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Incomplete functional differentiation of HL-60 leukemic cells by synthetic lipopeptides

Partial inhibition by pertussis toxin of enhanced superoxide formation

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In human neutrophils, the synthetic lipopeptide, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine [$Pam_3CysSer(Lys)_4$], activates NADPH-oxidase catalyzed superoxide (O_2^-) formation through pertussis-toxin-sensitive and pertussis-toxin-insensitive mechanisms (Seifert, R., Schultz, G., Richter-Freund, M., Metzger, J., Wiesmüller, K.-H., Jung, G., Bessler, W. G. & Hauschildt, S. (1990) *Biochem. J. 267*, 795–802). We studied the effects of lipopeptides on differentiation of HL-60 leukemic cells. $Pam_3CysSer(Lys)_4$ enhanced phorbol-12-myristate-13-acetate-induced O_2^- formation (presumably through the expression of components of NADPH oxidase) in a concentration-dependent manner with a half-maximal effect at 100 ng/ml and a maximum at 1 μg/ml. The effect of the lipopeptide was evident after 24 h and reached a plateau after 48 h. (2*S*,6*S*)-2-Palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine enhanced O_2^- formation as well. The effects of $Pam_3CysSer(Lys)_4$ were potentiated by dibutyryl cAMP, dimethyl sulfoxide, retinoic acid, 1,25-dihydroxyvitamin D_3, interferon-γ and tumor-necrosis-factor-α. Pertussis toxin, but not its B-oligomer, partially inhibited enhanced O_2^- formation induced by $Pam_3CysSer(Lys)_4$. O_2^- formation induced by arachidonic acid and γ-hexachlorocyclohexane were more sensitive to inhibition by pertussis toxin than O_2^- formation induced by phorbol 12-myristate 13-acetate. Enhanced O_2^- formation induced by dibutyryl cAMP was not affected by pertussis toxin. Unlike ATP, histamine, prostaglandin E_1 and the β-adrenergic agonist, isoproterenol, $Pam_3CysSer(Lys)_4$ did not increase cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in undifferentiated HL-60 cells. Histamine but not lipopeptides stimulated high-affinity GTPase of guanine-nucleotide-binding proteins in membranes of undifferentiated HL-60 cells. In $Pam_3CysSer(Lys)_4$-differentiated HL-60 cells, the responsiveness to the $[Ca^{2+}]_i$-increasing agonists, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, C5a and leukotriene B_4, was increased, whilst the responsiveness to prostaglandin E_1 and isoproterenol was decreased. $Pam_3CysSer(Lys)_4$ did not inhibit proliferation of HL-60 cells but decreased transferrin receptor expression and increased C3bi receptor expression. Pertussis toxin did not affect proliferation and expression of transferrin and C3bi receptors. Dibutyryl cAMP was considerably more effective than $Pam_3CysSer(Lys)_4$ at inducing alterations in the above parameters. Our results suggest that (a) $Pam_3CysSer(Lys)_4$ induces incomplete functional differentiation of HL-60 cells through a mechanism which does not depend on a rise in $[Ca^{2+}]_i$ and is different from that of other differentiation-inducing substances and (b) the mechanism by which $Pam_3CysSer(Lys)_4$ induces differentiation involves pertussis-toxin-sensitive and pertussis-toxin-insensitive mechanisms.

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Abbreviations. Bt_2cAMP, dibutyryl cAMP; C_{36}-Myc(Ste)Ala, *N*-(2-hexadecyl-3-octadecanoyloxyeicosanoyl)-alanyl; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; fMetLeuPhe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; G-protein, regulatory heterotrimeric guanine-nucleotide-binding protein; G_i, family of highly homologous G-proteins (G_{i1}–G_{i3}) involved in the receptor/agonist-mediated inhibition of adenylyl cyclase, activation of phospholipase C in some systems (e.g. in HL-60 cells) and stimulation of certain types of ion channels (e.g. voltage-dependent K^+ and Ca^{2+} channels); HMD, 1,6-diaminohexane; O_2^-, superoxide anion; Pam_3Adh, (2*S*)-palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl; Pam_3Ahh, (2*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxy-heptanoyl; Pam_3Cys, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-(*R*)-cysteinyl; PMA, phorbol 12-myristate 13-acetate.

Enzymes. GTPase (EC 3.6.1.–); NADPH oxidae (EC 1.6.99.6).

The human promyelocytic cell line, HL-60, is a widely employed model system to study various aspects of neutrophilic and monocytic differentiation. Among others, retinoic acid, dimethyl sulfoxide and dibutyryl cAMP (Bt_2cAMP) induce certain aspects of neutrophilic differentiation and 1,25-dihydroxyvitamin D_3, interferon-γ and tumor-necrosis-factor-α induce monocytic differentiation. These processes result, to different degrees, in decreases of proliferation and expression of the transferrin receptor, and in increases of expression of C3bi receptors, $[Ca^{2+}]_i$-increasing chemoattractant receptors and enhanced superoxide (O_2^-) formation (for review see Harris and Ralph, 1985; Collins, 1987). The precise mechanisms by which very heterogenous substances induce differentiation, are largely unknown. Interestingly, pertussis toxin which ADP ribosylates and functionally uncouples regulatory heterotrimeric guanine-nucleotide-binding proteins (G-proteins) of the G_i-family [family of highly homologous G-proteins ($G_{i1}-G_{i3}$) involved in the receptor/agonist-mediated inhibition of adenylyl cyclase and activation of phospholipase C in some systems (e.g. in HL-60 cells) and stimulation of certain types of ion channels (e.g. voltage-dependent K^+ and Ca^{2+} channels) (for review see Gilman, 1987; Birnbaumer et al., 1990)] from receptors, partially inhibits retinoic-acid-induced enhancement of O_2^- formation in HL-60 cells, suggesting that G_i-type G-proteins are involved in this process (Tohkin et al., 1989; Hemmi et al., 1989).

