Activation of superoxide formation and lysozyme release in human neutrophils by the synthetic lipopeptide Pam_3Cys-Ser-$(Lys)_4$

Involvement of guanine-nucleotide-binding proteins and synergism with chemotactic peptides

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Upon exposure to the bacterial chemotactic peptide fMet-Leu-Phe, human neutrophils release lysozyme and generate superoxide anions ($O_2^{\cdot-}$). The synthetic lipoamino acid *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam_3Cys), which is derived from the *N*-terminus of bacterial lipoprotein, when attached to Ser-$(Lys)_4$ [giving Pam_3Cys-Ser-$(Lys)_4$], activated $O_2^{\cdot-}$ formation and lysozyme release in human neutrophils with an effectiveness amounting to about 15% of that of fMet-Leu-Phe. Palmitic acid, muramyl dipeptide, lipopolysaccharide and the lipopeptides Pam_3Cys-Ala-Gly, Pam_3Cys-Ser-Gly, Pam_3Cys-Ser, Pam_3Cys-OMe and Pam_3Cys-OH did not activate $O_2^{\cdot-}$ formation. Pertussis toxin, which ADP-ribosylates guanine-nucleotide-binding proteins (G-proteins) and functionally uncouples formyl peptide receptors from G-proteins, prevented activation of $O_2^{\cdot-}$ formation by fMet-Leu-Phe and inhibited Pam_3Cys-Ser-$(Lys)_4$-induced $O_2^{\cdot-}$ formation by 85%. Lipopeptide-induced exocytosis was pertussis-toxin-insensitive. $O_2^{\cdot-}$ formation induced by Pam_3Cys-Ser-$(Lys)_4$ and fMet-Leu-Phe was enhanced by cytochalasin B, by a phorbol ester and by a diacylglycerol kinase inhibitor. Addition of activators of adenylate cyclase and removal of extracellular Ca^{2+} inhibited $O_2^{\cdot-}$ formation by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$ to different extents. Pam_3Cys-Ser-$(Lys)_4$ synergistically enhanced fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation and primed neutrophils to respond to the chemotactic peptide at non-stimulatory concentrations. Our data suggest the following. (1) Pam_3Cys-Ser-$(Lys)_4$ activates neutrophils through G-proteins, involving pertussis-toxin-sensitive and -insensitive processes. (2) The signal transduction pathways activated by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$ are similar but not identical. (3) In inflammatory processes, bacterial lipoproteins and chemotactic peptides may interact synergistically to activate $O_2^{\cdot-}$ formation, leading to enhanced bactericidal activity.

INTRODUCTION

Human neutrophils play a key role in the host defence against bacterial infections, with bactericidal activity depending largely on lysosomal enzyme release and on NADPH-oxidase-catalysed superoxide ($O_2^{\cdot-}$) formation (Rossi, 1986; Malech & Gallin, 1987). The bacterial chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe) binds to formyl peptide receptors and activates phospholipase C, catalysing polyphosphoinositide degradation to diacylglycerol and inositol phosphates (Rossi, 1986). Diacylglycerol activates protein kinase C, and inositol trisphosphate mobilizes intracellular Ca^{2+}. Formyl peptide receptors interact with guanine-nucleotide-binding proteins (G-proteins), whose ADP-ribosylation by pertussis toxin prevents cell activation. The mechanism by which chemotactic peptides activate NADPH oxidase may involve protein kinase C, Ca^{2+} mobilization and direct stimulation by G-proteins (Rossi, 1986; Seifert *et al.*, 1986; Seifert & Schultz, 1987*a*,*b*).

The outer cell wall of Gram-negative bacteria contains lipopolysaccharide and lipoprotein (Braun, 1975; Guthrie *et al.*, 1984; Forehand *et al.*, 1989). Lipoproteins and synthetic analogues derived from the *N*-terminus of lipoproteins are potent activators of macrophages and lymphocytes (Melchers *et al.*, 1975; Resch & Bessler, 1981; Bessler *et al.*, 1985; Hoffmann *et al.*, 1988; Hauschildt *et al.*, 1989). Lipopolysaccharide primes neutrophils for enhanced $O_2^{\cdot-}$ formation (Guthrie *et al.*, 1984; Forehand *et al.*, 1989), but the effects of lipoproteins on $O_2^{\cdot-}$ formation have not yet been studied. 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-$(Lys)_4$] contains two ester-bound and one amide-bound palmitoyl residues attached to *S*-glyceryl-cysteine (Fig. 1). In the murine macrophage cell line $P388D_1$, but not in murine B-lymphocytes or bone-marrow-derived macrophages, synthetic lipopeptides induce polyphosphoinositide degradation and translocation of protein kinase C (Hauschildt *et al.*, 1988; Steffens *et al.*, 1989). Recently it has been shown that synthetic viral

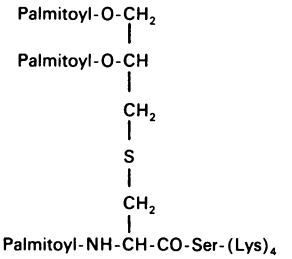

Fig. 1. Structural formula of Pam_3Cys-Ser-$(Lys)_4$

Abbreviations used: Pam_3Cys, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy-(2*RS*)-propyl]-(*R*)-cysteine; G-protein, guanine-nucleotide-binding protein; NEM, *N*-ethylmaleimide; CB, cytochalasin B; PMA, phorbol 12-myristate 13-acetate; PGE_1, prostaglandin E_1; PAF, platelet-activating factor; EC_{50}, concn. causing 50% of maximal stimulation; TNF, tumour necrosis factor.

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peptides covalently linked to lipopeptides, such as the influenza virus itself, induce high-affinity cytotoxic T-lymphocytes *in vivo* (Deres *et al.*, 1989). We report here that Pam_3Cys-Ser-$(Lys)_4$ activates $O_2^{\cdot-}$ formation and exocytosis in human neutrophils through pertussis-toxin-sensitive and -insensitive signal transduction pathways.

