulus (control) (●). (**a**) GTP hydrolysis as a function of time. Reactions were conducted for the indicated periods of time. Reaction mixtures contained 5.7 μg of protein (main panel) and 6.2 μg of protein (inset). (**b**) GTP hydrolysis as a function of the amount of protein. Reaction mixtures contained the indicated amounts of protein, and reactions were conducted for 15 min. The open circles indicate that at the designated incubation time and amount of protein no GTP was hydrolysed. The stimulatory effects of Pam_3AhhSK_4 on GTP hydrolysis were significant versus control ($P < 0.05$, Wilcoxon test) under all conditions studied.

corresponding to PA were scraped off after sublimation of iodine. Lipids were eluted from the silica gel with 1 M HCl/CH_3OH (1/1, v/v), and radioactivity was determined in a liquid-scintillation counter.

Assay for O_2^- formation

O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase, by using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) (Seifert et al., 1990). Bt_2cAMP-differentiated HL-60 cells (2.5×10^6 cells in 500 μl) were incubated for 3 min at 37 °C before addition of stimuli. The absolute amounts of O_2^- generated within 10 min were calculated.

Miscellaneous

Protein was determined by the method of Lowry et al. (1951). [γ-^{32}P]GTP was prepared as described by Walseth et al. (1991). [α-^{32}P]GTP azidoanilide was prepared as described by Offermanns et al. (1991). [^{32}P]NAD was synthesized as described by Cassel and Pfeuffer (1978). SDS/PAGE and autoradiography were performed as described by Rosenthal et al. (1986). $[Ca^{2+}]_i$ was determined by using the fluorescent dye fura-2, as described by Seifert et al. (1992a).

Data reproducibility

Data shown in Figures 1–3 and 5 and Tables 1–3 are the means of assay quadruplicates. Unless shown, the S.D. values were generally less than 5% (GTP hydrolysis) and 10% (O_2^- formation) of the means. Similar results were obtained with at least three different preparations of HL-60 membranes or intact HL-60 cells. Basal GTP hydrolysis and the extent of GTPase stimulation caused by fMLP and Pam_3AhhSK_4 (10 μM each) varied to some extent among different membrane preparations from Bt_2cAMP-differentiated HL-60 cells (see Figures 1–3 and Tables 1 and 2). Similar findings have been documented for basal and fMLP-stimulated GTP hydrolysis in membranes from dimethyl sulphoxide-differentiated HL-60 cells (McLeish et al., 1989). The autoradiographs shown in Figure 4 are representative of at least three independent experiments.

RESULTS

First, the time- and protein-dependence of high-affinity GTP hydrolysis in HL-60 membranes was studied. In the presence of 5.7 μg of protein per tube, Pam_3AhhSK_4 and fMLP (10 μM each) stimulated GTP hydrolysis in a linear manner for up to 30 min (Figure 1). Activation of GTPase by the lipopeptide and fMLP occurred without measurable delay (see Figure 1a, inset). At an incubation time of 15 min, basal, lipopeptide- and fMLP-stimulated GTP hydrolyses were linear up to 10 μg of protein per tube.

Figure 2 shows typical Lineweaver–Burk plots of basal, Pam_3AhhSK_4- and fMLP-stimulated GTP hydrolyses in HL-60 membranes. The K_m of basal high-affinity GTPase in HL-60 membranes was 0.45 ± 0.12 μM (mean ± S.D., $n = 6$). This value corresponds to the K_m of high-affinity GTPase in human and rabbit neutrophil membranes (Feltner et al., 1986; Kupper et al., 1992). The lipopeptide and the formyl-peptide increased $V_{max.}$ of GTP hydrolysis in HL-60 membranes without affecting K_m.

The influence of Pam_3AhhSK_4 and fMLP on Mg^{2+}-ATPase and Na^+/K^+-ATPase was studied. The activities of these enzymes in HL-60 membranes were 0.26 ± 0.06 and 0.15 ± 0.02 μmol/min per mg respectively. Pam_3AhhSK_4 and fMLP (10 μM each) did not alter these enzyme activities (results not shown).

