Biochem. J. (1992) 282, 551–557 (Printed in Great Britain)

Lipopeptides are effective stimulators of tyrosine phosphorylation in human myeloid cells

Stefan OFFERMANNS,*§ Roland SEIFERT,* Jörg W. METZGER,† Günther JUNG†, Albrecht LIEBERKNECHT‡, Ulrich SCHMIDT‡ and Günter SCHULTZ*

*Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, †Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen, and ‡Institut für Organische Chemie, Biochemie und Isotopenforschung, Universität Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart 80, Federal Republic of Germany

Synthetic lipopeptide analogues of the *N*-terminus of bacterial lipoprotein are effective activators of macrophages, neutrophils and lymphocytes. We studied the effect of the lipopeptide *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine [Pam_3Cys-Ser-$(Lys)_4$] on tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells, using anti-phosphotyrosine antibodies. Pam_3Cys-Ser-$(Lys)_4$ concentration-dependently stimulated tyrosine phosphorylation of 100/110 kDa and 60 kDa proteins and, to a lesser extent, of 55 kDa and 70/75 kDa proteins. Half-maximal and maximal effects were observed at concentrations of 1–6 and 5–50 μg/ml respectively. The lipopeptide-induced increase in phosphorylation was rapid and transient, with a peak response after 30–60 s. The lipopeptide (2*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoyl-Ser-$(Lys)_4$ [Pam_3Ahh-Ser-$(Lys)_4$] was as potent as Pam_3Cys-Ser$(Lys)_4$, whereas (2*S*,6*S*)-2-palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl-Ser-$(Lys)_4$ [Pam_3Adh-Ser-$(Lys)_4$] and Pam_3Cys-Ser-Gly did not induce tyrosine phosphorylation. Lipopeptide-induced tyrosine phosphorylation was not affected by treatment of cells with pertussis toxin. Neither phorbol 12-myristate 13-acetate nor A23187 induced tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells. In HL-60 promyelocytes, Pam_3Cys-Ser-$(Lys)_4$ had no effect on tyrosine phosphorylation, whereas the lipopeptide also induced tyrosine phosphorylation in 1,25-dihydroxyvitamin-D_3-differentiated HL-60 cells and in human neutrophils. These results show that lipopeptides are effective stimulators of tyrosine phosphorylation in mature human myeloid cells.

INTRODUCTION

Lipoprotein from the outer membrane of Gram-negative bacteria *N*-terminally contains one amide-linked and two ester-linked fatty acids attached to *S*-(2,3-dihydroxypropyl)-cysteine (Braun, 1975). Synthetic lipopeptide analogues of the *N*-terminus of bacterial lipoprotein are effective activators of lymphocytes (Bessler *et al.*, 1985), macrophages (Hauschildt *et al.*, 1990*a*) and neutrophils (Seifert *et al.*, 1990). 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_3Cys-Ser-$(Lys)_4$] induces superoxide formation and exocytosis in human neutrophils (Seifert *et al.*, 1990) and causes incomplete functional differentiation of promyelocytic HL-60 cells (Seifert *et al.*, 1991). The molecular mechanisms by which lipopeptides induce cellular activation are not known. Stimulation of superoxide formation by lipopeptide and lipopeptide-induced enhancement of stimulated superoxide formation in HL-60 cells was partially inhibited by pertussis toxin, suggesting the involvement of pertussis-toxin-sensitive G-proteins. However, in membranes of HL-60 promyelocytes and dibutyryl-cyclic-AMP-differentiated HL-60 cells, lipopeptides failed to stimulate high-affinity GTPase, the enzymic activity ascribed to G-proteins (Seifert *et al.*, 1991). Neither in macrophages nor in B-lymphocytes do lipopeptides show an effect on intracellular levels of cyclic AMP and cyclic GMP, phosphoinositide metabolism or protein kinase C activity (Steffens *et al.*, 1989; Hauschildt *et al.*, 1990*b*). Increase in cytosolic Ca^{2+} by lipopeptides has been observed in macrophages (Hauschildt *et al.*, 1990*b*), whereas lipopeptides show no effect on cytosolic Ca^{2+} in promyelocytic HL-60 cells (Seifert *et al.*, 1991).

Protein tyrosine kinase activities, first described in several products of viral transforming genes and growth-factor receptors, are considered to be involved in transformation and proliferation of cells (Hunter & Cooper, 1985; Ullrich & Schlessinger, 1990). The finding of high levels of normal cellular protein tyrosine kinases in non-proliferating post-mitotic cells (Brugge *et al.*, 1985; Golden *et al.*, 1986; Toyoshima *et al.*, 1990) suggests that the role of these enzymes is not restricted to transformation and proliferation of cells. Protein tyrosine kinase activity as well as phosphotyrosine phosphatase activity have been demonstrated in human neutrophils and HL-60 cells (Kraft & Berkow, 1987; Berkow *et al.*, 1989). Recently, the cytokine, granulocyte/monocyte colony-stimulating factor, and the chemoattractants, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), leukotriene B_4 and platelet-activating factor, have been shown to stimulate tyrosine phosphorylation of several protein substrates in rabbit and human neutrophils (Huang *et al.*, 1988; Gomez-Cambronero *et al.*, 1989; Huang *et al.*, 1990; Gomez-Cambronero *et al.*, 1991). In addition, studies on permeabilized neutrophils suggest a relationship between tyrosine phosphorylation and superoxide production (Nasmith *et al.*, 1989; Grinstein & Furuya, 1991), indicating a functional relevance of tyrosine phosphorylation in neutrophils. Taken together, protein tyrosine phosphorylation may play an important role in transducing signals of neutrophil-activating substances to the cell interior.