The outer cell wall of Gram-negative bacteria contains lipoprotein (for review see Braun, 1975). Lipoprotein and synthetic analogues derived from its *N* terminus show various biological activities. First, they are effective activators of macrophages and lymphocytes (Bessler et al., 1985; Hoffmann et al., 1989; Hauschildt et al., 1990). Second, immunization of guinea pigs with a synthetic low-molecular-mass vaccine consisting of a B-cell and T-cell epitope corresponding to a partial sequence of foot-and-mouth-disease virus-protein VP1 covalently linked to *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-serine [Pam_3Cys-$(Ser)_2$] protects the host against virus challenge (Wiesmüller et al., 1989). Third, synthetic lipopeptides carrying killer cell epitopes from influenza nucleoprotein prime virus-specific killer cells *in vivo* (Deres et al., 1989). Finally, we recently reported that the synthetic lipopeptide, $Pam_3CysSer(Lys)_4$, induced O_2^- formation and exocytosis in human neutrophils (Seifert et al., 1990). The $Ser(Lys)_4$ moiety was an important structural prerequisite for the biological activity of this lipopeptide, as substances devoid of positively charged amino acids did not stimulate O_2^- formation. Lipopeptide-induced O_2^- formation was partially inhibited by pertussis toxin, and exocytosis was not affected by the toxin at all, suggesting that both pertussis-toxin-sensitive and pertussis-toxin-insensitive signal-transduction pathways are involved in neutrophil activation by $Pam_3CysSer(Lys)_4$. All these findings prompted us to study the effects of various lipopeptides with positively charged amino acid residues on differentiation of HL-60 cells. The structural formulae of the lipopeptides studied are shown in Fig. 1. We report that $Pam_3CysSer(Lys)_4$ induces enhanced responsiveness to stimulated O_2^- formation in HL-60 cells (presumably through the expression of components of NADPH oxidase) which is partially inhibited by pertussis toxin. It also induces increased responsiveness to chemoattractants and changes in expression of transferrin and C3bi receptors, but in comparison to Bt_2cAMP, functional differentiation by lipopeptides is incomplete.

MATERIALS AND METHODS

Materials

$Pam_3CysSer(Lys)_4$, 2*R*,6*R*-$Pam_3CysSer(Lys)_4$, 2*R*,6*S*-$Pam_3CysSer(Lys)_4$, $Pam_3CysSer$-tuftsin, $Pam_3CysSer$-1,6-hexamethylene diamine-tuftsin ($Pam_3CysSer$-HMD-tuftsin), (2*S*)-palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl-Ser-$(Lys)_4$ [$Pam_3AdhSer(Lys)_4$], (2*S*,6*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoylSer$(Lys)_4$ [Pam_3-$AhhSer(Lys)_4$] and *N*-(2-hexadecyl-3-octadecanoyloxyeicosanoyl)-$AlaSer(Lys)_4$ [C_{36}-Myc(Ste)$AlaSer(Lys)_4$] were prepared by chemical synthesis and were unequivocally characterized by various analytical and spectroscopic methods such as ion-spray mass spectrometry, ^{13}C-NMR spectroscopy and chiral-phase amino acid analysis (Metzger and Jung, 1987; Metzger et al., 1991a, b; Schmidt et al., 1991). Stock solutions of lipopeptides (100 or 500 μg/ml) were prepared in 145 mM NaCl and stored at −20 °C. Research quantities of lipopeptides are commercially available from Rapp Polymere, Tübingen, Germany. Pertussis toxin and its B-oligomer were kind gifts of Dr. M. Yajima (Kyoto, Japan). Leukotriene B_4 was provided by Prof. W. Bartmann (Hoechst, Frankfurt/Main, Germany) and 1,25-dihydroxyvitamin D_3 was a gift from Drs H. Gutmann and U. Fischer (Hoffmann-La Roche, Basel, Switzerland). Tuftsin was obtained from Sigma Chemie (Taufkirchen, Germany). Human recombinant interferon-γ and tumor-necrosis-factor-α were from Boehringer Mannheim (Mannheim, Germany). Sources of other materials have been described elsewhere (Rosenthal et al., 1986; Seifert and Schächtele, 1988; Seifert and Schultz, 1987; Seifert et al., 1989a, b, c, 1990; Offermanns et al., 1989; Serke et al., 1991).

Cell culture

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (by vol.) horse serum, 1% (by vol.) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere with 7% CO_2 at 37 °C as described (Seifert and Schächtele, 1988; Seifert et al., 1989a, b). To induce differentiation, HL-60 cells were seeded at 0.3 cells/nl and were cultured for different times in the presence of various substances. Preliminary studies showed that lipopeptides up to 10 μg/ml did not induce staining of HL-60 cells with trypan blue and release of lactate dehydrogenase, suggesting that they were not toxic (data not shown). Unlike phorbol esters, lipopeptides did not induce adherence of HL-60 cells to tissue culture plastic flasks suggesting that protein kinase C was not involved in lipopeptide-induced differentiation of cells (data not shown). Undifferentiated HL-60 cells consisted of <90% promyelocytes and incubation with $Pam_3CysSer(Lys)_4$ (1 μg/ml) for 48 h did not result in morphological maturation as assessed by evaluation of Pappenheim-stained cytospin preparations of cells (data not shown). Cell counts were performed in a hemocytometer. For pertussis-toxin treatment, HL-60 cells were seeded at 0.3 cells/nl and were cultured for 24 h in the presence of the toxin (0.5 μg/ml), its B-oligomer (0.5 μg/ml) or solvent (control). After that time, pertussis-toxin-induced ADP ribosylation of >98% of the cellular G_i-type G-proteins (Cowen et al., 1990; Wenzel-Seifert and Seifert, 1990). Thereafter, HL-60 cells were treated with different substances for various times without changing the culture medium. Subsequently, HL-60 cells were harvested and functional assays were performed. Similar results as those shown in Tables 3 and 4 were obtained when pertussis toxin was added to the

cells simulataneously with the lipopeptides (data not shown). Thus, recovery of G_i from ADP ribosylation through *de novo* synthesis of G_i and/or inactivation of pertussis toxin did not satisfactorily explain the differential effects of the toxin on stimulated O_2^- formation. Treatment of HL-60 cells with pertussis toxin or its B-oligomer did not affect cell viability as assessed by trypan-blue-dye exclusion and lactate dehydrogenase release (data not shown). Treatment of HL-60 cells with pertussis toxin or its B-oligomer (0.5 μg/ml) for 24–96 h did not result in differentiation of HL-60 cells as measured by phorbol-12-myristate-13-acetate-(PMA)-stimulated O_2^- formation (data not shown). The lipopeptide did not inhibit cell growth as measured by proliferation curves. By contrast, Bt_2cAMP effectively induced cessation of cell growth. Pertussis toxin and its B-oligomer showed no effect on growth of HL-60 cells in the absence or presence of Bt_2cAMP or $Pam_3CysSer(Lys)_4$. Analysis of cell-cycle phases revealed a considerably greater percentage of control cells and $Pam_3CysSer(Lys)_4$-treated cells in the S-phase than of Bt_2cAMP-treated cells (data not shown).