MATERIALS AND METHODS

Materials

The lipopeptides Pam_3Cys-Ser (Prass *et al.*, 1987), Pam_3Cys-Ser-Gly, Pam_3Cys-Ala-Gly and Pam_3Cys-Ser-$(Lys)_4$ (Reitermann *et al.*, 1989), and the lipoamino acid Pam_3Cys-OH (Wiesmüller *et al.*, 1983) and its methyl ester Pam_3Cys-OMe (Jung *et al.*, 1983) were prepared by chemical synthesis and were unequivocally characterized by various analytical and spectroscopic methods such as mass spectroscopy, ^{13}C n.m.r. spectroscopy and chiral-phase amino acid analysis. None of the lipopeptides studied induced the release of lactate dehydrogenase, and staining of human neutrophils with Trypan Blue (results not shown) suggesting that lipopeptides are not cytotoxic in the concentrations supplied. Pertussis toxin was a gift from Dr. M. Yajima (Kyoto, Japan). Staurosporine was from Fluka (Buchs, Switzerland), and 6-[2{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl]-7-methyl-5*H*-thiazolo-[3,2-*al*]pyrimidin-5-one (R 59 022) was from Janssen (Olen, Belgium). *N*-Ethylmaleimide (NEM), lipopolysaccharide from *Escherichia coli* (serotype 055:B5), muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) and lyophilized *Micrococcus lysodeikticus* were obtained from Sigma Chemie (Taufkirchen, Germany). Recombinant human tumour necrosis factor-α (TNFα) was from Boehringer Mannheim (Mannheim, Germany). Sources of other materials have been described elsewhere (Seifert *et al.*, 1986, 1989*a*,*b*,*c*; Seifert & Schultz, 1987*a*,*b*; Seifert & Schächtele, 1988; Burde *et al.*, 1989).

Isolation of neutrophils

Human neutrophils were prepared as described previously in detail (Markert *et al.*, 1984; Seifert *et al.*, 1989*b*). Briefly, blood was obtained by venous puncture from healthy volunteers. Neutrophils were isolated by Dextran sedimentation and centrifugation through Ficoll–Hypaque. Cell preparations consisted of more than 98% viable neutrophils as judged by Trypan Blue dye exclusion.

Assay for $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ formation was monitored at 550 nm by continuous measurement of ferricytochrome *c* reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual-beam spectrophotometer (Kontron, Eching, Germany) (Markert *et al.*, 1984; Seifert *et al.*, 1989*b*). Reaction mixtures (0.5 ml) contained 100 μM-ferricytochrome *c* and a buffer consisting of 138 mM-NaCl, 6 mM-KCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5.5 mM-glucose and 20 mM-Hepes/NaOH, pH 7.4. Cells (1.0×10^6 neutrophils) were preincubated for 3 min in the absence or presence of various additives at 37 °C. $O_2^{\cdot-}$ formation was initiated by the addition of the stimuli. Reference cuvettes contained all of the components listed above, plus superoxide dismutase (50 μg).

Assay for lysozyme and β-glucuronidase release

Exocytosis was assessed as described (Absolom, 1986; Seifert *et al.*, 1989*c*; Burde *et al.*, 1989). Briefly, neutrophils (5×10^6 cells) were incubated in buffer (0.5 ml) for 5 min in the presence of cytochalasin B (CB) (1 μg/ml). At 10 min after addition of stimuli, the tubes were cooled to 0 °C and were centrifuged for 10 min at 250 *g*. Determination of the activities of lysozyme, β-glucuronidase and lactate dehydrogenase in the supernatant fluids of reaction mixtures and of cell lysates was performed as described (Absolom, 1986; Seifert *et al.*, 1989*c*; Burde *et al.*, 1989). The release of lactate deydrogenase generally amounted to < 5% of the cellular content (results not shown).

Statistics

Data shown in Tables 1–6 are the means ± S.D. of five to seven experiments. The significance of the effects was assessed using the Wilcoxon test.

RESULTS

Activation of $O_2^{\cdot-}$ formation by Pam_3Cys-Ser-$(Lys)_4$ in human neutrophils: kinetics and concentration-dependency

fMet-Leu-Phe at a submaximally stimulatory concentration (30 nM) induced $O_2^{\cdot-}$ formation with a lagtime of < 10 s, ceasing after 4–5 min (Fig. 2, trace 3). In contrast, Pam_3Cys-Ser-$(Lys)_4$ at submaximally and maximally stimulatory concentrations (30 μg/ml and 100 μg/ml respectively) induced $O_2^{\cdot-}$ formation with a lagtime of > 1 min, not ceasing until 8–9 min (Fig. 2, traces 1 and 2). Pam_3Cys-Ser-$(Lys)_4$ at a maximally stimulatory concentration was a less effective activator of $O_2^{\cdot-}$ formation than the chemotactic peptide at 30 nM (compare traces 2 and 3). The addition of Pam_3Cys-Ser-$(Lys)_4$ (30 μg/ml) to neutrophils 3 min before or together with fMet-Leu-Phe resulted in synergistic activation of NADPH oxidase with respect to the maximum rate of $O_2^{\cdot-}$ formation and the absolute amount of $O_2^{\cdot-}$ generated (trace 6). $O_2^{\cdot-}$ formation synergistically induced by the chemotactic peptide and the lipopeptide was characterized by a period lasting about 2 min with a high rate of $O_2^{\cdot-}$ formation and was followed by a period lasting about 9 min during which $O_2^{\cdot-}$ formation declined slowly. The lipopeptide re-initiated $O_2^{\cdot-}$ formation when added to neutrophils after the fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation had ceased (Fig. 2, traces 4 and 5). However, the extent of NADPH oxidase activation induced by the lipopeptide in fMet-Leu-Phe-stimulated neutrophils was not greater than in unstimulated cells (compare trace 1 with trace 4 and trace 2 with trace 5).