In this paper, we show that lipopeptides are effective stimu-

Abbreviations used: Pam_3Cys, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2-*RS*)-propyl]-(*R*)-cysteinyl; Adh, (2*S*,6*S*)-2-amino-6,7-dihydroxyheptanoyl; Ahh, (2*S*)-2-amino-6-hydroxymethyl-7-hydroxyheptanoyl; Pam_3Adh, (2*S*)-2-palmitoylamino-6,7-bis(palmitoyloxy)heptanoyl; Pam_3Ahh, (2*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoyl; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; G-protein, regulatory heterotrimeric guanine-nucleotide-binding protein; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride.

§ To whom correspondence should be addressed.

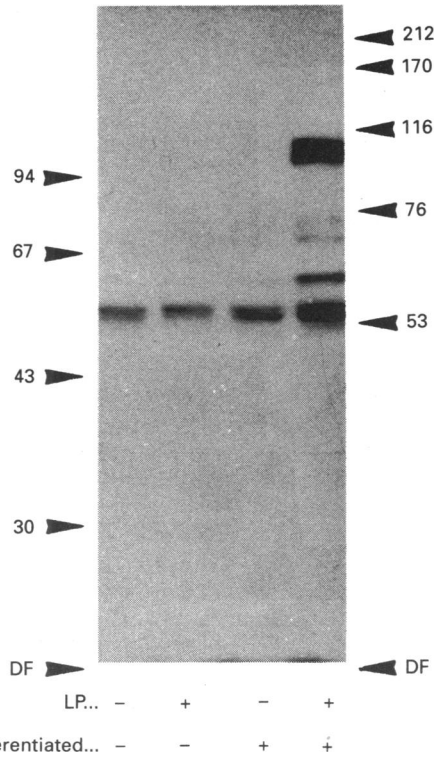

Fig. 1. Tyrosine phosphorylation stimulated by Pam_3Cys-Ser-$(Lys)_4$ in HL-60 cells differentiated with dibutyryl cyclic AMP

For this, 1×10^6 undifferentiated (two left-hand lanes) and dibutyryl-cyclic-AMP-differentiated HL-60 cells (two right-hand lanes) were incubated in the absence (LP−) or presence of 50 μg of Pam_3Cys-Ser-$(Lys)_4$/ml (LP+) for 1 min. Proteins were analysed as described in the Experimental section. The autoluminogram of a blot is shown. Numbers in the left and right margins indicate molecular masses of standard proteins (kDa); DF, dye front.

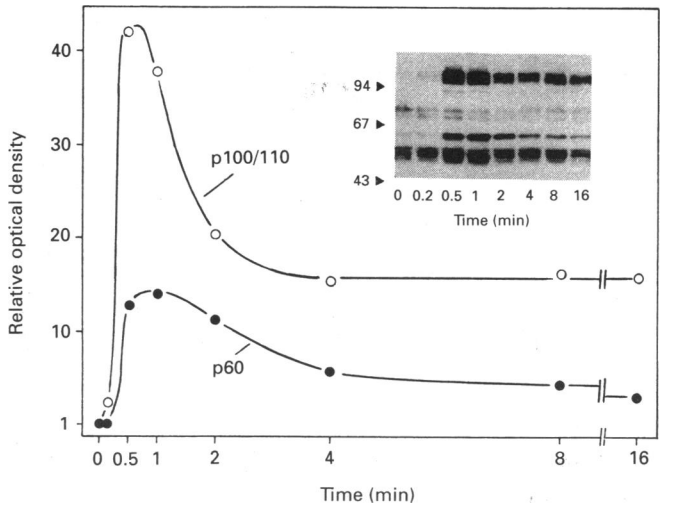

Fig. 2. Time course of Pam_3Cys-Ser-$(Lys)_4$-induced tyrosine phosphorylation in HL-60 cells differentiated with dibutyryl cyclic AMP

Cells (1×10^6/tube) were incubated with 50 μg of Pam_3Cys-Ser-$(Lys)_4$/ml for the indicated time periods. Samples were processed as described in the Experimental section. The obtained films were quantified by laser scanning densitometry. The optical density at zero time was arbitrarily taken as 1.0. Densitometric analyses of p60 (●) and p100/110 (○) are shown. Values are mean values of three experiments varying by less than 15%. The inset shows a representative autoluminogram, with the molecular masses (kDa) on the left margin.

lators of tyrosine phosphorylation in differentiated human myeloid cells.

EXPERIMENTAL

Materials

FMLP and phorbol 12-myristate 13-acetate (PMA) were from Sigma (Deisenhofen, Germany). Ca^{2+} ionophore A23187 was purchased from Calbiochem (Frankfurt, a. M., Germany), genistein and 1-(5-isoquinolinesulphonyl-2-methylpiperazine dihydrochloride (H-7) were from Gibco (Berlin, Germany), and monoclonal anti-phosphotyrosine antibody PY-20 IgG 2B was from ICN (Meckenheim, Germany). Pertussis toxin was given by Dr. M. Yajima (Kyoto, Japan). Electrophoresis calibration standards for molecular-mass determination were from Pharmacia (Freiburg, Germany).