Table 1. Potentiation by various lipopeptides of Bt_2cAMP-induced enhancement of O_2^- formation in HL-60 cells. HL-60 cells were incubated for 48 h in the presence of Bt_2cAMP (200 μM) and various substances (1 μg/ml each) or solvent (control). HL-60 cells were harvested and O_2^- formation was activated with PMA (100 ng/ml). Data shown are the means ± S.D. of six experiments performed with different batches of HL-60 cells.

Additives	O_2^- formation
	nmol/10^6 cells
None	4.5 ± 0.3
$Pam_3CysSer(Lys)_4$	10.3 ± 0.5
2*R*,6*R*-$Pam_3CysSer(Lys)_4$	10.5 ± 0.6
2*R*,6*S*-$Pam_3CysSer(Lys)_4$	10.2 ± 0.7
$Pam_3AdhSer(Lys)_4$	11.2 ± 0.5
$Pam_3CysSer(Lys)_4$ + $Pam_3AdhSer(Lys)_4$	11.5 ± 0.9
$Pam_3AhhSer(Lys)_4$	4.3 ± 0.6
C_{36}-Myc(Ste)AlaSer(Lys)$_4$	4.2 ± 0.5
$Pam_3CysSer$-tuftsin	4.2 ± 0.5
$Pam_3CysSer$-HMD-tuftsin	4.1 ± 0.5
Tuftsin	4.0 ± 0.7

Assay for O_2^- generation

O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase as described (Seifert et al., 1989a, b). Unless stated otherwise O_2^- formation was activated by PMA (100 ng/ml). In the absence of stimuli, HL-60 cells did not generate O_2^-. The total extent of O_2^- formation was calculated.

Measurement of GTPase activity

GTPase activity was measured according to Offermanns et al. (1989) with modifications. Briefly, assay mixtures contained membranes from HL-60 cells (10 μg protein/tube), 0.5 μM [γ-^{32}P]GTP (4 kBq/tube), 0.5 mM $MgCl_2$, 0.1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 0.1 mM ATP, 1 mM adenosine 5′-[β,γ-imido]triphosphate, 5 mM creatine phosphate, 40 μg creatine kinase, 1 mM dithiothreitol and 0.2% (mass/vol.) bovine serum albumin in 50 mM triethanolamine/HCl, pH 7.4. After the addition of substances, the reaction mixtures were incubated for 3 min at 25 °C. Measurement of GTPase activity was initiated by the addition of [γ-^{32}P]GTP. Assays were conducted for 10 min. Stopping of the reaction and isolation of P_i were carried out as described (Aktories et al., 1983). Low-affinity GTPase activity was determined in the presence of GTP (50 μM) and was subtracted from total GTPase activity measured in the presence of GTP (0.5 μM). Low-affinity GTPase activity in membranes from undifferentiated and Bt_2cAMP-differentiated HL-60 cells amounted to < 5% of total GTPase activity (data not shown).

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

$[Ca^{2+}]_i$ was determined using the dye, fura-2 acetoxymethyl ester, according to the protocol described by Wenzel-Seifert and Seifert (1990). Basal $[Ca^{2+}]_i$ in undifferentiated and $Pam_3CysSer(Lys)_4$-differentiated or Bt_2cAMP-differentiated HL-60 cells amounted to 90–120 nM and there were no significant differences among these groups (data not shown).

Flow cytometry

Staining of washed cells and flow cytometry were performed as described recently (Serke et al., 1991). For detection of the C3bi receptor and transferrin receptor, HL-60 cells were first incubated with monoclonal antibodies (Leu15, anti-transferrin receptor) (Becton-Dickinson, Heidelberg, Germany) and subsequently with fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (An der Grub, Kaumberg, Austria). Cell-cycle phase analysis was performed on ethanol-fixed cells stained with propidium iodide following RNAse treatment. Analyses were performed in a Facscan flow cytometer (Becton-Dickinson). Data were recorded in list mode using the Consort 30 software and cell-cycle phase analysis was performed using the DNA software (Becton-Dickinson).

Miscellaneous

Membranes from HL-60 cells were prepared as described (Seifert and Schultz, 1987). [γ-^{32}P]GTP was synthesized according to Johnson and Walseth (1979). Protein was measured according to Lowry et al. (1951) using bovine serum albumin as standard.

Statistics

The statistical significance of the effects of substances was assessed using the Wilcoxon test.

RESULTS

$Pam_3CysSer(Lys)_4$-enhanced PMA-induced O_2^- formation (presumably through the expression of components of NADPH oxidase) in a concentration-dependent manner with a half-maximal effect at 100 ng/ml and a maximum at 1 μg/ml (Fig. 2). The effect of the lipopeptide was evident after 24 h and reached a maximum after 48 h. Incubations of HL-60 cells with the lipopeptide for up to 96 h (data shown only for up to 72 h) did not further increase PMA-stimulated O_2^- formation (data not shown). $Pam_3CysSer(Lys)_4$ was considerably less effective than Bt_2cAMP at enhancing O_2^- formation. However, $Pam_3CysSer(Lys)_4$ enhanced the effect of Bt_2cAMP on stimulated O_2^- formation by up twofold. In the presence of Bt_2cAMP, the effect of the lipopeptide was half-maximal at 100 ng/ml and was maximal at 1 μg/ml.

The effects of various lipopeptides on PMA-stimulated O_2^- formation were compared (Table 1). The diastereomers,

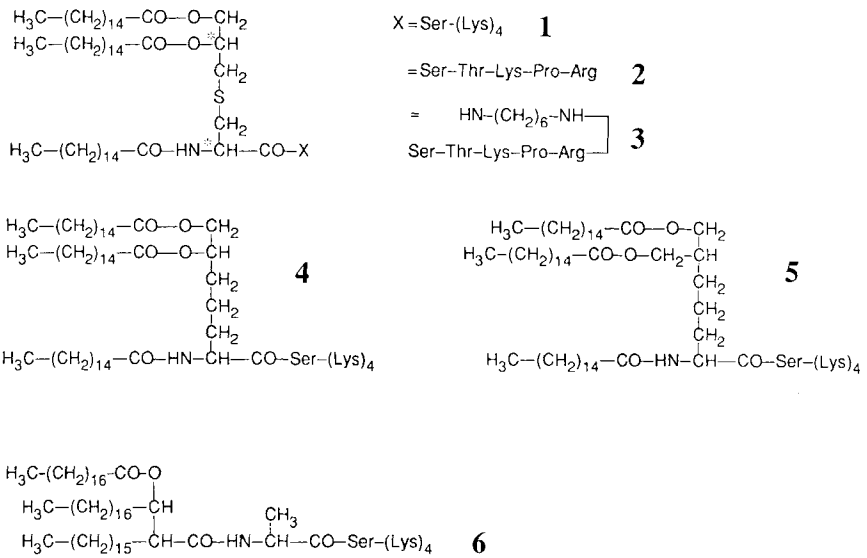

Fig. 1. Structural formulae of lipopeptides. 1, $Pam_3CysSer(Lys)_4$ (a, 2*R*,6*R*; b, 2*R*,6*S*; a/b, mixture of both diastereomers). **2**, $Pam_3CysSer$-tuftsin. **3**, $Pam_3CysSer$-HMD-tuftsin. **4** $Pam_3AdhSer(Lys)_4$. **5**, $Pam_3AhhSer(Lys)_4$. **6**, C_{36}-Myc(Ste)AlaSer$(Lys)_4$.