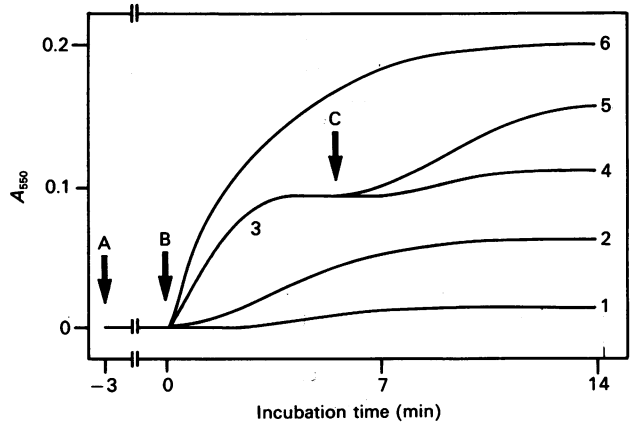

Fig. 2. Kinetics of $O_2^{\cdot-}$ formation in human neutrophils

Trace 1, addition of Pam_3Cys-Ser-$(Lys)_4$ (30 μg/ml) at time B; trace 2, addition of Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml) at time B; trace 3, addition of fMet-Leu-Phe (30 nM) at time B; trace 4, addition of Pam_3Cys-Ser-$(Lys)_4$ (30 μg/ml) at time C; trace 5, addition of Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml) at time C; trace 6, addition of fMet-Leu-Phe (30 nM) at time B plus Pam_3Cys-Ser-$(Lys)_4$ (30 μg/ml) at time A or B. Experiments were carried out in the absence of CB. Superimposed original registrations from one single experiment are shown. Similar results were obtained in six identical experiments.

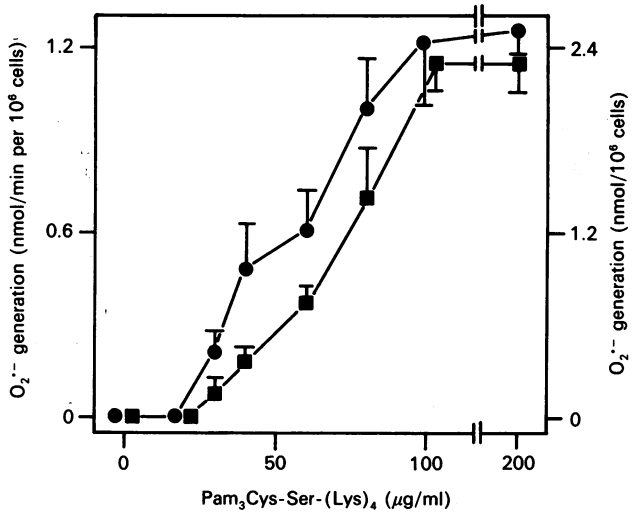

Fig. 3. Concentration–response curve of Pam_3Cys-Ser-(Lys)$_4$-induced $O_2^{\cdot-}$ formation in human neutrophils

Experiments were carried out in the presence of CB (1 μg/ml). ●, Maximum rate of $O_2^{\cdot-}$ formation; ■, total amount of $O_2^{\cdot-}$ generated. Data shown are the means ± S.D. of three experiments.

Fig. 3 shows the concentration–response curve for activation of NADPH oxidase by Pam_3Cys-Ser-(Lys)$_4$. The experiments were carried out in the presence of CB, which potentiates receptor-mediated $O_2^{\cdot-}$ formation by inhibiting sequestration of plasma membrane receptors (Jesaitis *et al.*, 1986). The lipopeptide activated $O_2^{\cdot-}$ formation in a concentration-dependent manner with respect to the maximum rate and the absolute amount of $O_2^{\cdot-}$ generated. The effect of Pam_3Cys-Ser-(Lys)$_4$ was half-maximal at 60–70 μg/ml and reached a plateau at 100 μg/ml. In contrast, muramyl dipeptide and the lipopeptides Pam_3Cys-OH, Pam_3Cys-OMe, Pam_3Cys-Ser, Pam_3Cys-Ser-Gly and Pam_3Cys-Ala-Gly at 1–100 μg/ml did not activate $O_2^{\cdot-}$ formation in the absence or presence of CB (results not shown). Palmitic acid and lipopolysaccharide at 1–100 μg/ml did not activate $O_2^{\cdot-}$ formation in suspended neutrophils (results not shown).

Activation of $O_2^{\cdot-}$ formation by Pam_3Cys-Ser-(Lys)$_4$: role of G-proteins

In order to elucidate the role of G-proteins in the activation of $O_2^{\cdot-}$ formation by Pam_3Cys-Ser-(Lys)$_4$, neutrophils were treated with pertussis toxin. Pertussis toxin inhibited $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe and Pam_3Cys-Ser-(Lys)$_4$ but not by phorbol 12-myristate 13-acetate (PMA), which circumvents receptor stimulation by activating protein kinase C directly (Fig. 4). The inhibitory effect of pertussis toxin on fMet-Leu-Phe- and lipopeptide-induced $O_2^{\cdot-}$ formation was half-maximal at 5–10 ng/ml and maximal at 1 μg/ml. Pertussis toxin completely abolished the stimulatory effect of fMet-Leu-Phe, whereas the effect of Pam_3Cys-Ser-(Lys)$_4$ was inhibited by up to 85%. The effect of pertussis toxin on $O_2^{\cdot-}$ formation was time-dependent. Half-maximal inhibition of fMet-Leu-Phe- and lipopeptide-induced $O_2^{\cdot-}$ formation was observed after 60–75 min of toxin treatment. Inhibition of chemotactic-peptide-induced $O_2^{\cdot-}$ formation was complete after an incubation time of 3 h. Again, pertussis toxin did not completely abolish the stimulatory effect of Pam_3Cys-Ser-(Lys)$_4$ on $O_2^{\cdot-}$ formation.