Synthesis of lipopeptides

The synthesis of the lipopeptides Pam_3Cys-Ser-$(Lys)_4$, Pam_3Cys-Ser-Gly and (2*S*,6*S*)-2-palmitoylamino-6,7-bis-(palmitoyloxy)heptanoyl-Ser-$(Lys)_4$ [Pam_3Adh-Ser-$(Lys)_4$] were described previously (Metzger *et al.*, 1990, 1991). The amphiphilic lipohexapeptide (2*S*)-2-palmitoylamino-6-palmitoyloxymethyl-7-palmitoyloxyheptanoyl-Ser-$(Lys)_4$ [Pam_3Ahh-Ser-$(Lys)_4$] was obtained by coupling of Pam_3Ahh-OH (for preparation of this diastereomerically pure lipoamino acid see Schmidt *et al.*, 1991) to resin-bound Ser(tBu)-[Lys(Boc)]$_4$ (tBu = t-butyl; Boc = t-butyloxycarbonyl; for details of the synthesis of this resin-bound pentapeptide see Metzger *et al.*, 1991). Pam_3Ahh-OH (200 mg; 0.22 mmol) was activated with *NN'*-dicyclohexylcarbodi-imide (45 mg; 0.22 mmol) and 1-hydroxybenzotriazole (31 mg; 0.22 mmol) in chloroform/dimethylformamide (1:1, v/v; 5 ml) for 10 min. This mixture was added to a reaction vessel containing resin-bound Ser(tBu)-[Lys(Boc)]$_4$ (0.4 g of polystyrene/1% divinylbenzene resin with Wang linker; substitution 0.34 mmol/g). The suspension was shaken for 12 h and the reaction mixture finally removed by filtration. The resin was washed with dimethylformamide (3 times) and dried *in vacuo*. Pam_3Ahh-Ser-$(Lys)_4$ was cleaved from the resin with trifluoroacetic acid and purified by repeated precipitations from acetone at −20 °C. The lipopeptide was converted into its trihydrochloride as previously described for Pam_3Cys-Ser-$(Lys)_4$ (Metzger *et al.*, 1991) and freeze-dried from 2-methylpropan-2-ol (yield 173 mg; 79%). T.l.c. $R_F = 0.46$ (butan-1-ol/acetic acid/water, 2:1:1, by vol.); ion-spray mass spectrum: m/z 1507 $[M+H]^+$, 754 $[M+2H]^{2+}$.

Cell culture

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) horse serum, 2 mM-glutamine, 1% (v/v) non-essential amino acids, 50 units of penicillin/ml and 50 μg of streptomycin/ml in a humidified atmosphere with 7% CO_2 at 37 °C. To induce granulocyte differentiation, cells were seeded at a density of 10^6 cells/ml and were grown for 48 h in the presence of 200 μM dibutyryl cyclic AMP (Seifert *et al.*, 1989*a*). Monocytic differentiation was achieved by growing the cells for 120 h in the presence of 10 nM-1,25-dihydroxyvitamin D_3 (Jungblut & Seifert, 1990). For

pertussis-toxin treatment, cells were cultured in the presence of 100 ng of toxin/ml for 24 h.

Isolation of neutrophils

Human neutrophils were isolated from buffy-coat preparations obtained from the local blood bank by dextran sedimentation and centrifugation through Ficoll/Hypaque (Seifert *et al.*, 1989*b*).

Preparation of cell lysates

Cells were harvested by centrifugation for 10 min at 250 ***g***. Cells were resuspended in a buffer containing 138 mM-NaCl, 6 mM-KCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 1 mM-Na_2HPO_4, 5 mM-$NaHCO_3$, 5.5 mM-glucose and 20 mM-Hepes, pH 7.4. About 1×10^6 cells were preincubated for 3 min at 37 °C in a final volume of 40 μl. Incubation was initiated by addition of stimuli. If not stated otherwise, the reaction was stopped after 1 min by adding 20 μl of lysis buffer [6% (w/v) SDS, 18% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, 1 mM-Na_3VO_4, and a trace amount of Bromophenol Blue dye in 200 mM-Tris/HCl (pH 7.5)]. Samples were immediatedly incubated for 5 min at 100 °C.

Immunoblotting

The samples were subjected to SDS/PAGE on gels containing 9% (w/v) acrylamide. The separated proteins were then transferred on to nitrocellulose filters at 170 mA for 15 h in a buffer consisting of 300 mM-glycine, 40 mM-Tris/HCl (pH 8.3), 0.01% SDS and 20% (v/v) methanol. After blocking the filters with 3% (w/v) ovalbumin in 10 mM-Tris/HCl (pH 8.0)/150 mM-NaCl (TBS) for 1 h, proteins were incubated with 1 μg of monoclonal anti-phosphotyrosine antibodies/ml for 1 h in TBS, supplemented with 0.1% (w/v) BSA, followed by four 5 min washes in TBS containing 0.05% (v/v) Tween 20. Filters were then incubated with a goat anti-mouse IgG–peroxidase conjugate (Sigma) for 1 h and subsequently washed as described above. Bound antibodies were detected by the chemiluminescence (ECL) Western-blotting detection system (Amersham, Braunschweig, Germany). Phosphotyrosine-containing proteins were detected by exposure of blots to medical X-ray films (autoluminography). In some experiments, the autoluminograms obtained were analysed by a laser densitometer (LKB 2202 Ultroscan).

Reproducibility

The experiments shown are representative of three or more independently performed experiments.