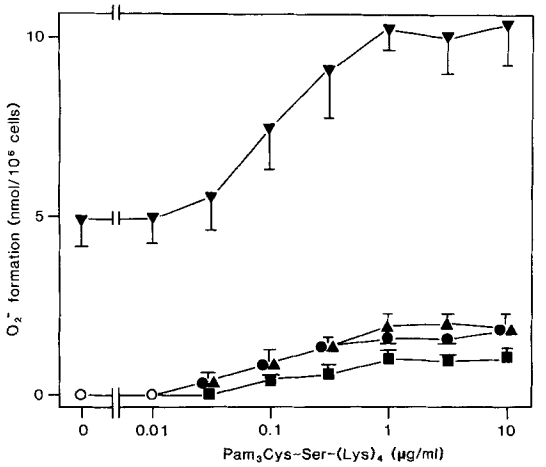

Fig. 2. Concentration-dependence and time-dependence of $Pam_3CysSer(Lys)_4$-induced enhancement of O_2^- formation in HL-60 cells, synergism with Bt_2cAMP. HL-60 cells were incubated for different times with $Pam_3CysSer(Lys)_4$ at various concentrations in the absence or presence of Bt_2cAMP (200 μM). HL-60 cells were harvested and O_2^- formation was activated with PMA (100 ng/ml). (■) $Pam_3CysSer(Lys)_4$ at an incubation time of 24 h; (●) $Pam_3CysSer(Lys)_4$ at an incubation time of 48 h; (▲) $Pam_3CysSer(Lys)_4$ at an incubation time of 72 h; (▼) $Pam_3CysSer(Lys)_4$ plus Bt_2cAMP at an incubation time of 48 h. (○) Indicates that $Pam_3CysSer(Lys)_4$ at the designated concentrations did not significantly enhance O_2^- formation. Data shown are the means ± S.D. of for experiments performed with different batches of HL-60 cells.

2*R*,6*R*-$Pam_3CysSer(Lys)_4$ and 2*R*,6*S*-$Pam_3CysSer(Lys)_4$ with a different configuration of the asymmetric carbon atom of the S-glyceryl part (Fig. 1), were similarly effective potentiators of Bt_2cAMP-induced enhancement of O_2^- formation as a mixture of the two diastereomers. $Pam_3AdhSer(Lys)_4$ had the same activity as $Pam_3CysSer(Lys)_4$ on O_2^- formation. The concentration-response function of $Pam_3AdhSer(Lys)_4$-induced potentiation of O_2^- formation resembled that of $Pam_3CysSer(Lys)_4$ (see Fig. 2; data not shown). The combination of $Pam_3CysSer(Lys)_4$ plus $Pam_3AdhSer(Lys)_4$ was not more effective than each substance alone in enhancing O_2^- formation. $Pam_3AhhSer(Lys)_4$, C_{36}-Myc(Ste)AlaSer$(Lys)_4$, $Pam_3CysSer$-tuftsin, $Pam_3CysSer$-HMD-tuftsin and tuftsin did not enhance O_2^- formation in Bt_2cAMP-treated HL-60 cells. The effects of various lipopeptides (1 μg/ml each for 48 h) on O_2^- formation were also studied in the absence of Bt_2cAMP. Under these conditions, $Pam_3AdhSer(Lys)_4$ was similarly effective as $Pam_3CysSer(Lys)_4$ and $Pam_3AhhSer(Lys)_4$, C_{36}-Myc(Ste)AlaSer$(Lys)_4$, $Pam_3CysSer$-tuftsin, $Pam_3CysSer$-HMD-tuftsin and tuftsin were ineffective (data not shown).

The interaction of $Pam_3CysSer(Lys)_4$ with various well-known differentiation-inducing substances on O_2^- formation was studied (Table 2). $Pam_3CysSer(Lys)_4$ potentiated stimulated O_2^- formation in HL-60 cells treated with Bt_2cAMP,

Table 2. Synergistic enhancement by $Pam_3CysSer(Lys)_4$ and various other substances of O_2^- formation in HL-60 cells. HL-60 cells were incubated for 48 h in the presence of various substances at maximally effective concentrations and with combinations of substances. HL-60 cells were harvested and O_2^- formation was activated with PMA (100 ng/ml). Undifferentiated HL-60 cells did not generate O_2^- upon stimulation with PMA. Data shown are the means ± S.D. from five experiments performed with different batches of HL-60 cells.

Additives	O_2^- formation
	nmol/10^6 cells
$Pam_3CysSer(Lys)_4$ (1 μg/ml)	1.6 ± 0.3
Bt_2cAMP (200 μM)	4.8 ± 0.6
Dimethylsulfoxide (160 mM)	3.2 ± 0.4
Retinoic acid (10 nM)	1.6 ± 0.3
1,25-Dihydroxyvitamin D_3 (10 nM)	2.4 ± 0.5
Interferon-γ (300 U/ml)	0.8 ± 0.2
Tumor-necrosis-factor-α (300 U/ml)	1.5 ± 0.4
$Pam_3CysSer(Lys)_4$ + Bt_2cAMP	10.2 ± 1.0
$Pam_3CysSer(Lys)_4$ + dimethylsulfoxide	5.3 ± 0.5
$Pam_3CysSer(Lys)_4$ + retinoic acid	5.4 ± 0.3
$Pam_3CysSer(Lys)_4$ + 1,25-dihydroxyvitamin D_3	6.4 ± 0.7
$Pam_3CysSer(Lys)_4$ + interferon-γ	5.5 ± 0.4
$Pam_3CysSer(Lys)_4$ + tumor-necrosis-factor-α	9.6 ± 0.5

Table 3. Effect of pertussis toxin and its B-oligomer on enhancement of O_2^- formation in HL-60 cells in the presence of Bt_2cAMP without or with $Pam_3CysSer(Lys)_4$. HL-60 cells were incubated for 24 h with pertussis toxin or its B-oligomer (0.5 μg/ml each) or solvent (control). Thereafter, Bt_2cAMP (100 μM) with or without $Pam_3CysSer(Lys)_4$ (1 μg/ml) was added to cell cultures and cells were grown for an additional 24 h or 48 h without changing the culture medium. Subsequently, cells were harvested and O_2^- formation was activated with PMA (100 ng/ml). Undifferentiated HL-60 cells did not generate O_2^- upon stimulation with PMA. Data shown are the means ± S.D. of five experiments performed with different batches of HL-60 cells. Comparison of pertussis-toxin-treated or B-oligomer-treated cells versus control cells is shown in the footnotes.