Concentration/response curves for the stimulatory effects of Pam_3AhhSK_4 and fMLP on high-affinity GTPase in HL-60 membranes are shown in Figure 3. Pam_3AhhSK_4 activated GTP hydrolysis with an EC_{50} of about 2 μM and a maximum at 10 μM. fMLP activated GTPase with an EC_{50} of 0.5 μM and a plateau at 30–100 μM. The effectiveness of Pam_3AhhSK_4 (100 μM) to activate GTPase was about 30% of that of fMLP (100 μM).

The effects of four lipopeptides (10 μM each) on GTP hydrolysis were compared (see Figure 3, inset). Pam_3CSK_4RPQASGVYMGNLTAQ contains the epitope of lymphocytic choriomeningitis virus nucleoprotein, RPQASGVYMGNLTAQ, used for induction of cytotoxic T-lymphocytes *in vivo* (Schulz et al.,

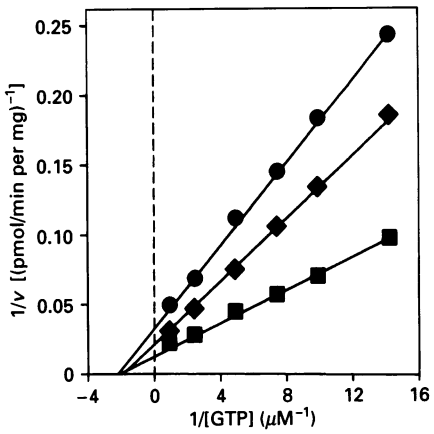

Figure 2 Kinetic analysis of high-affinity GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells

GTP hydrolysis was determined as described in the Materials and methods section with GTP (0.07–1.0 μM) in the presence of Pam_3AhhSK_4 (10 μM) (◆), fMLP (10 μM) (■), or water added instead of stimulus (control) (●). Lineweaver–Burk plots of typical experiments are shown. The stimulatory effects of Pam_3AhhSK_4 on GTP hydrolysis were significant versus control ($P < 0.05$, Wilcoxon test) under all conditions studied.

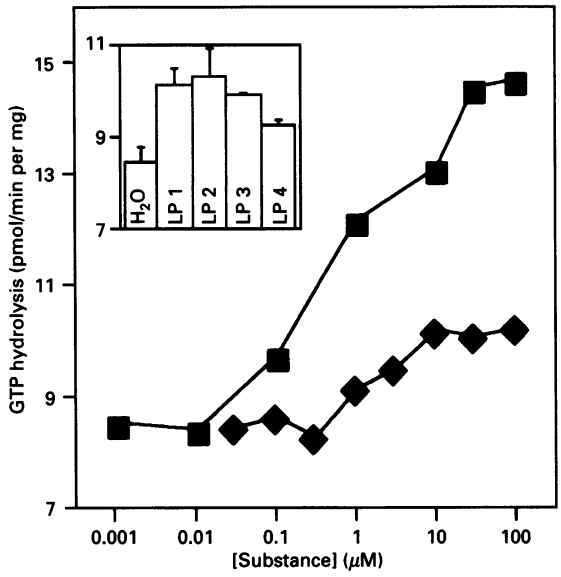

Figure 3 Concentration/response curves for effects of Pam_3AhhSK_4 and fMLP on high-affinity GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells: comparison of the effects of various lipopeptides

High-affinity GTPase activity in HL-60 membranes was determined as described in the Materials and methods section. Main panel: effects of Pam_3AhhSK_4 (◆) and fMLP (■) at various concentrations on GTP hydrolysis. Inset shows effects of various lipopeptides (10 μM each) on GTP hydrolysis: Pam_3AhhSK_4 (LP1), Pam_3CSK_4RPQASVYMNLTAQ (LP2), Pam_3CSK_4RPQASGVYMGNLTAQ (LP3), Pam_3CSK_4YGGFL (LP4).