RESULTS

In HL-60 cells differentiated towards neutrophils with dibutyryl cyclic AMP, the lipopeptide Pam_3Cys-Ser-$(Lys)_4$ markedly stimulated tyrosine phosphorylation of 100/110 kDa and 60 kDa proteins (p100/110, p60). To a lesser extent, enhanced phosphorylation of 70/75 kDa proteins was observed (see Fig. 1). Prolonged exposure time of the blots revealed that these proteins contained phosphotyrosine also in the absence of stimuli (see inset of Fig. 3). A 55 kDa protein, which showed a considerable level of constitutive phosphorylation, was further phosphorylated after addition of lipopeptide (see Fig. 1). Interactions of the antibodies with proteins were blocked by phosphotyrosine (1 mM) but not by either phosphothreonine (1 mM) or phosphoserine (1 mM), confirming the specificity of the antibodies used (results not shown). Addition of Pam_3Cys-Ser-$(Lys)_4$ to undifferentiated HL-60 cells did not cause tyrosine phosphorylation of any protein (see Fig. 1). Thus, increased tyrosine phosphorylation was dependent on induction of granulocytic differentiation of HL-60 cells.

The stimulation of tyrosine phosphorylation of p60 and p100/110 was rapid and transient (Fig. 2). Enhanced phosphorylation of both proteins was clearly detectable as early as 0.5 min after addition of the stimulus and reached a maximum after 0.5–1 min. Thereafter phosphorylation declined quickly to about 30–40% of maximum effects and remained at this level for up to at least 16 min. Maximal phosphorylation of p100/110 was about 2-fold higher than that of p60.

The effect of Pam_3Cys-Ser-$(Lys)_4$ on tyrosine phosphorylation of p60 and p100/110 was concentration-dependent and was evident at concentrations as low as 0.3–1 μg/ml (Fig. 3). Stimulation of tyrosine phosphorylation of p60 was half-maximal and maximal at concentrations of 1 and 5 μg/ml respectively, whereas somewhat higher concentrations were required for half-maximal and maximal effects on p100/110 (6 and about 50 μg/ml).

We further studied the effect of various other lipopeptides on tyrosine phosphorylation (Fig. 4). In contrast with Pam_3Cys-Ser-$(Lys)_4$, the lipopeptides Pam_3Adh-Ser-$(Lys)_4$ and Pam_3Cys-Ser-Gly showed no effect, whereas Pam_3Ahh-Ser-$(Lys)_4$ was equally effective as Pam_3Cys-Ser-$(Lys)_4$. Stimulation of Pam_3Ahh-Ser-$(Lys)_4$ and Pam_3Cys-Ser-$(Lys)_4$, employed at maximally effective concentrations, was not additive, indicating that both lipopeptides acted through a common mechanism (results not shown).

The chemotactic peptide fMLP has been shown to stimulate tyrosine phosphorylation of several proteins in neutrophils (Huang *et al.*, 1988; Berkow & Dodson, 1990). This effect was

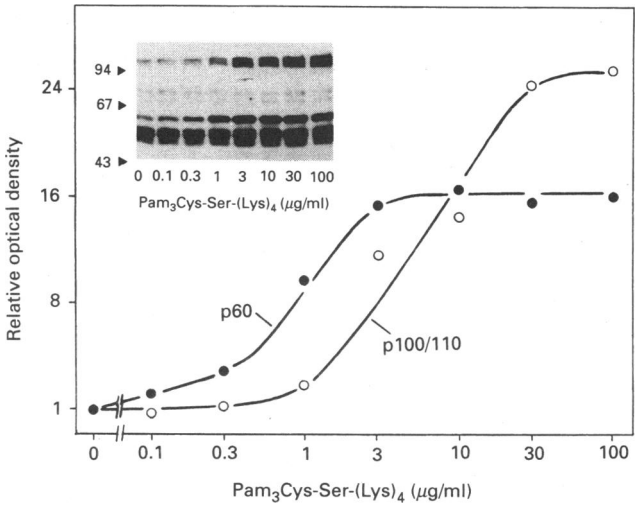

Fig. 3. Concentration-dependence of Pam_3Cys-Ser-$(Lys)_4$-stimulated tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells

Cells (1×10^6/tube) were incubated with Pam_3Cys-Ser-$(Lys)_4$ at the indicated final concentrations for 1 min and were analysed for tyrosine phosphorylation as described in the Experimental section. Autoluminograms of the blots were quantified by laser scanning densitometry. The optical density in the absence of Pam_3Cys-Ser-$(Lys)_4$ was arbitrarily taken as 1.0. Densitometric analyses of p60 (●) and p100/110 (○) are shown. Values are mean values of three experiments varying by less than 15%. The inset shows a representative autoluminogram, with the molecular masses (kDa) on the left margin.

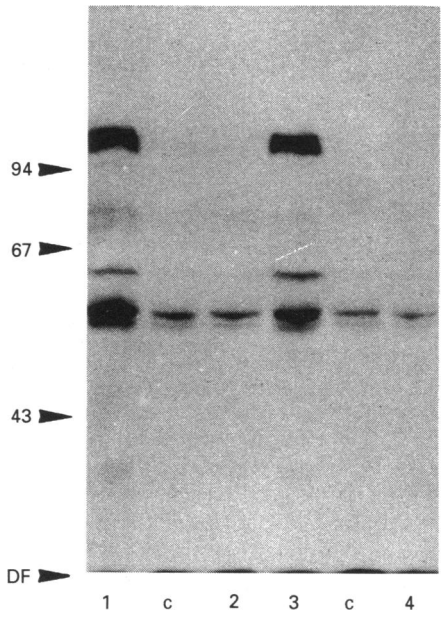

Fig. 4. Stimulation of tyrosine phosphorylation by different lipopeptides in dibutyryl-cyclic-AMP-differentiated HL-60 cells

Cells (1×10^6/tube) were incubated with different lipopeptides at a concentration of 100 μg/ml for 1 min. Proteins were analysed as described in the Experimental section. The autoluminogram of a blot is shown. Lipopeptides used were Pam_3Cys-Ser-$(Lys)_4$ (1), Pam_3Cys-Ser-Gly (2), Pam_3Ahh-Ser-$(Lys)_4$ (3) and Pam_3Adh-Ser-$(Lys)_4$ (4); c, control (without stimulus). Numbers on the left represent molecular masses of marker proteins (kDa); DF, dye front.