Additive	Incubation time	O_2^- formation	
		Bt_2cAMP	Bt_2cAMP + $Pam_3CysSer(Lys)_4$
	h	nmol/10^6 cells	
Control	24	0.9 ± 0.3	3.0 ± 0.4
B-oligomer	24	0.8 ± 0.2[a]	2.8 ± 0.3[a]
Pertussis toxin	24	0.9 ± 0.4[a]	1.5 ± 0.2[b]
Control	48	2.8 ± 0.4	6.3 ± 0.6
B-oligomer	48	2.7 ± 0.3[a]	6.1 ± 0.5[a]
Pertussis toxin	48	2.6 ± 0.3[a]	6.2 ± 0.4[a]

[a] Not significant.
[b] $p < 0.05$.

dimethyl sulphoxide, retinoic acid, 1,25-dihydroxyvitamin D_3, tumor-necrosis-factor-α or interferon-γ for 48 h. Qualitatively the same results were obtained when O_2^- formation was assessed after 24 h and 72 h (data not shown).

The effects of pertussis toxin and its B-oligomer, which does not catalyze ADP ribosylation of G-proteins (Tamura et al., 1983), on O_2^- formation were assessed. Neither pertussis toxin nor its B-oligomer affected Bt_2cAMP-induced enhancement of O_2^- formation regardless of whether cells were treated with Bt_2cAMP for 24 h or 48 h (Table 3). By contrast, pertussis toxin reduced potentiation by $Pam_3CysSer(Lys)_4$ of O_2^- formation after an incubation time of 24 h by about 50%. However, after an incubation time of 48 h, pertussis toxin failed to inhibit potentiation by $Pam_3CysSer(Lys)_4$. Its B-oligomer did not affect potentiation by $Pam_3CysSer(Lys)_4$.

The inhibitory effects of pertussis toxin on O_2^- formation induced by PMA, arachidonic acid or γ-hexachlorocyclohexane in $Pam_3CysSer(Lys)_4$-differentiated HL-60 cells were compared. In agreement with the experiments performed in the presence of Bt_2cAMP (Table 3), pertussis toxin did not significantly inhibit PMA-induced O_2^- formation in HL-60 cells treated with the lipopeptide for 48 h in the absence of Bt_2cAMP (Table 4). By contrast, pertussis toxin inhibited O_2^- formation induced by arachidonic acid and γ-hexachlorocylohexane at maximally effective concentrations in $Pam_3CysSer(Lys)_4$-differentiated cells by more than 50%.

Agonists at G-protein-coupling receptors increase high-affinity GTPase activity of G-proteins (for review see Gilman, 1987; Birnbaumer et al., 1990). Regulation by various substances of high-affinity GTPase in membranes of undifferentiated and Bt_2cAMP-differentiated HL-60 cells was studied (Table 5). Differentiation of HL-60 cells with Bt_2cAMP increased basal GTPase activity by about 60%. In membranes of undifferentiated and differentiated HL-60 cells, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMetLeuPhe) stimulated GTPase activity by 15% and 130%, respectively, and histamine stimulated enzyme activity in both types of membranes by about 20%. $Pam_3CysSer(Lys)_4$ at 1, 10 and 100 μg/ml (data shown only for 100 μg/ml) showed no effect on high-affinity GTPase activity in membranes of undifferentiated and differentiated HL-60 cells. $Pam_3AdhSer(Lys)_4$ (100 μg/ml) inhibited high-affinity GTPase activity in membranes of undifferentiated HL-60 cells by 13% and this lipopeptide at 1 μg/ml and 10 μg/ml was without effect (data not shown).

The effects of various substances on $[Ca^{2+}]_i$ in undifferentiated HL-60 cells were assessed. ATP induced a large and sustained increase in $[Ca^{2+}]_i$ (Fig. 3). In comparison to ATP, histamine and prostaglandin E_1 induced considerably smaller and more transient increases in $[Ca^{2+}]_i$. Pertussis

Table 4. Differential pertussis toxin-sensitivity of $Pam_3CysSer(Lys)_4$-induced enhancement of O_2^- formation in HL-60 cells. HL-60 cells were incubated for 24 h with pertussis toxin (0.5 μg/ml) or solvent (control). Thereafter, $Pam_3CysSer(Lys)_4$ (1 μg/ml) was added to cell cultures, and cells were grown for an additional 48 h without changing the culture medium. Subsequently, cells were harvested and O_2^- formation was stimulated with arachidonic acid (160 μM), γ-hexachlorocyclohexane (0.5 mM) or PMA (100 ng/ml). Undifferentiated HL-60 cells did not generate O_2^- upon stimulation with these substances. Data shown are the means ± S.D. of five experiments performed with different batches of HL-60 cells. Comparison of pertussis-toxin-treated cells versus control cells is shown in the footnotes.

Stimulus	O_2^- formation	
	control	pertussis toxin
	(nmol/10^6 cells)	
None (control)	0	0
Arachidonic acid	0.9 ± 0.2	0.4 ± 0.1[b]
γ-Hexachlorocyclohexane	1.0 ± 0.3	0.4 ± 0.2[b]
PMA	1.7 ± 0.3	1.5 ± 0.3[a]

[a] Not significant.
[b] $p < 0.05$.

Table 5. Effects of various substances on high-affinity GTPase activity in membranes of undifferentiated and Bt_2cAMP-differentiated HL-60 cells. Membranes from undifferentiated HL-60 cells and HL-60 cells treated with Bt_2cAMP (200 μM) for 48 h were prepared and high-affinity GTPase activity was measured in the absence or presence of various substances. Data shown are the means ± S.D. of assay quintuplicates. Similar results were obtained in four independent experiments. Comparison of the effects of substances versus control is shown in the footnotes. n.d., not determined.