Synergistic activation of $O_2^{\cdot-}$ formation by fMet-Leu-Phe and Pam_3Cys-Ser-(Lys)$_4$

The synergistic effects of fMet-Leu-Phe and Pam_3Cys-Ser-(Lys)$_4$ were studied in detail. The experiments shown in Fig. 5

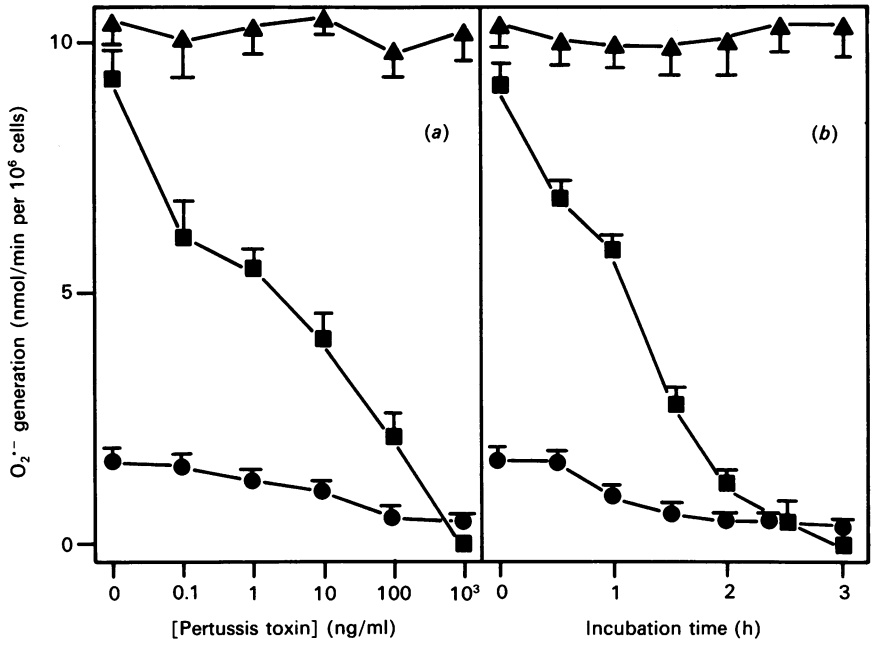

Fig. 4. Inhibition of $O_2^{\cdot-}$ formation by pertussis toxin in human neutrophils

Neutrophils were suspended at a concentration of 10^7 cells/ml in the assay buffer for determination of $O_2^{\cdot-}$ formation not supplemented with $CaCl_2$ and were incubated with pertussis toxin or its vehicle. Following toxin treatment, cells were transferred into cuvettes and assayed for $O_2^{\cdot-}$ formation. (*a*) Concentration-dependence. Neutrophils were incubated for 3 h in the presence of pertussis toxin at the indicated concentrations. (*b*) Time dependence. Cells were incubated in the presence of pertussis toxin (1 μg/ml) for the indicated periods of time. The concentrations of stimuli were as follows: ▲, PMA (100 ng/ml); ■, 1 μM-fMet-Leu-Phe plus CB (1 μg/ml); ●, Pam_3Cys-Ser-(Lys)$_4$ (100 μg/ml) plus CB (1 μg/ml). Data shown are the means ± S.D. of six experiments. The inhibitory effect of pertussis toxin on Pam_3Cys-Ser-(Lys)$_4$-induced $O_2^{\cdot-}$ formation was significant ($P < 0.05$ or $P < 0.01$) at toxin concentrations of 1–1000 ng/ml and incubation times of 1–3 h.

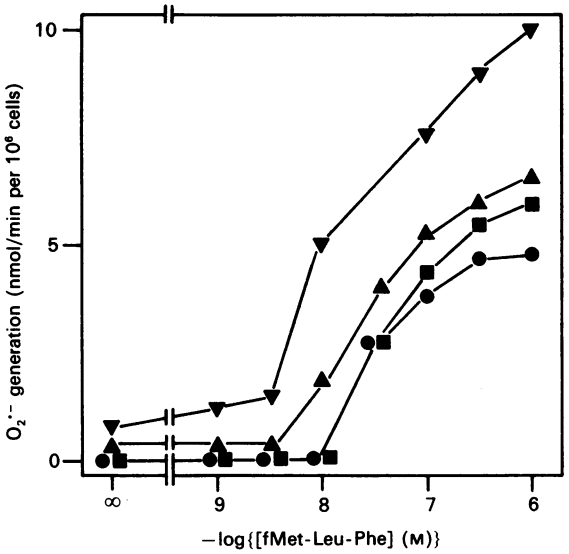

Fig. 5. Synergistic activation of $O_2^{\cdot-}$ formation by Pam_3Cys-Ser-$(Lys)_4$ and fMet-Leu-Phe in human neutrophils

The effects of fMet-Leu-Phe at various concentrations were studied in the presence of Pam_3Cys-Ser-$(Lys)_4$ at three fixed concentrations. Experiments were carried out in the absence of CB. ●, Solvent (control); ■, Pam_3Cys-Ser-$(Lys)_4$ (10 μg/ml); ▲, Pam_3Cys-Ser-$(Lys)_4$ (30 μg/ml); ▼, Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml). Pam_3Cys-Ser-$(Lys)_4$ plus fMet-Leu-Phe were added simultaneously to reaction mixtures. Data shown are the means of assay duplicates, which varied by less than 5%. Similar results were obtained in three experiments.

were carried out in the absence of CB. fMet-Leu-Phe activated $O_2^{\cdot-}$ formation with an EC_{50} (concn. causing 50% of maximal stimulation) of 40 nM and maximum stimulation at 300 nM. Pam_3Cys-Ser-$(Lys)_4$ at a non-stimulatory concentration (10 μg/ml) enhanced the $O_2^{\cdot-}$ formation induced by a fMet-Leu-Phe concentration above 30 nM by up to 30%. The lipopeptide at a submaximally stimulatory concentration (30 μg/ml) primed neutrophils to respond to the chemotactic peptide at a non-stimulatory concentration (10 nM) and enhanced $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe at submaximally and maximally stimulatory concentrations by up to 45% without significantly lowering the EC_{50} for the formyl peptide. Pam_3Cys-Ser-$(Lys)_4$ at a maximally effective concentration (100 μg/ml) primed neutrophils for a fMet-Leu-Phe concentration as low as 1 nM and enhanced the effect of fMet-Leu-Phe at submaximally and maximally stimulatory concentrations by up to 2-fold.

Table 1. Effects of various lipopeptides on fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation in human neutrophils

The effects of various lipopeptides on $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe (1 μM) were assessed. Lipopeptides at 100 μg/ml or solvent (control) were added to reaction mixtures simultaneously with the chemotactic peptide. Experiments were carried out in the absence of CB. Significant differences from control are indicated by $*P < 0.05$; $**P < 0.01$.