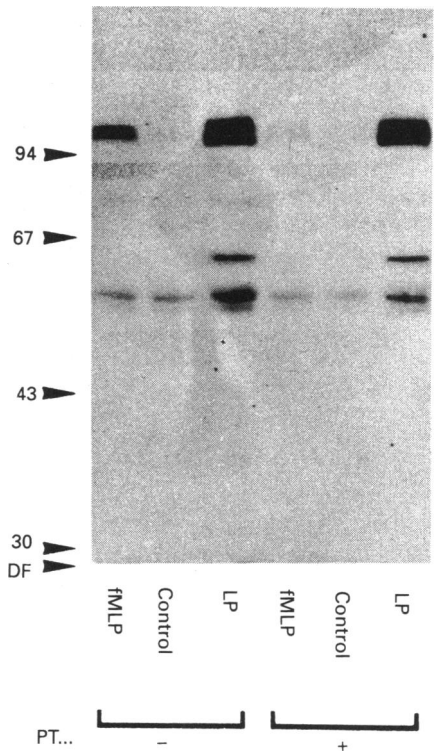

Fig. 5. Effect of pertussis toxin on fMLP- and lipopeptide-induced tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells

For this, 1×10^6 control (PT−) and pertussis-toxin-treated cells (PT+) were stimulated with 1 μM-fMLP or 50 μg of Pam_3Cys-Ser-$(Lys)_4$/ml (LP) for 1 min and were subsequently analysed for phosphotyrosine content as described in the Experimental section. Pertussis-toxin treatment was for 24 h with 100 ng of toxin/ml. An autoluminogram of a blot is shown. Values on the left indicate molecular masses of marker proteins (kDa); DF, dye front.

sensitive to pertussis toxin (Huang *et al.*, 1988), indicating the involvement of G-proteins which are substrates of the toxin. In order to evaluate the role of G-proteins in the stimulation of tyrosine phosphorylation by lipopeptides, HL-60 cells were pretreated with pertussis toxin (Fig. 5). fMLP, which weakly stimulated tyrosine phosphorylation of p100/110, but not of p60, had no effect in cells treated with pertussis toxin. In contrast, tyrosine phosphorylation induced by Pam_3Cys-Ser-$(Lys)_4$ was not affected by the toxin.

We also examined the role of protein kinase C and cytosolic Ca^{2+} in the lipopeptide-stimulated tyrosine phosphorylation, since both have been shown to be involved in tyrosine phosphorylation events induced by several agonists (Huang *et al.*, 1990; Huckle *et al.*, 1990; Takayama *et al.*, 1991) (Fig. 6). Stimulation of protein kinase C by PMA (100 ng/ml) for 5 min, as well as for 1 and 10 min (results not shown), did not change tyrosine phosphorylation of cellular proteins. In addition, the inhibitor of various serine and threonine kinases, H-7 (100 μM), did not affect lipopeptide-induced increase in tyrosine phosphorylation (results not shown).

Incubation of cells with A23187 (10 μM) for 5 min as well as for 1 and 10 min (results not shown) also had no effect on tyrosine phosphorylation. Additionally, incubation of HL-60 cells in a buffer devoid of Ca^{2+}, but containing 0.1 mM-EGTA, did not affect the lipopeptide-induced tyrosine phosphorylation (results not shown).

The isoflavone compound genistein has been shown to inhibit tyrosine kinases specifically *in vitro* and in intact cells (Akiyama *et al.*, 1987). Preincubation of cells with genistein (100 μM for 15 min) inhibited Pam_3Cys-Ser-$(Lys)_4$-stimulated tyrosine phosphorylation by more than 90% (Fig. 7).

We finally studied the effect of Pam_3Cys-Ser-$(Lys)_4$ on tyrosine phosphorylation in monocytic-differentiated HL-60 cells and in human neutrophils (Fig. 8). In monocytic-differentiated HL-60 cells, the lipopeptide induced tyrosine phosphorylation of a similar protein pattern to that in granulocytic-differentiated HL-60 cells. However, stimulation by the lipopeptide was much less pronounced. In human neutrophils, Pam_3Cys-Ser-$(Lys)_4$ weakly stimulated tyrosine phosphorylation of 110 and 62 kDa proteins. Proteins of 70/75 kDa, which showed substantial constitutive phosphorylation, were additionally phosphorylated by the lipopeptide.

DISCUSSION

In this paper, we show that lipopeptides increase tyrosine phosphorylation of several proteins in granulocytic-differentiated HL-60 cells. Stimulated tyrosine phosphorylation was mainly found in 60 kDa and 100/110 kDa bands (see Fig. 1). Depending on the individual gel, some experiments revealed that the 100/110 kDa band actually represented at least two proteins. Whether they represented distinct proteins or one protein with different covalent modifications is at present unknown. The fact that the 100/110 kDa proteins, but not the 60 kDa protein, were also phosphorylated in response to fMLP (see Fig. 5) suggests that p100/110 play a role in cellular signalling of different mediators.