1991). The peptide RPQASVYMNLTAQ, contained in Pam_3CSK_4RPQASVYMNLTAQ, lacks two glycine residues and was prepared as control substance for induction of cytotoxic T-lymphocytes (Schulz et al., 1991). Pam_3CSK_4YGGFL contains the Leu-enkephalin pentapeptide, YGGFL. Pam_3CSK_4RPQASGVYMGNLTAQ and Pam_3CSK_4RPQASVYMNLTAQ were similarly effective as Pam_3AhhSK_4 to stimulate GTPase, whereas Pam_3CSK_4YGGFL was by about 50% less effective.

Table 1 Effect of PTX on high-affinity GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells

Treatments with carrier (control) and PTX were performed as described in the Materials and methods section. High-affinity GTPase activity in HL-60 membranes was determined as described in the Materials and methods section. For determination of basal GTP hydrolysis, water (solvent) was added instead of stimulus.

	GTP hydrolysis (pmol/min per mg)	
Stimulus	Control	PTX
Water (solvent)	26.0 ± 0.5	15.4 ± 0.7
fMLP (10 μM)	61.4 ± 2.1	14.8 ± 0.5
Pam_3AhhSK_4 (10 μM)	35.2 ± 1.0	15.5 ± 0.6

Table 2 Effect of NEM on high-affinity GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells: interaction of Pam_3AhhSK_4 with fMLP

Treatments with solvent (control) and NEM were performed as described in the Materials and methods section. High-affinity GTPase activity in HL-60 membranes was determined as described in the Materials and methods section. For determination of basal GTP hydrolysis, water (solvent) was added instead of stimulus.

	GTP hydrolysis (pmol/min per mg)	
Stimulus	Control	NEM
Water (solvent)	12.8 ± 0.2	6.5 ± 0.1
fMLP (10 μM)	21.4 ± 0.8	9.1 ± 0.2
Pam_3AhhSK_4 (10 μM)	17.0 ± 0.5	9.5 ± 0.2
fMLP + Pam_3AhhSK_4	23.3 ± 0.4	13.3 ± 0.4

Table 1 compares the effects of Pam_3AhhSK_4 and fMLP on GTP hydrolysis in control membranes and in membranes from PTX-treated cells. In membranes from PTX-treated cells, the stimulatory effects of fMLP and Pam_3AhhSK_4 on GTP hydrolysis were abolished. Similarly to Pam_3AhhSK_4, Pam_3CSK_4RPQASGVYMGNLTAQ, Pam_3CSK_4RPQASVYMNLTAQ and Pam_3CSK_4YGGFL (10 μM each) had no effect on GTP hydrolysis in membranes from PTX-treated cells (results not shown).

NEM alkylates G_i-protein α-subunits, thereby uncoupling receptors from G-proteins in a manner similar to that of PTX (Jakobs et al., 1982; McLeish et al., 1989). In control and NEM-treated membranes, fMLP stimulated GTPase by 67% and 40% respectively (Table 2). The corresponding values for Pam_3AhhSK_4 were 33% and 46% respectively. In control membranes, Pam_3AhhSK_4 and fMLP activated GTP hydrolysis in a sub-additive manner (82% stimulation), and in NEM-treated membranes they interacted in an over-additive manner (105% stimulation).

In addition to the determination of GTP hydrolysis, photolabelling with the reactive GTP analogue GTP azidoanilide is an established method to assess activation of G_i-protein α-subunits by formyl-peptide receptors. As reported for membranes from dimethyl sulphoxide-differentiated HL-60 cells (Offermanns et al., 1990), fMLP (10 μM) increased incorporation of GTP azidoanilide into 40/41 kDa proteins, corresponding to the α-subunits of G_{i2} and G_{i3}, in membranes from Bt_2cAMP-differentiated HL-60 cells, but Pam_3AhhSK_4 (10 μM) had no stimulatory effect (Figure 4).