Lipopeptide	$O_2^{\cdot-}$ formation (nmol/min per 10^6 cells)
None	5.2±0.8
Pam_3Cys-Ser-$(Lys)_4$	12.2±1.4**
Pam_3Cys-Ala-Gly	3.8±0.9
Pam_3Cys-Ser-Gly	4.1±0.7
Pam_3Cys-Ser	3.5±0.6*
Pam_3Cys-OMe	3.6±0.4*
Pam_3Cys-OH	1.2±0.3**

The interaction of fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$ was markedly affected by CB (results not shown). In the presence of CB, neutrophils generated $O_2^{\cdot-}$ at similar rates upon exposure to fMet-Leu-Phe at 3 nM and to Pam_3Cys-Ser-$(Lys)_4$ at 100 μg/ml. The lipopeptide and the chemotactic peptide at these concentrations activated $O_2^{\cdot-}$ formation additively. However, with fMet-Leu-Phe at a maximally stimulatory concentration (1 μM), no synergism with Pam_3Cys-Ser-$(Lys)_4$ was apparent.

Table 1 summarizes the effects of various lipopeptides at 100 μg/ml on fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation in the absence of CB. In contrast with Pam_3Cys-Ser-$(Lys)_4$, the lipotripeptides Pam_3Cys-Ala-Gly and Pam_3Cys-Ser-Gly did not significantly affect $O_2^{\cdot-}$ formation, whereas Pam_3Cys-Ser and Pam_3Cys-OMe inhibited $O_2^{\cdot-}$ formation by up to 35% and Pam_3Cys-OH inhibited $O_2^{\cdot-}$ formation by more than 75%.

PMA, UTP, platelet-activating factor (PAF) and TNFα prime neutrophils for enhanced $O_2^{\cdot-}$ formation upon exposure to fMet-Leu-Phe (Dewald & Baggiolini, 1985; Tyagi *et al.*, 1988; Seifert *et al.*, 1989*a*,*b*,*c*; Yuo *et al.*, 1989). Therefore we compared the interaction of these priming agents with fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$ (Table 2). Neither UTP nor TNFα *per se* induced $O_2^{\cdot-}$ formation. PMA at 1 ng/ml induced $O_2^{\cdot-}$ formation at a low rate, and PAF (1 μM) activated $O_2^{\cdot-}$ formation to an extent amounting to 30% of that induced by fMet-Leu-Phe (1 μM). PMA, TNFα, UTP and PAF interacted synergistically with fMet-Leu-Phe to activate $O_2^{\cdot-}$ formation. In contrast, only PMA but not TNFα, UTP or PAF interacted synergistically with Pam_3Cys-Ser-$(Lys)_4$ to activate NADPH oxidase.

Table 2. Priming of human neutrophils for enhanced $O_2^{\cdot-}$ formation with PMA, TNFα, UTP and PAF

The effects of PMA, TNFα, UTP and PAF on $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe (1 μM), Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml) or solvent (control) were studied. PAF was added to reaction mixtures together with fMet-Leu-Phe or Pam_3Cys-Ser-$(Lys)_4$. PMA and UTP were added to reaction mixtures 3 min before addition of stimuli, and TNFα was added 15 min before addition of stimuli. Experiments were carried out in the absence of CB. Significant differences from control (no addition) are indicated by $*P < 0.05$, $**P < 0.01$.

Addition	$O_2^{\cdot-}$ formation (nmol/min per 10^6 cells)		
	Control	fMet-Leu-Phe	Pam_3Cys-Ser-$(Lys)_4$
None	0	4.9±0.4	1.3±0.4
PMA (1 ng/ml)	0.3±0.1	7.7±0.8**	2.2±0.3*
TNFα (6 nM)	0	8.8±0.5**	1.3±0.5
UTP (100 μM)	0	9.2±0.9**	1.2±0.4
PAF (1 μM)	1.5±0.2	11.3±1.0**	0.9±0.2

Table 3. Effects of various drugs on $O_2^{\cdot-}$ formation in human neutrophils induced by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$

The effects of various drugs on $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe (1 μM) and Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml) were studied. Drugs or solvent (control) were added to reaction mixtures 3 min before addition of stimuli. Significant differences from control values (no addition) are indicated by $*P < 0.05$; $**P < 0.01$.

Addition	$O_2^{\cdot-}$ formation (nmol/min per 10^6 cells)	
	fMet-Leu-Phe	Pam_3Cys-Ser-$(Lys)_4$
None	4.8±0.4	0.6±0.2
Staurosporine (1 μM)	0**	0**
NEM (1 mM)	0**	0**
EDTA (1 mM), no Ca^{2+} added	0.7±0.3**	0.4±0.2*
CB (1 μg/ml)	9.9±1.2**	1.3±0.3**
R 59 022 (10 μM)	10.4±1.2**	1.4±0.2**

Modulation of $O_2^{\cdot-}$ formation by various drugs

The effects of various drugs on chemotactic peptide- and lipopeptide-induced $O_2^{\cdot-}$ formation were compared (Table 3). Staurosporine, a potent inhibitor of protein kinases (Schächtele *et al.*, 1988; Rüegg & Burgess, 1989), and the thiol reagent NEM abolished $O_2^{\cdot-}$ formation induced by both Pam_3Cys-Ser-$(Lys)_4$ and fMet-Leu-Phe. The removal of extracellular Ca^{2+} decreased fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation by more than 80%, whereas the effect of Pam_3Cys-Ser-$(Lys)_4$ was diminished only by about 30%. CB and R 59 022, the inhibitor of diacylglycerol kinase (de Chaffoy de Courcelles *et al.*, 1985), enhanced chemotactic peptide- and lipopeptide-induced $O_2^{\cdot-}$ formation by up to 2.5-fold.