The potency of lipopeptides to induce tyrosine phosphorylation in HL-60 cells resembles that to activate lymphocytes,

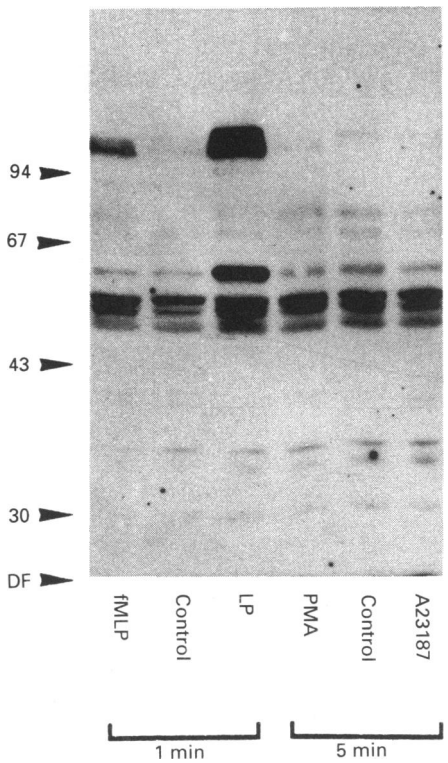

Fig. 6. Effects of PMA and A23187 on tyrosine phosphorylation in HL-60 cells differentiated with dibutryl cyclic AMP

Cells (1×10^6/tube) were incubated for the indicated time periods (1 or 5 min) with 10 μM-fMLP, 50 μg of Pam_3Cys-Ser-$(Lys)_4$/ml (LP), 100 ng of PMA/ml or 10 μM-A23187. Samples were processed as described in the Experimental section. The autoluminogram of a blot is shown. Numbers on the left indicate molecular masses of marker proteins (kDa); DF, dye front.

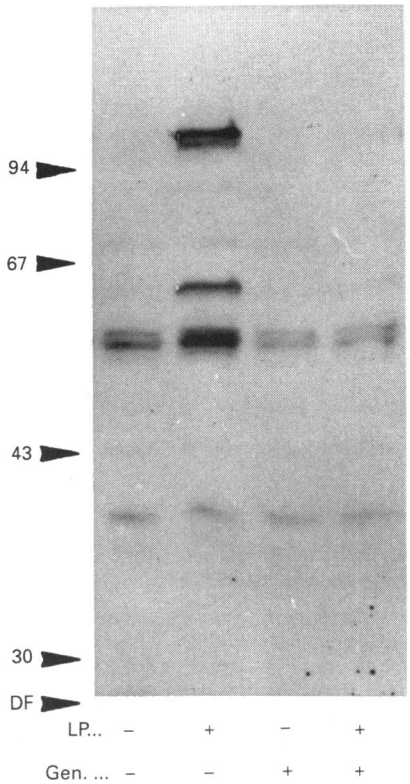

Fig. 7. Influence of genistein on lipopeptide-induced tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells

Cells (1×10^6/cell) were preincubated for 15 min in the absence (Gen.−) or presence of 100 μM-genistein (Gen.+) and were then incubated for 1 min with 50 μg of Pam_3Cys-Ser-$(Lys)_4$/ml (LP+) or vehicle (LP−). Proteins were analysed as described in the Experimental section. The autoluminogram of a blot is shown, with the molecular masses of marker proteins on the left; DF, dye front.

macrophages and neutrophils (Bessler *et al.*, 1985; Hoffmann *et al.*, 1989; Hauschildt *et al.*, 1990*c*; Seifert *et al.*, 1990). Stimulated tyrosine phosphorylation was detectable after a lag time of less than 30 s and quickly reached maximal value (see Fig. 2). A longer lag time (> 1 min) was observed for lipopeptide-induced superoxide formation in neutrophils (Seifert *et al.*, 1990). Thus tyrosine phosphorylation is an early response to lipopeptides and may precede activation of various cell functions.

In order to exclude that lipopeptides acted in an unspecific manner, we compared the effects of various lipopeptides differing in the lipid moiety and in the peptide chain (see Fig. 4). In contrast with Pam_3Cys-Ser-$(Lys)_4$, Pam_3Cys-Ser-Gly, which lacks the four positively charged amino groups of the lysyl residues, had no effect on phosphotyrosine levels. Replacement of the sulphur atom in Pam_3Cys-Ser-$(Lys)_4$ by a methylene group led to a loss of activity. However, both analogues, Pam_3Cys-Ser-$(Lys)_4$ and Pam_3Adh-Ser-$(Lys)_4$, had shown comparable stimulating effects in the proliferation test of splenic cells *in vitro* (Metzger *et al.*, 1991). Interestingly, Pam_3Ahh-Ser-$(Lys)_4$, an analogue of Pam_3Adh-Ser-$(Lys)_4$ with a further methylene group between the 6-palmitoyloxy residue and the asymmetric carbon atom C-6, displayed pronounced activity. Presumably Pam_3Ahh requires more space when incorporated into the cell membrane than Pam_3Adh. These biophysical diffences may be responsible for the observed differences in biological activity. These data indicate that both the positively charged amino acids of the peptide chain and the structure of the lipid moiety are necessary for stimulation of tyrosine phosphorylation by lipopeptides.

The finding that Pam_3Cys-Ser-$(Lys)_4$ had no effect on tyrosine phosphorylation in promyelocytic HL-60 cells raises the question of which changes occur during granulocytic differentiation to permit lipopeptide-induced phosphorylation. One attractive possibility is that stimulation by lipopeptides depends on the expression of specific protein tyrosine kinases. Several reports show that granulocytic differentiation of HL-60 cells is accompanied by increased expression of the *hck*, *c-fgr* and *c-fes* gene products (Quintrell *et al.*, 1987; Smithgall *et al.*, 1988; Notario *et al.*, 1989; Katagiri *et al.*, 1991), all of which are protein tyrosine kinases.