Addition	GTPase activity	
	undifferentiated	Bt_2cAMP-differentiated
	pmol $P_i \cdot mg^{-1} \cdot min^{-1}$	
None (control)	11.4 ± 0.3	18.2 ± 0.4
fMetLeuPhe (10 μM)	13.1 ± 0.5[b]	41.9 ± 1.5[c]
Histamine (1 mM)	13.7 ± 0.4[c]	22.0 ± 0.4[c]
$Pam_3CysSer(Lys)_4$ (100 μg/ml)	11.3 ± 0.5[a]	17.9 ± 0.7[a]
$Pam_3AdhSer(Lys)_4$ (100 μg/ml)	9.9 ± 0.3[b]	n.d.

[a] Not significant.
[b] $p < 0.05$.
[c] $p < 0.01$.

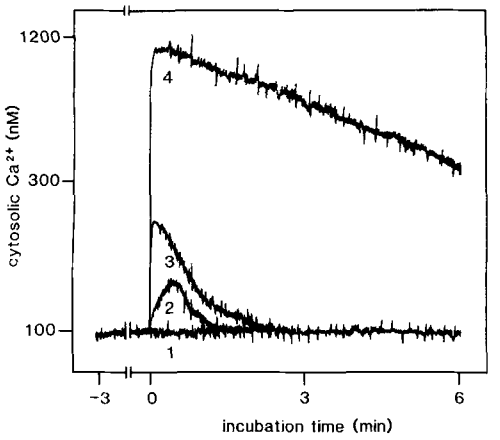

Fig. 3. Effect of various substances on $[Ca^{2+}]_i$ in undifferentiated HL-60 cells. Undifferentiated HL-60 cells were loaded with fura-2-acetoxymethylester, and $[Ca^{2+}]_i$ was determined. At $t = 0$ min, various substances were added to cells. Trace 1, pertussis toxin or its B-oligomer (0.5 μg/ml each), $Pam_3CysSer(Lys)_4$, $Pam_3AdhSer(Lys)_4$ or $Pam_3AhhSer(Lys)_4$ (1 and 10 μg/ml each); trace 2, prostaglandin E_1 (10 μM); trace 3, histamine (100 μM); trace 4, ATP (100 μM). Superimposed original tracings from one experiment are shown. Similar results were obtained in three experiments with different batches of HL-60 cells.

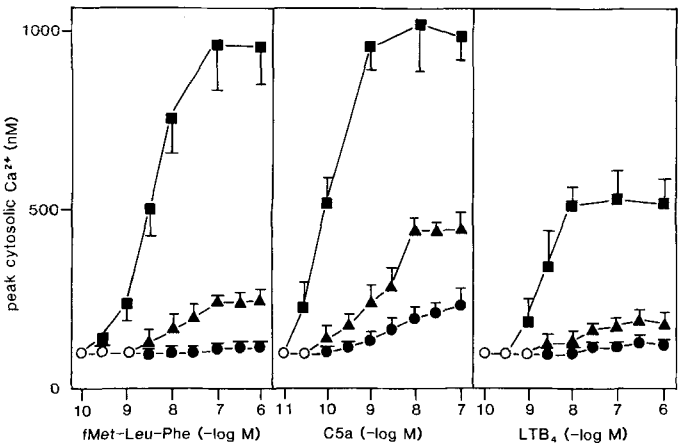

Fig. 4. Effect of chemoattractants on $[Ca^{2+}]_i$ in HL-60 cells. HL-60 cells were incubated for 48 h in the presence of $Pam_3CysSer(Lys)_4$ (1 μg/ml), Bt_2cAMP (200 μM) or solvent (control). Cells were harvested, loaded with fura-2-acetoxymethylester and $[Ca^{2+}]_i$ was determined. Chemoattractants were added to cells and the increase in $[Ca^{2+}]_i$ was determined. (●) Undifferentiated HL-60 cells; (▲) $Pam_3CysSer(Lys)_4$-differentiated cells; (■) Bt_2cAMP-differentiated HL-60 cells. (○) Indicates that chemoattractants at the designated concentrations did not significantly increase $[Ca^{2+}]_i$. Data shown are the means ± S.D. of four experiments performed with different batches of HL-60 cells. LTB_4, leukotriene B_4.

toxin, its B-oligomer, $Pam_3CysSer(Lys)_4$, $Pam_3AdhSer(Lys)_4$ and $Pam_3AhhSer(Lys)_4$ showed no effect on $[Ca^{2+}]_i$.

In undifferentiated HL-60 cells, fMetLeuPhe and leukotriene B_4 were almost ineffective to increase $[Ca^{2+}]_i$ and C5a was only weakly effective (Fig. 4). Differentiation of HL-60 cells by Bt_2cAMP was accompanied by a large enhancement in potency and effectiveness of these chemoattractants to increase $[Ca^{2+}]_i$. $Pam_3CysSer(Lys)_4$ also induced increased responsiveness to chemoattractants, especially to fMetLeuPhe and C5a, but its effects were less pronounced than those of Bt_2cAMP.

Table 6. Effect of various substances on $[Ca^{2+}]_i$ in HL-60 cells. HL-60 cells were incubated for 48 h in the presence of $Pam_3CysSer(Lys)_4$ (1 μg/ml), Bt_2cAMP (200 μM) or solvent (control). Cells were harvested and the increase in $[Ca^{2+}]_i$ induced by various substances was assessed. Data shown are the means ± S.D. of five experiments performed with different batches of HL-60 cells. Comparison of differentiated versus undifferentiated cells is shown in the footnotes.

Stimulus	Increase in $[Ca^{2+}]_i$		
	control	$Pam_3CysSer(Lys)_4$	Bt_2cAMP
	nM		
ATP (100 μM)	1105 ± 112	1098 ± 98[a]	1204 ± 102[a]
Concanvalin A (100 μg/ml)	49 ± 10	52 ± 8[a]	152 ± 25[b]
Prostaglandin E_1 (10 μM)	56 ± 18	27 ± 4[b]	11 ± 5[b]
Isoproterenol (10 μM)	39 ± 8	19 ± 7[b]	17 ± 10[b]

[a] Not significant.
[b] $p < 0.05$.