$O_2^{\cdot-}$ formation induced by various receptor agonists is differentially inhibited by the activators of adenylate cyclase, prostaglandin E_1 (PGE_1), isoprenaline, histamine and cholera toxin (Seifert *et al.*, 1989*a*; Burde *et al.*, 1989). PGE_1, isoprenaline and histamine inhibited fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation by up to 55%, whereas the effect of Pam_3Cys-Ser-$(Lys)_4$ was inhibited by up to 85% (Table 4). In agreement with a recent report, cholera toxin did not significantly inhibit fMet-Leu-Phe-induced $O_2^{\cdot-}$ formation (Gabler *et al.*, 1989). In contrast, cholera toxin inhibited the effect of Pam_3Cys-Ser-$(Lys)_4$ by about 70%.

Activation of exocytosis by Pam_3Cys-Ser-$(Lys)_4$

The role of lipopeptides in the regulation of exocytosis was

Table 4. Inhibition by activators of adenylate cyclase of $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$ in human neutrophils

The effects of various activators of adenylate cyclase on $O_2^{\cdot-}$ formation induced by fMet-Leu-Phe (1 μM) or Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml) plus CB (1 μg/ml) were studied. PGE_1, isoprenaline, histamine or solvent (control) were added to reaction mixtures 3 min before addition of stimuli. Cells were treated with cholera toxin or its vehicle for 3 h according to the procedure described in the legend to Fig. 4. Significant differences from control values (no additions) are indicated by $*P < 0.01$.

Addition	$O_2^{\cdot-}$ generation (nmol/min per 10^6 cells)	
	fMet-Leu-Phe	Pam_3Cys-Ser-$(Lys)_4$
None	8.9±0.5	1.2±0.3
PGE_1 (10 μM)	4.1±0.6*	0.2±0.1*
Isoprenaline (1 μM)	5.6±1.3*	0.4±0.2*
Histamine (1 mM)	5.4±0.9*	0.2±0.1*
Cholera toxin (1 μg/ml)	7.7±0.9	0.4±0.2*

Table 5. Synergistic activation of lysozyme release in human neutrophils by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$

The effects of fMet-Leu-Phe and of Pam_3Cys-Ser-$(Lys)_4$ and combination of both stimuli on lysozyme release in human neutrophils were studied. Neutrophils were treated with CB (1 μg/ml) for 5 min before addition of stimuli. Assays were conducted for a period of 10 min. Comparison of the effectiveness of fMet-Leu-Phe or Pam_3Cys-Ser-$(Lys)_4$ versus solvent (control): $*P < 0.01$. Comparison of fMet-Leu-Phe alone versus fMet-Leu-Phe plus Pam_3Cys-Ser-$(Lys)_4$: $\dagger P < 0.05$.

Stimulus	Lysozyme release (% of cellular content)
None	4.3±1.5
fMet-Leu-Phe (30 nM)	12.8±2.1*
fMet-Leu-Phe (1 μM)	34.0±3.9*
Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml)	10.3±2.7*
fMet-Leu-Phe (30 nM) + Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml)	19.0±3.0†
fMet-Leu-Phe (1 μM) + Pam_3Cys-Ser-$(Lys)_4$ (100 μg/ml)	38.6±5.2

Table 6. Effect of pertussis toxin on exocytosis in human neutrophils induced by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$

Neutrophils were treated with pertussis toxin (PTX) at 1 μg/ml or its vehicle under the conditions described in the legend to Fig. 4. Cells were preincubated with CB (1 μg/ml) for 3 min before the addition of stimuli or solvent (control). The concentrations of stimuli were as follows: fMet-Leu-Phe, 1 μM; Pam_3Cys-Ser-$(Lys)_4$, 100 μg/ml; A23187, 10 μM. Effectiveness of stimuli to induce exocytosis in control cells: $*P < 0.01$. Effectiveness of PTX to inhibit exocytosis versus control cells: $\dagger P < 0.01$.

Stimulus	Treatment...	β-Glucuronidase release (% of cellular content) None	β-Glucuronidase release (% of cellular content) PTX	Lysozyme release (% of cellular content) None	Lysozyme release (% of cellular content) PTX
None		5.6±0.6	6.2±0.6	4.1±0.2	3.8±1.8
fMet-Leu-Phe		30.7±2.1*	5.1±0.5†	32.1±1.9*	10.1±1.0†
Pam_3Cys-Ser-$(Lys)_4$		5.9±0.4	4.9±1.6	11.1±2.8*	12.0±2.3
A23187		44.3±2.8*	43.0±5.6	41.0±5.4*	38.0±5.5

studied (Tables 5 and 6). Lysozyme is localized in the azurophilic and specific granules, and β-glucuronidase is present only in the azurophilic granules (Absolom, 1986). Pam_3Cys-Ser-$(Lys)_4$ at 100 μg/ml induced lysozyme release to a similar extent as did the chemotactic peptide at 30 nM. In addition, the lipopeptide and fMet-Leu-Phe (30 nM) induced lysozyme release additively. However, fMet-Leu-Phe at 1 μM and Pam_3Cys-Ser-$(Lys)_4$ showed no additive effect on lysozyme release. Pam_3Cys-Ser-$(Lys)_4$ did not induce β-glucuronidase release, suggesting that the lipopeptide induced only exocytosis of specific granules.

Human neutrophils were treated with pertussis toxin (1 μg/ml) for 3 h (see Fig. 4). Pertussis toxin completely inhibited fMet-Leu-Phe-induced β-glucuronidase release and did not inhibit exocytosis induced by the Ca^{2+} ionophore, A23187, which circumvents receptor stimulation by increasing the intracellular Ca^{2+} concentration directly. In addition, pertussis toxin inhibited fMet-Leu-Phe-induced lysozyme release by 75%, whereas Pam_3Cys-Ser-$(Lys)_4$-induced exocytosis was not affected by the toxin treatment.