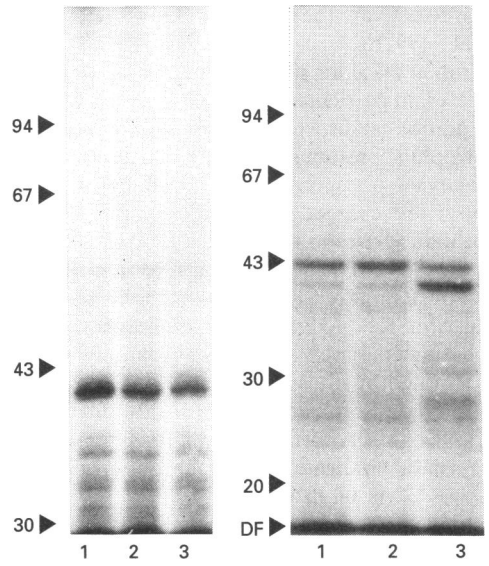

Figure 4 Effects of Pam_3AhhSK_4 and fMLP on incorporation of GTP azidoanilide into, and CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in membranes from Bt_2cAMP-differentiated HL-60 cells

(**a**) Photolabelling was performed as described in the Materials and methods section. The autoradiogram of an SDS gel containing 4 M urea and 9% (w/v) acrylamide is shown. Lane 1, fMLP (10 μM); lane 2, water added instead of stimulus (control); lane 3, Pam_3AhhSK_4 (10 μM). (**b**) ADP-ribosylation was performed as described in the Materials and methods section. The autoradiogram of an SDS gel containing 10% (w/v) acrylamide is shown. Lane 1, Pam_3AhhSK_4 (10 μM); lane 2, water added instead of stimulus (control); lane 3, fMLP (10 μM). Numbers on the left are molecular masses of marker proteins (kDa). DF, dye front.

Table 3 Stimulation by Pam_3AhhSK_4 and fMLP of PA formation in Bt_2cAMP-differentiated HL-60 cells: effect of PTX

Treatments with carrier (control) and PTX were performed as described in the Materials and methods section. PA formation was determined as described in the Materials and methods section. The concentration of fMLP was 1 μM and that of Pam_3AhhSK_4 was 10 μM. For determination of basal PA formation, water (solvent) was added instead of stimulus.

	PA formation (d.p.m.)	
Stimulus	Control	PTX
Water (solvent	652 ± 36	458 ± 6
fMLP	924 ± 66	492 ± 27
Pam_3AhhSK_4	799 ± 33	497 ± 21

Moreover, CTX-catalysed ADP-ribosylation of G_i-protein α-subunits was studied. By analogy to dimethyl sulphoxide-differentiated HL-60 cells (Gierschik et al., 1989), fMLP (10 μM) enhanced CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in membranes from Bt_2cAMP-differentiated HL-60 cells (see Figure 4). Unlike fMLP, Pam_3AhhSK_4 (10 μM) did not enhance ADP-ribosylation of G_i-protein α-subunits.

We investigated the effects of Pam_3AhhSK_4 on $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells. The lipopeptide (0.3–10 μM) did not induce rises in $[Ca^{2+}]_i$ in HL-60 cells (results not shown). By contrast, fMLP potently and effectively increases $[Ca^{2+}]_i$ in Bt_2cAMP-differentiated HL-60 cells (Seifert et al., 1992a,b).

The effects of lipopeptides and fMLP on PA formation are shown in Table 3. Pam_3AhhSK_4 (10 μM) and fMLP (1 μM) increased PA formation by 23% and 42% respectively. In PTX-treated cells, the stimulatory effects of Pam_3AhhSK_4 and fMLP on PA formation were greatly diminished. In the presence of ethanol (0.5%, v/v), Pam_3AhhSK_4 (10 μM) and fMLP (1 μM) stimulated phosphatidylethanol formation to a similar extent as PA formation (results not shown).