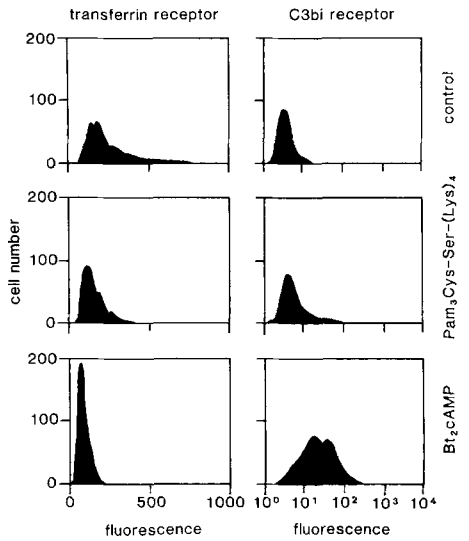

Fig. 5. Expression of transferrin and C3bi receptors in HL-60 cells. HL-60 cells were incubated for 48 h in the presence of $Pam_3CysSer(Lys)_4$ (1 μg/ml), Bt_2cAMP (200 μM) or solvent (control). Cells were harvested and the expression of transferrin and C3bi receptors was determined by flow cytometry. In each sample, 5000 cells were analyzed. Typical fluorescence diagrams from one experiment are shown. Similar results were obtained in five experiments performed with different batches of HL-60 cells.

The effects of various substances employed at maximally effective concentrations in undifferentiated and $Pam_3CysSer(Lys)_4$-differentiated or Bt_2cAMP-differentiated HL-60 cells on $[Ca^{2+}]_i$ were studied (Table 6). Differentiation by either substance was not associated with alterations in responsiveness to ATP. Treatment with Bt_2cAMP, but not with the lipopeptide, increased the responsiveness to concanavalin A, whereas differentiation by both substances decreased rsponsiveness to prostaglandin E_1 and isoproterenol.

Differentiation of HL-60 cells with $Pam_3CysSer(Lys)_4$ and Bt_2cAMP decreased the percentage of transferrin-receptor-positive cells from 44% to 30% and 3%, respectively (Fig. 5).

In the presence of the lipopeptide and Bt_2cAMP, the percentage of C3bi-receptor-positive cells increased from 2% to 7% and 26%, respectively. Pertussis toxin and its B-oligomer did not affect the expression of transferrin and C3bi receptors in undifferentiated and lipopeptide-differentiated or Bt_2cAMP-differentiated HL-60 cells (data not shown). Treatment of HL-60 cells with Bt_2cAMP (100 μM) plus $Pam_3CysSer(Lys)_4$ (1 μg/ml) for 48 h resulted in synergistic expression of the C3bi receptor (data not shown).

DISCUSSION

In this report, we show that the synthetic lipopeptide, $Pam_3CysSer(Lys)_4$, in comparison to Bt_2cAMP, induces incomplete functional differentiation of HL-60 cells as assessed by enhanced O_2^- formation (Fig. 2, Table 1), altered responsiveness to $[Ca^{2+}]_i$-increasing substances (Fig. 4, Table 6) and decreased expression of transferrin- and increased expression of C3bi receptors (Fig. 5).

In order to study the mechanism by which lipopeptides induce functional differentiation, we compared the effects of $Pam_3CysSer(Lys)_4$ with those of other lipopeptides containing positively charged amino acids (Fig. 1). One group of lipopeptides studied differs in the structure of the lipid moiety but not in the pentapeptide chain, $Ser(Lys)_4$. This group comprises 2*R*,6*R*-*S*-Pam_3Cys-$Ser(Lys)_4$, 2*R*,6*R*-$Pam_3CysSer(Lys)_4$, 2*R*,6*S*-$Pam_3CysSer(Lys)_4$, $Pam_3AdhSer(Lys)_4$, Pam_3Ahh-$Ser(Lys)_4$ and C_{36}-Myc(Ste)AlaSer$(Lys)_4$. The other group comprises the tuftsinyl derivatives, Pam_3CysSer-tuftsin and Pam_3CysSer-HMD-tuftsin. Unlike some of the $Ser(Lys)_4$-containing lipopeptides, neither tuftsin nor its lipopeptide derivatives enhanced O_2^- formation (Table 1). Our results suggest that the presence of two positively charged amino acid residues with or without $Pam_3CysSer$ is not sufficient for activation and that $Ser(Lys)_4$ plays a crucial role in differentiation of HL-60 cells by lipopeptides. However, the presence of $Ser(Lys)_4$ is also not sufficient for lipopeptide-induced activation of HL-60 cells, as only $Pam_3CysSer(Lys)_4$ and Pam_3-$AdhSer(Lys)_4$ induced differentiation, whereas $Pam_3AhhSer$-$(Lys)_4$ and C_{36}-Myc(Ste)Ala-$Ser(Lys)_4$ are ineffective. Thus, the structure of the lipid moiety of lipopeptides also determines their effectiveness to enhance O_2^- formation. The configuration of the asymmetric carbon atom of the glyceryl moiety of $Pam_3CysSer(Lys)_4$ is apparently not critical, since the mixture of diastereomers and the pure 2*R*,6*R* and 2*R*,6*S* diastereomers of $Pam_3CysSer(Lys)_4$ had comparable effects on O_2^- formation. In addition, substitution for the sulfur in $Pam_3CysSer(Lys)_4$ by a methylene group in $Pam_3AdhSer$-$(Lys)_4$ did not alter the potency and effectiveness of the lipopeptide to enhance O_2^- formation, but the insertion of a methylene group between the palmitoyloxy residue and the asymmetric carbon atom of the glyceryl residue in Pam_3Ahh-$Ser(Lys)_4$ resulted in complete loss of differentiation-inducing activity. The mycoloyl amino acid derivative, C_{36}-Myc(Ste)-$AlaSer(Lys)_4$, which differs substantially from $Pam_3CysSer$-$(Lys)_4$ in the lipid moiety, did not enhance O_2^- formation. All these results suggest that the structure of both the lipid moiety and of the positively charged amino acid chain are important for activation of HL-60 cells by lipopeptides and that these substances do not act in an unspecific manner. The lack of synergism between $Pam_3CysSer(Lys)_4$ and $Pam_3AdhSer$-$(Lys)_4$ indicates that both lipopeptides act through a mechanism they have in common (Table 1).

The fact that $Pam_3CysSer(Lys)_4$ potentiated enhanced O_2^- formation induced by Bt_2cAMP, retinoic acid, dimethyl sulphoxide, 1,25-dihydroxyvitamin D_3, tumor-necrosis-factor-α and interferon-γ, suggests that the lipopeptide-induces differentiation by a mechanism distinct from that of the above substances (Table 2). Synergistic interaction of different substances to induce differentiation of HL-60 cells was also observed by other groups (Olsson et al., 1982; Weinberg and Larrick, 1987; Breitman and He, 1990). Unlike, ATP, histamine and prostaglandin E_1, $Pam_3CysSer(Lys)_4$ did not increase $[Ca^{2+}]_i$ in undifferentiated HL-60 cells indicating that calcium is not an early signal in lipopeptide-induced differentiation (Fig. 3). These results also suggest that lipopeptides induces differentiation of HL-60 cells through a mechanism different from that of ATP, histamine and prostaglandin E_1 (Chaplinski and Niedel, 1982; Olsson et al., 1982; Tasaka et al., 1990; Cowen et al., 1991).