DISCUSSION

Similarities and dissimilarities between lipopeptides and other activators of neutrophils: role of plasma membrane receptors and G-proteins

Lipoproteins are major constituents of bacterial cell walls (Braun, 1975). We report here on the activation of $O_2^{\cdot-}$ formation and lysozyme release in human neutrophils by the synthetic lipohexapeptide Pam_3Cys-Ser-$(Lys)_4$. The identification of a 35 kDa lipopeptide-binding protein in murine B-lymphocytes by Biesert *et al.* (1987) raises the possibility that activation of NADPH oxidase by Pam_3Cys-Ser-$(Lys)_4$ is mediated through specific binding proteins as well. In order to answer this question we compared activation of $O_2^{\cdot-}$ formation by fMet-Leu-Phe and Pam_3Cys-Ser-$(Lys)_4$. Activation of NADPH oxidase by chemotactic peptides and lipopeptides has several characteristics in common, i.e. the ceasing of $O_2^{\cdot-}$ formation after some minutes, the (at least partial) dependency on extracellular Ca^{2+}, sensitivity to NEM, pertussis toxin, activators of adenylate cyclase and staurosporine, and potentiation by PMA, CB and R 59 022 (Figs. 2 and 4; Tables 2–4). In addition, activation of $O_2^{\cdot-}$ formation by lipopeptides shows specificity for the amino acid chain (see Table 2). These data suggest that activation of NADPH oxidase by Pam_3Cys-Ser-$(Lys)_4$ is a receptor-mediated process which involves activation of pertussis-toxin-sensitive G-proteins.

How can the partial pertussis-toxin-sensitivity of lipopeptide-induced $O_2^{\cdot-}$ formation and pertussis-toxin-insensitivity of lipopeptide-induced exocytosis (Fig. 4; Table 6) be explained? It is well known that activation of human myeloid cells by various classes of receptor agonists, e.g. IgG, purine nucleotides and cytokines, shows partial pertussis-toxin-insensitivity (Blackburn & Heck, 1988; Dubyak *et al.*, 1988; Richter *et al.*, 1989; Seifert *et al.*, 1989*a*). These data suggest that receptors for these intercellular signal molecules interact, at least in part, with ADP-ribosylated G-proteins, and/or that pertussis-toxin-insensitive G-proteins are involved in the signal transduction pathway, including GTP-binding proteins with molecular masses of approx. 20 kDa (Bokoch & Parkos, 1988). In addition, there is recent evidence that different types of receptors interact specifically with pertussis-toxin-sensitive or -insensitive G-proteins, ultimately leading to activation of the same effector system (Ashkenazi *et al.*, 1989). Furthermore, McLeish *et al.* (1989) reported that formyl peptide and leukotriene B_4 receptors in human myeloid cells interact differently with G-proteins. Another explanation for the partial pertussis-toxin-insensitivity of the effects of Pam_3Cys-Ser-$(Lys)_4$ is outlined below.

There are some dissimilarities between $O_2^{\cdot-}$ formation induced by chemotactic peptides and by Pam_3Cys-Ser-$(Lys)_4$, i.e. the differential sensitivity to pertussis toxin and cyclic AMP-increasing agents and dependency on extracellular Ca^{2+} (Fig. 4; Tables 3 and 4). In addition, TNFα, UTP and PAF prime neutrophils for fMet-Leu-Phe but not for Pam_3Cys-Ser-$(Lys)_4$ (see Table 2). Furthermore, the onset of Pam_3Cys-Ser-$(Lys)_4$-induced $O_2^{\cdot-}$ formation is delayed, suggesting that the lipopeptide reaches its target of action more slowly than does fMet-Leu-Phe (see Fig. 2) (Seifert *et al.*, 1989*b*). Finally, the kinetics of synergistic activation of $O_2^{\cdot-}$ formation by fMet-Leu-Phe and UTP or PAF or Pam_3Cys-Ser-$(Lys)_4$ are quite different (Fig. 2) (Seifert *et al.*, 1989*b*,*c*), and UTP and PAF, but not Pam_3Cys-Ser-$(Lys)_4$, interacted synergistically with fMet-Leu-Phe when added to neutrophils after the chemotactic-peptide-induced $O_2^{\cdot-}$ formation had ceased (Fig. 1; Dewald & Baggiolini, 1985; Seifert *et al.*, 1989*c*). These data suggest that activation of NADPH oxidase by lipopeptides involves mechanisms additional to those by which fMet-Leu-Phe, UTP and PAF activate this effector system. This assumption is also supported by our finding that Pam_3Cys-Ser-$(Lys)_4$, unlike fMet-Leu-Phe, does not induce β-glucuronidase release (see Table 6).

There are some functional dissimilarities between lipopolysaccharide and lipopeptides with respect to NADPH oxidase regulation. First, lipopolysaccharide *per se* does not activate $O_2^{\cdot-}$ formation in suspended neutrophils but only in adherent cells (Dahinden *et al.*, 1983), whereas Pam_3Cys-Ser-$(Lys)_4$ is effective in suspended neutrophils (see Fig. 2). Lipopolysaccharide primes neutrophils for enhanced $O_2^{\cdot-}$ formation in a pertussis-toxin-insensitive manner (Forehand *et al.*, 1989). By contrast, the effects of lipopeptides on $O_2^{\cdot-}$ formation are, at least in part, pertussis-

toxin-sensitive (Fig. 4). In addition, the priming effect of lipopolysaccharide on $O_2^{\cdot-}$ formation becomes evident only with incubation times longer than 30 min, whereas the priming effect of Pam_3Cys-Ser-$(Lys)_4$ is evident immediately (Fig. 2). These data indicate that lipopolysaccharide and lipopeptides are functionally non-equivalent and play different roles in the regulation of NADPH oxidase.

Structure–activity studies with lipopeptides

The analysis of the structure–activity relationship for lipopeptide-induced activation of $O_2^{\cdot-}$ formation provides important information concerning the mode of action of these compounds (Table 1). Among a variety of lipopeptides studied, Pam_3Cys-Ser-$(Lys)_4$ is the only one with stimulatory effects on neutrophils. In contrast, Pam_3Cys-Ala-Gly and Pam_3Cys-Ser-Gly are inactive and Pam_3Cys-Ser, Pam_3Cys-OMe and Pam_3Cys-OH are inhibitory. The difference between Pam_3Cys-Ser-$(Lys)_4$ and the other lipopeptides is that the former possesses four positively charged amino groups in the lysyl residues of the hexapeptide chain (see Fig. 1). Apparently these positive charges, which facilitate the interaction with negatively charged domains of phospholipids and proteins, are important for the stimulatory action of Pam_3Cys-Ser-$(Lys)_4$. In murine lymphocytes, Pam_3Cys-Ser-$(Lys)_4$, Pam_3Cys-Ser and Pam_3Cys-Ala-Gly are similarly effective cell activators, and Pam_3Cys-OH and Pam_3Cys-OMe are only weakly active (Bessler *et al.*, 1985; Reitermann *et al.*, 1989). These data suggest that the interaction of lipopeptides with plasma membranes is cell-type-specific.