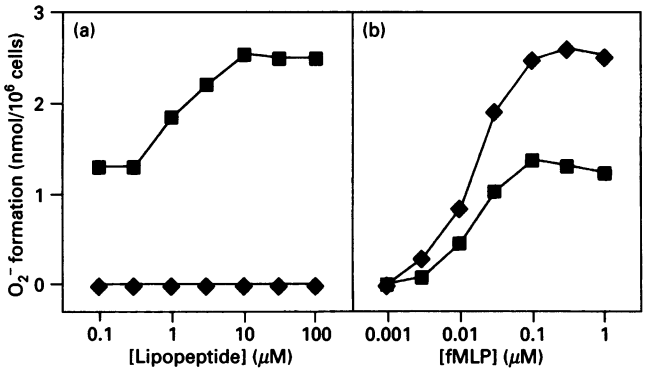

Figure 5 Effects of Pam_3AhhSK_4 on O_2^- formation in Bt_2cAMP-differentiated HL-60 cells: synergism with fMLP

O_2^- formation was determined as described in the Materials and methods section. (**a**) Cells were exposed to Pam_3AhhSK_4 at various concentrations in the presence of fMLP (1 μM) (■) or water instead of fMLP (◆). (**b**) Cells were exposed to fMLP at various concentrations in the presence of Pam_3AhhSK_4 (10 μM) (◆) or water instead of Pam_3AhhSK_4 (■).

Regulation by lipopeptides of O_2^- formation in HL-60 cells is depicted in Figure 5. Pam_3AhhSK_4 itself up to 100 μM did not activate O_2^- formation. However, Pam_3AhhSK_4 potentiated O_2^- formation induced by fMLP (1 μM) with an EC_{50} of about 2 μM and a maximum at 10 μM. fMLP activated O_2^- formation with an EC_{50} of 15 nM and a maximum at 100 nM. Pam_3AhhSK_4 (10 μM) substantially enhanced the effect of fMLP at sub-maximally and maximally effective concentrations.

DISCUSSION

Lipopeptides activate O_2^- formation in human neutrophils in a PTX-sensitive manner, suggesting the involvement of G_i-proteins in the signal-transduction pathway (Seifert et al., 1990). So far, however, a heptahelical receptor for lipopeptides has not yet been identified. In addition, lipopeptides, unlike chemoattractants, do not induce rises in $[Ca^{2+}]_i$ in HL-60 cells (Wenzel-Seifert and Seifert, 1993; Seifert et al., 1992a,b). Thus it is unlikely that lipopeptides activate Bt_2cAMP-differentiated HL-60 cells through a chemoattractant receptor with known ligands or through the newly cloned formyl-peptide-related receptor with as-yet unknown ligands (Murphy et al., 1992). Therefore we have put forward the hypothesis that lipopeptides activate G_i-proteins directly, i.e. in a receptor-independent manner (Seifert et al., 1990). This assumption is supported by the finding that only lipopeptides bearing positive charges activate O_2^- formation in human neutrophils (Seifert et al., 1990). By analogy to lipopeptides, the stimulatory effects of various direct G-protein activators in neutrophils are inhibited, at least in part, by PTX (Serra et al., 1988; Perianin and Snyderman, 1989; Seifert et al., 1990, 1992b; Norgauer et al., 1992; Kanaho et al., 1992). To corroborate our suggestion further, we have now studied the effects of lipopeptides in Bt_2cAMP-differentiated HL-60 cells, as these cells are a useful model system for the analysis of G_i-protein-mediated signal-transduction processes at the

cellular and membrane level (Krautwurst et al., 1992; Seifert et al., 1992a; Wenzel-Seifert and Seifert, 1993).

We found that the lipopeptide Pam_3AhhSK_4 activated high-affinity GTP hydrolysis in HL-60 membranes in a time-, membrane-protein- and stimulus-concentration-dependent manner (see Figures 1 and 3). Most importantly, the stimulatory effects of lipopeptides on GTPase were inhibited by PTX, as were those of fMLP (see Table 1). These data indicate that lipopeptides increase the GTPase activity of G_i-proteins. Pam_3AhhSK_4 increased $V_{max.}$ of GTP hydrolysis, suggesting that it stimulated the catalytic rate of GTP turnover (see Figure 2). By analogy with lipopeptides, formyl-peptides also increase $V_{max.}$ of GTP hydrolysis (see Figure 2) (Feltner et al., 1986). The stimulatory effect of Pam_3AhhSK_4 on high-affinity GTPase was not unspecific, inasmuch as the lipopeptide did not affect the activities of other nucleotide-metabolizing enzymes, i.e. Mg^{2+}-ATPase and Na^+/K^+-ATPase, in HL-60 membranes.