Whether lipopeptide-stimulated tyrosine phosphorylation is due to increased protein kinase activity or to a decrease in phosphotyrosine phosphatase activity, which are both present in HL-60 cells (Kraft & Berkow, 1987), remains to be determined. Sensitivity of the lipopeptide effect to the protein tyrosine kinase inhibitor genistein (see Fig. 7) does not rule out an influence of lipopeptides on phosphotyrosine phosphatases, since protein tyrosine kinase activity may be necessary for basal phosphorylation levels which may actually be regulated by specific phosphatases.

In addition to granulocytic differentiated HL-60 cells, Pam_3Cys-Ser-$(Lys)_4$ also stimulated tyrosine phosphorylation in monocytic-differentiated HL-60 cells as well as in human neutrophils (see Fig. 8). Thus tyrosine phosphorylation is an intracellular event common to various types of differentiated human myeloid cells in response to lipopeptides. Pam_3Cys-Ser-$(Lys)_4$ was considerably more effective to induce tyrosine

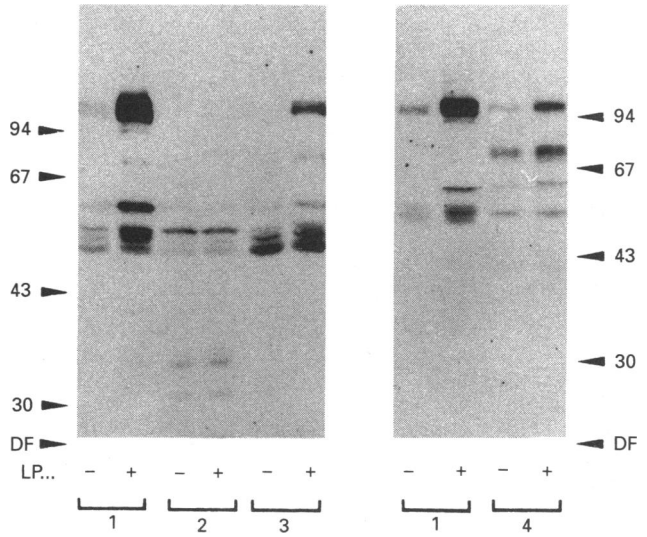

Fig. 8. Tyrosine phosphorylation stimulated by Pam_3Cys-Ser-$(Lys)_4$ in different types of human myeloid cells

Different myeloid cells (1×10^6 cells/tube) were incubated in the absence (LP−) and presence of 100 μg of Pam_3Cys-Ser-$(Lys)_4$/ml (LP+) for 1 min, and proteins were analysed for phosphotyrosine content as described in the Experimental section. An autolumiogram of a blot is shown. Cells used were dibutyryl-cyclic-AMP-differentiated HL-60 cells (1), undifferentiated HL-60 cells (2), 1,25-dihydroxyvitamin-D_3-differentiated HL-60 cells (3) and human neutrophils (4). Values on the left and right indicate molecular masses of marker proteins (kDa); DF, dye front.

phosphorylation in granulocytic-differentiated HL-60 cells than in monocytic-differentiated HL-60 cells and neutrophils. The reason for this difference is not known, but it may point to granulocytic-differentiated HL-60 cells as a suitable system for studies on lipopeptide-induced cellular activation.

Identification of a lipopeptide-binding protein in mouse spleen B-lymphocytes (Biesert *et al.*, 1987) may lead to the speculation that lipopeptide-induced tyrosine phosphorylation in HL-60 cells is also mediated by a specific binding protein. Studies carried out by electron-energy-loss spectroscopy (Wolf *et al.*, 1988) indicate that lipopeptides can quickly enter the cell, raising the possibility that lipopeptides exert their effects independently of a plasma-membrane receptor.

Recent studies using non-hydrolysable analogues of GTP (Nasmith *et al.*, 1989; Grinstein & Furuya, 1991) or pertussis toxin (Huang *et al.*, 1988; Gomez-Cambronero *et al.*, 1991) point to an involvement of G-proteins in some stimulations of tyrosine phosphorylation in neutrophils. In contrast with fMLP, lipopeptide-stimulated tyrosine phosphorylation in dibutyryl-cyclic-AMP-differentiated HL-60 cells was not affected by pertussis toxin (see Fig. 5), indicating that both substances exerted their effects by different mechanisms. If G-proteins are involved in lipopeptide-induced tyrosine phosphorylation, involvement may be restricted to pertussis-toxin-insensitive G-proteins or may be based on direct interaction of lipopeptides with G-proteins, a mechanism which could by-pass pertussis-toxin modification of G-proteins.

In some cases, activation of protein kinase C or elevation of cytosolic Ca^{2+} seems to be involved in the stimulation of tyrosine phosphorylation by neutrophil-activating agents (Huang *et al.*, 1990; Berkow & Dodson, 1990). Since an increase in cytosolic Ca^{2+} after addition of lipopeptide was observed in macrophages (Hauschildt *et al.*, 1990*b*), we examined the role of elevated cytosolic Ca^{2+} and additionally of activated protein kinase C in stimulation of tyrosine phosphorylation (see Fig. 6). Our results indicate that neither protein kinase C nor levels of cytosolic Ca^{2+} are involved in the lipopeptide-induced tyrosine phosphorylation.

Although the mechanisms by which cells respond to lipopeptides are as yet not understood, our findings clearly show that protein tyrosine phosphorylation is an early intracellular event effectively induced by lipopeptides in differentiated HL-60 cells.

We thank M. Bigalke and I. Reinsch for cell culture and Dr. M. Yajima (Kyoto, Japan) for providing pertussis toxin. This work was supported by the DFG and the Fonds der Chemischen Industrie.