There are some structural and functional similarities between Pam_3Cys-Ser-$(Lys)_4$ and the tetradecapeptide mastoparan, which is found in wasp venom. Both Pam_3Cys-Ser-$(Lys)_4$ and mastoparan are amphiphilic and bear positive charges (Fig. 1) (Higashijima *et al.*, 1988). Thus both molecules may cross the plasma membrane to reach intracellular targets of action. In addition, mastoparan mimicks certain aspects of agonist-induced cell activation (Okano *et al.*, 1985; Wilson, 1989; Wojcikiewicz & Nahorski, 1989; Yokokawa *et al.*, 1989) as does Pam_3Cys-Ser-$(Lys)_4$. Furthermore, Higashijima *et al.* (1988) have shown that mastoparan directly interacts with G-proteins, possibly by mimicking the effects of basic loops of plasma membrane receptors. Finally, studies with intact cells and purified G-proteins showed that mastoparan-induced activation of G-proteins is partially pertussis-toxin-resistant (Higashijima *et al.*, 1988; Yokokawa *et al.*, 1989). By analogy, the effects of Pam_3Cys-Ser-$(Lys)_4$ on $O_2^{\cdot-}$ formation are also partially resistant to inhibition by pertussis toxin (Fig. 4), and its effects on lysozyme release are not inhibited by the toxin at all (Table 6).

Lipopeptides as potentiators of neutrophil activation

Another aspect of neutrophil activation by Pam_3Cys-Ser-$(Lys)_4$ is its synergism with chemotactic peptides (Figs. 2 and 5; Tables 1 and 5). The synergism between the two classes of stimuli on $O_2^{\cdot-}$ formation is still evident in the presence of the agonists at maximally stimulatory concentrations, suggesting that they activate neutrophils by different mechanisms. This assumption is supported by the differential sensitivity of lipopeptide- and chemotactic-peptide-induced $O_2^{\cdot-}$ formation to various inhibitory drugs and by the different kinetics of $O_2^{\cdot-}$ formation (see Figs. 2 and 4; Tables 3 and 4). Pam_3Cys-Ser-$(Lys)_4$ primes neutrophils for fMet-Leu-Phe at non-stimulatory concentrations but does not substantially shift the concentration–response function to chemotactic peptide-induced $O_2^{\cdot-}$ formation to the left, suggesting that it does not alter the affinity state of formyl peptide receptors. However, CB blunts synergistic interaction of Pam_3Cys-Ser-$(Lys)_4$ with fMet-Leu-Phe, indicating that the lipopeptide and CB act, at least in part, by a mechanism they have in common, i.e. by preventing receptor sequestration and by expressing new plasma membrane formyl peptide receptors (Jesaitis *et al.*, 1986). Additional mechanisms by which lipopeptides potentiate chemotactic-peptide-induced activation of NADPH oxidase may involve synergistic activation of stimulatory G-proteins, amplification of the activation of effector systems and generation of intracellular signal molecules, and translocation of cytosolic components of NADPH oxidase to the plasma membrane.

Physiological role of lipopeptides as activators of human neutrophils

Finally, what may be the physiological relevance of neutrophil activation by lipoproteins? As lipoproteins are present in Gram-negative bacteria, they would be expected to serve as intercellular signal molecules for neutrophils in inflammatory processes, as is the case for chemotactic peptides (Braun, 1975; Rossi, 1986; Malech & Gallin, 1987). A major obstacle to the proper interpretation of experiments with purified lipoprotein is its potential contamination with small amounts of other bacterial products, e.g. lipopolysaccharide, and its poor solubility in aqueous media (Bessler *et al.*, 1985). These problems are overcome by the use of chemically synthesized lipopeptides containing the pure biologically active *N*-terminal moiety of native lipoproteins. Pam_3Cys-Ser-$(Lys)_4$ *per se* is a much less effective activator of $O_2^{\cdot-}$ formation and exocytosis than fMet-Leu-Phe, but potentiates the effects of chemotactic peptides (Figs. 2 and 5; Table 5). These data suggest that lipopeptides function predominantly as priming agents, as do purine and pyrimidine nucleotides, PAF and TNFα (Table 3). Apparently, all of these potentiators of neutrophil activation are functionally non-equivalent. Of potential physiological relevance is the finding that Pam_3Cys-Ser-$(Lys)_4$ induces the release of lysozyme which degrades murein, to which lipoproteins are covalently attached (Braun, 1975; Absolom, 1986) (Tables 5 and 6). Lipoprotein-induced lysozyme release from neutrophils would thus provide a feed-forward signal for enhanced bactericidal activity, a process which is accompanied by an increased release of lipoprotein from bacteria.

It should be noted that the lipopeptides used in our present study, in particular Pam_3Cys-Ser-$(Lys)_4$, are not secretory products of Gram-negative bacteria. It is likely that lipopeptides bearing positively charged amino acids are released from native lipoprotein during its proteolytic degradation, but this process has not yet been studied (Braun, 1975). The activation *in vivo* of $O_2^{\cdot-}$ formation by chemotactic peptides is modulated substantially by various factors such as adherence and cytokines (Dahinden *et al.*, 1983; Nathan, 1987). In what way these factors may affect lipopeptide-induced $O_2^{\cdot-}$ formation *in vivo* is not known. Thus at present it is difficult to finally assess the physiological relevance of neutrophil activation by lipopeptides.

In summary, we provide evidence that the synthetic lipopeptide Pam_3Cys-Ser-$(Lys)_4$ is a neutrophil activator which possesses properties both similar and dissimilar to other classes of neutrophil activators, suggesting that it activates neutrophils through a unique transmembrane signalling process. In addition, our data suggest that Pam_3Cys-Ser-$(Lys)_4$ may be a useful experimental tool to study activation of G-proteins in human neutrophils.

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