NEM, through alkylation of G_i-protein α-subunits, disrupts the interaction of heptahelical receptors with G-proteins in a manner similar to that of PTX-catalysed ADP-ribosylation (Jakobs et al., 1982; McLeish et al., 1989). With respect to the interaction of formyl-peptide receptors with G_i-protein α-subunits, alkylation and ADP-ribosylation have similar consequences, i.e they are inhibitory (see Tables 1 and 2) (McLeish et al., 1989). Concerning the interaction of lipopeptides with G_i-proteins, ADP-ribosylation is also inhibitory (see Table 1). Unexpectedly, alkylation was found to enhance the relative extent of lipopeptide-stimulated GTP hydrolysis (see Table 2). These data suggest that alkylation and ADP-ribosylation of α-subunits may affect G-protein function in different manners. Alkylation of G_i-protein α-subunits, unlike ADP-ribosylation, may increase their lipophilicity, and thereby may facilitate their interaction with lipophilic portions of lipopeptides. It is also possible that alkylation and ADP-ribosylation induce different conformations of G_i-protein α-subunits, resulting in opposite effects concerning their interaction with lipopeptides, but not with regard to receptors. Additionally, Pam_3AhhSK_4 and fMLP activated GTPase in NEM-treated membranes in a synergistic manner. Taken together, these results suggest that lipopeptides and formyl-peptides differently activate the GTPase of G_i-proteins.

Certain lipopeptides, i.e. Pam_3CSK_4 and Pam_3AdhSK_4, do not stimulate GTP hydrolysis in membranes from Bt_2cAMP-differentiated HL-60 cells (Seifert et al., 1992b). It is possible that the extent of activation of G_i-proteins induced by these lipopeptides is too small to be detected in the GTPase assay. Pam_3CSK_4, Pam_3AdhSK_4 and Pam_3AhhSK_4 have in common the positively charged peptide chain, but they differ in the structure of the lipid moiety (Seifert et al., 1992b). With respect to GTP hydrolysis, only Pam_3AhhSK_4 is stimulatory (see Figure 3) (Seifert et al., 1992b). These differential effects of lipopeptides on GTP hydrolysis support the view that these substances do not activate the GTPase of G_i-proteins in an unspecific manner. Intriguingly, the results of a recent study indicate that lipophilic amino acids in the third cytoplasmic loop of heptahelical receptors may be more important for G-protein activation than are positively charged amino acids (Cheung et al., 1992). By analogy, the structure of the lipid moiety of lipopeptides is important for their G-protein-activating properties (see Figure 3) (Seifert et al., 1992b). However, the peptide chain is also of relevance for G-protein activation by lipopeptides. This assumption is supported by the findings that the lipopeptides Pam_3CSK_4, $Pam_3CSK_4RPQASGVYMGNLTAQ$, Pam_3CSK_4-$RPQASVYMNLTAQ$ and Pam_3CSK_4YGGFL have in common the lipid portion, but differ in the peptide chain and their effectiveness to activate the GTPase of G_i-proteins (see Figure 3) (Seifert et al., 1992b).