REFERENCES

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. & Fukami, Y. (1987) J. Biol. Chem. **262**, 5592–5595
Berkow, R. L. & Dodson, R. W. (1990) Blood **75**, 2445–2452
Berkow, R. L., Dodson, R. W. & Kraft, A. S. (1989) Biochim. Biophys. Acta **997**, 292–301
Bessler, W., Cox, M., Lex, A., Suhr, B., Wiesmüller, K.-H. & Jung, G. (1985) J. Immunol. **135**, 1900–1905
Biesert, L., Scheuer, W. & Bessler, W. G. (1987) Eur. J. Biochem. **162**, 651–657
Braun, V. (1975) Biochim. Biophys. Acta **415**, 335–377
Brugge, J. S., Cotton, P. C., Queral, A. E., Barrett, J. N., Nonner, D. & Keane, R. W. (1985) Nature (London) **316**, 554–557
Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 852–856
Gomez-Cambronero, J., Huang, C.-K., Bonak, V. A., Wang, E., Casnelli, J. E., Shiraishi, T. & Sha'afi, R. I. (1989) Biochem. Biophys. Res. Commun. **162**, 1478–1485
Gomez-Cambronero, J., Wang, E., Johnson, G., Huang, C.-K. & Sha'afi, R. I. (1991) J. Biol. Chem. **266**, 6240–6245
Grinstein, S. & Furuya, W. (1991) Am. J. Physiol. **260**, C1019–C1027
Hauschildt, S., Hoffman, P., Beuscher, H. U., Dufhues, G., Heinrich, P., Wiesmüller, K.-H., Jung, G. & Bessler, W. G. (1990*a*) Eur. J. Immunol. **20**, 63–68
Hauschildt, S., Wolf, B., Lückhoff, A. & Bessler, W. G. (1990*b*) Mol. Immunol. **27**, 473–479
Hauschildt, S., Lückhoff, A., Mülsch, A, Kohler, J., Bessler, W. & Busse, R. (1990*c*) Biochem. J. **270**, 351–356
Hoffmann, P., Wiesmüller, K.-H., Metzger, J., Jung, G. & Bessler, W. G. (1989) Biol. Chem. Hoppe-Seyler **370**, 575–582
Huang, C.-K., Laramee, G. R. & Casnelli, J. E. (1988) Biochem. Biophys. Res. Commun. **151**, 794–801
Huang, C.-K., Bonak, V., Laramee, G. R. & Casnelli, J. E. (1990) Biochem. J. **269**, 431–436
Huckle, W. R., Prokop, C. A., Dy, R. C., Herman, B. & Earp, S. (1990) Mol. Cell. Biol. **10**, 6290–6298
Hunter, T. & Cooper, J. A. (1985) Annu. Rev. Biochem. **54**, 897–930
Jungblut, P. R. & Seifert, R. (1990) J. Biochem. Biophys. Methods **21**, 47–58
Katagiri, K., Katagiri, T., Koyama, Y., Morikawa, M., Yamamoto, T. & Yoshida, T. (1991) J. Immunol. **146**, 701–707
Kraft, A. S. & Berkow, R. L. (1987) Blood **70**, 356–362
Metzger, J., Jung, G., Bessler, W. G., Hoffmann, P., Strecker, M., Lieberknecht, A. & Schmidt, U. (1990) J. Med. Chem. **34**, 1969–1974
Metzger, J., Wiesmüller, K.-H., Schaude, R., Bessler, W. G. & Jung, G. (1991) Int. J. Pept. Protein Res. **37**, 46–57
Nasmith, P. E., Mills, G. B. & Grinstein, S. (1989) Biochem. J. **257**, 893–897
Notario V., Gutkind, J. S., Imaizumi, M., Katamine, S. & Robbins, K. C. (1989) J. Cell Biol. **109**, 3129–3136
Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M. Pettenati, M. J., Le Beau, M. M., Diaz, M. O. & Rowley, J. D. (1987) Mol. Cell. Biol. **7**, 2267–2275
Schmidt, U., Lieberknecht, A., Kazmaier, U., Griesser, H., Jung, G. & Metzger, J. (1991) Synthesis 49–55
Seifert, R., Burde, R. & Schultz, G. (1989*a*) Biochem. J. **259**, 813–819
Seifert, R., Burde, R. & Schultz, G. (1989*b*) Naunyn-Schmiedeberg's Arch. Pharmacol. **340**, 101–106

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
Seifert, R., Serke, S., Huhn, D., Bessler, W. G., Hauschildt, S., Metzger, J., Wiesmüller, K.-H. & Jung, G. (1991) Eur. J. Biochem., in the press
Smithgall, T. E., Yu, G. & Glazer, R. I. (1988) J. Biol. Chem. **263**, 15050–15055
Steffens, U., Bessler, W. & Hauschildt, S. (1989) Mol. Immunol. **26**, 897–904
Takayama, H., Nakamura, T., Yanagi, S., Taniguchi, T., Nakamura, S. & Yamamura, H. (1991) Biochem. Biophys. Res. Commun. **174**, 922–927
Toyoshima, K., Yamanashi, Y., Inoue, K., Katagiri, T., Sukegawa, J., Semba, K. & Yamamoto, T. (1990) Adv. Second Messenger Phosphoprotein Res. **24**, 284–289
Ullrich, A. & Schlessinger, J. (1990) Cell **61**, 203–212
Wolf, B., Uhl, B., Hauschildt, S., Metzger, J., Jung, G. & Bessler, W. G. (1988) Immunobiology **180**, 93–100

Received 10 September 1991; accepted 15 October 1991