We investigated the question whether pertussis-toxin-sensitive G-proteins are involved in lipopeptide-induced enhancement of O_2^- formation in HL-60 cells. Unlike in various other systems, pertussis toxin and its B-oligomer neither increased $[Ca^{2+}]_i$ nor affected proliferation (Fig. 3, and Materials and Methods) (Tamura et al., 1983; Strnad and Carchman, 1987; Sommermeyer and Resch, 1990). Pertussis toxin may increase cAMP levels through a blockade of the function of G_i (Katada and Ui, 1979; Murayama and Ui, 1983). Unlike Bt_2cAMP, pertussis toxin did not induce differentiation of HL-60 cells and did not inhibit cell growth (see Materials and Methods). Additionally, Bt_2cAMP potentiated lipopeptide-induced enhancement of O_2^- formation, whereas pertussis toxin was inhibitory or without effect (Fig. 2, Tables 3 and 4). Hemmi et al. (1989) reported that pertussis toxin did not increase cAMP levels in HL-60 cells. All these results suggest that the effects of pertussis toxin on differentiation of HL-60 cells were not due to Ca^{2+} influx from the extracellular space but rather due to ADP ribosylation of G_i-proteins which did not result in the imitation of the effects of Bt_2cAMP.

Both, retinoic-acid-induced and $Pam_3CysSer(Lys)_4$-induced enhancements of O_2^- formation were partially inhibited by pertussis toxin (Tables 3 and 4) (Tohkin et al., 1989; Hemmi et al., 1989). The notion that the effects of pertussis toxin on lipopeptide-induced enhancement of O_2^- formation were mediated through ADP ribosylation of G-proteins is supported by the finding that its B-oligomer did not mimick its effects (Table 3). Intriguingly, the effects of $Pam_3CysSer(Lys)_4$ on differentiation were not uniformly inhibited by pertussis toxin. With PMA as the stimulus, O_2^- formation was less sensitive to inhibition by pertussis toxin than with γ-hexachlorocyclohexane or arachidonic acid as stimuli (Table 4) and pertussis toxin did not affect alterations in expression of C3bi and transferrin receptors induced by $Pam_3CysSer(Lys)_4$. This differential and only partial sensitivity to inhibition by pertussis toxin of differentiation is reminiscent of the effects of pertussis toxin on lipopeptide-induced O_2^- formation and exocytosis in human neutrophils (Seifert et al., 1990). The stimulus specificity of the effects of pertussis toxin on lipopeptide-induced enhancement of O_2^- formation may be due, at least in part, to the fact that PMA, γ-hexachlorocyclohexane and arachidonic acid activate O_2^- formation through different mechanisms (Badwey et al., 1984, English et al., 1986; Tauber, 1987), possibly involving different regulatory and/or structural components of NADPH oxidase. At present, it is not yet known whether lipopeptides interact with ADP ribosylated G-proteins of the G_i family and/or pertussis-toxin-insensitive G-proteins (Strathmann and Simon, 1990) and/or low-molecular-mass GTP-binding proteins (Bokoch and Parkos, 1988). Similar to the effects of purine and pyrimidine nucleotides in

HL-60 cells, the ones of lipopeptides are partially pertussis-toxin-insensitive (Tables 3 and 4) (Dubyak et al., 1988; Seifert et al., 1989a; Cowen et al., 1990; Wenzel-Seifert and Seifert, 1990). Additionally, pertussis toxin inhibits ionophore A-23187-induced but not PMA-induced prostaglandin and leukotriene release in murine macrophages treated with lipopolysaccharide (Matsunaga et al. 1990).

The increases in basal high-affinity GTPase activity and stimulation by fMetLeuPhe in membranes from Bt_2cAMP-differentiated HL-60 cells may be explained by both increased expression of G_i-type G-proteins and formyl peptide receptors (Table 5) (Chaplinski and Niedel, 1982; Murphy et al., 1987). In membranes of undifferentiated and differentiated HL-60 cells, histamine substantially stimulated high-affinity GTPase. Unlike fMetLeuPhe and histamine, $Pam_3CysSer(Lys)_4$ did not affect GTPase activity in HL-60 membranes and $Pam_3AdhSer(Lys)_4$ significantly reduced basal high-affinity GTPase activity. Thus, the failure of lipopeptides to stimulate GTPase in membranes of undifferentiated HL-60 cells cannot be explained by the absence of G-proteins. These results and those on the regulation of $[Ca^{2+}]_i$ (Fig. 3) suggest that in HL-60 cells, lipopeptides interact with G-proteins in a manner different from that of agonists at various G-protein-linked receptors, i.e. formyl peptide-, nucleotide, histamine, prostaglandin and β-adrenergic receptors.

There are structural and functional similarities between lipopeptides, mastoparan of wasp venom and substance P (for review see Mousli et al., 1990b). All these substances bear positive charges and possess hydrophobic domains. In neutrophils, mastoparan, substance P and $Pam_3CysSer(Lys)_4$ mimick certain aspects of agonist-induced cell activation and the effects of either substance are only partially inhibited by pertussis toxin (Serra et al., 1988; Perianin and Snyderman, 1989; Seifert et al., 1990). It has been shown that substance P and mastoparan directly activate G-proteins, possibly by mimicking the effects of basic loops of plasma-membrane receptors (Higashijima et al., 1988; Mousli et al., 1990a). These results raise the possibility that lipopeptides do not necessarily act through plasma-membrane receptors but through the direct activation of G-proteins. However, unlike mastoparan and substance P, $Pam_3CysSer(Lys)_4$ did not stimulate high-affinity GTPase of G_i-type G-proteins and $Pam_3AdhSer(Lys)_4$ was moderately inhibitory in this respect (Table 5) (Higashijima et al., 1988; Mousli et al., 1990a). Finally, it cannot be excluded that the effects of lipopeptides on differentiation of HL-60 cells, by analogy with the ones of retinoic acid (Nervi et al., 1989), are mediated through nuclear receptor proteins, as the former substances rapidly enter the nucleus (Wolf et al. 1989). Thus, our results clearly emphasize the need for additional work to reveal the precise mechanism by which lipopeptides induce functional differentiation of HL-60 cells.

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