The microbial alkaloid staurosporine is a potent inhibitor of protein kinases, in particular of protein kinase C (Tamaoki et al., 1986). In addition, staurosporine stimulates phospholipase D in rabbit neutrophils, presumably via direct activation of G_i, but it does not activate phospholipase C (Kanaho et al., 1992). By analogy, positively charged lipopeptides inhibit protein kinase C and do not induce rises in $[Ca^{2+}]_i$ in HL-60 cells (Bessler, 1990; Seifert et al., 1992b). These findings prompted us to study the effects of Pam_3AhhSK_4 on phospholipase D in HL-60 cells. Pam_3AhhSK_4 activated the formation of PA and of phosphatidylethanol, a transphosphatidylation product specifically formed by phospholipase D (see Table 3) (Pai et al., 1988; Bourgoin and Grinstein, 1992). Additionally, stimulation by Pam_3AhhSK_4 of PA formation was PTX-sensitive (see Table 3). These data indicate that the lipopeptide activates phospholipase D via G_i-proteins.

In this context, the question arises whether the effects of Pam_3AhhSK_4 on phospholipase D were mediated via G_{i2}, G_{i3} or both G_i-proteins. Therefore we studied the effects of Pam_3AhhSK_4 on photolabelling and CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in HL-60 membranes. Both methods have been shown to be useful for the analysis of G_i-protein activation by formyl-peptide receptors in membranes from dimethyl sulphoxide-differentiated HL-60 cells (Gierschik et al., 1990; Offermanns et al., 1990). In agreement with the aforementioned studies, we found prominent stimulatory effects of fMLP on photolabelling and CTX-catalysed ADP-ribosylation of G_i-protein α-subunits in membranes from Bt_2cAMP-differentiated HL-60 cells (see Figure 4). However, Pam_3AhhSK_4 was devoid of any stimulatory effect in these regards. Thus we cannot yet answer the question as to which G_i-protein subtypes are involved in the activation of phospholipase D by lipopeptides. Interestingly, some lipopeptides do not measurably stimulate the GTPase of G_i-proteins (Seifert et al., 1992b). Moreover, even the most effective lipopeptides with regard to GTPase activation, i.e. Pam_3AhhSK_4, Pam_3CSK_4-$RPQASGVYMGNLTAQ$ and $Pam_3CSK_4RPQASVYLMNLT$-AQ, are still much less effective than fMLP (see Figure 3). Thus the sensitivity of photolabelling and CTX-catalysed ADP-ribosylation may be too low to detect the relatively small extent of G_i-protein activation by Pam_3AhhSK_4. However, it cannot be excluded that the lack of stimulatory effect of lipopeptides on photolabelling and CTX-catalysed ADP-ribosylation reflects also qualitative differences in activation of G_i-proteins by these substances and by formyl-peptides (McLeish et al., 1989).

The data obtained with Pam_3AhhSK_4 concerning stimulation of PA formation imply that an increase in $[Ca^{2+}]_i$ via activation of phospholipase C and/or non-selective cation channels is not a prerequisite for stimulation of phospholipase D (see Table 3) (Seifert et al., 1992b). In agreement therewith, Rosoff et al. (1988), Bourgoin et al. (1990) and Kanaho et al. (1992) suggested that phospholipase D is activated in a Ca^{2+}/phospholipase C-independent manner. However, in human neutrophils, rises in $[Ca^{2+}]_i$ may play a part in the activation of phospholipase D (Kessels et al., 1991; Bauldry et al., 1992).

The effectiveness of Pam_3AhhSK_4 to activate PA formation was about 50% of that of fMLP (see Table 3). Nonetheless, the lipopeptide itself did not activate O_2^- formation (see Figure 5). Thus stimulation of phospholipase D is not sufficient for the activation of NADPH oxidase. Our data are in agreement with those obtained by Kessels et al. (1991) and Bauldry et al. (1992). By analogy to the cytokines, granulocyte/macrophage-colony-stimulating factor and tumour necrosis factor-α, activation of

PA formation by lipopeptides may be important for potentiation of fMLP-induced O_2^- formation (see Table 3 and Figure 5) (Bourgoin et al., 1990; Bauldry et al., 1990).

In conclusion, we have shown that lipopeptides activate the GTPase of G_i-proteins and that lipopeptides and fMLP activate G_i-proteins differently. Lipopeptides activate phospholipase D via G_i-proteins, but PA formation is not sufficient for the activation of O_2^- formation.

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