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Induction and activity of NO synthase in bone-marrow-derived macrophages are independent of Ca^{2+}

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The aim of the present study was to analyse whether an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a role as a signal mediating synthesis of nitric oxide (NO) in bone-marrow-derived macrophages, either by stimulating induction of NO synthase or by regulating the activity of the enzyme. Therefore we compared the effects of various synthetic analogues of bacterial lipopeptide and of lipopolysaccharide (LPS) on NO production (assessed as nitrite formation during an incubation for 24 h) and on $[Ca^{2+}]_i$ [measured with the fluorescent probe indo-1 (1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*NNN′N′*-tetra-acetic acid)]. Strongly dissociating effects were evoked on nitrite formation and on $[Ca^{2+}]_i$ by the stimuli. LPS was preferentially effective on nitrite formation, whereas the Ca^{2+} ionophore ionomycin and AlF_3 induced increases only in $[Ca^{2+}]_i$. The lipopeptides *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylalanylglycine, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylseryl-lysyl-lysyl-lysyl-lysine and (*S*)-(1,2-dicarboxyhexadecyl)ethyl-*N*-palmitoylcysteinylseryl-lysyl-lysyl-lysyl-lysine stimulated both parameters, but the maximal effects on nitrite formation and the shape of the dose–response curves did not parallel the effects on $[Ca^{2+}]_i$. Reduction of extracellular Ca^{2+} with EGTA significantly inhibited increases in $[Ca^{2+}]_i$, but did not change nitrite formation. Furthermore, NO synthesis in the cytosolic fraction of stimulated macrophages was not affected by Ca^{2+} over the concentration range 10 nM–2 μM. We conclude that increases in $[Ca^{2+}]_i$ are not required for NO production in bone-marrow-derived macrophages. Thus the cellular regulation of NO production strikingly differs from that in the vascular endothelium, brain and adrenal gland.

INTRODUCTION

It has become apparent during the last few years that nitric oxide (NO), synthesized from L-arginine, is an important paracrine and autocrine signal used by many different cell types (Marletta, 1989; Moncada *et al.*, 1989). In macrophages, NO mediates the L-arginine-dependent cytostasis and respiratory inhibition involved in the host defence (Hibbs *et al.*, 1987; Stuehr & Nathan, 1989), whereas in endothelial cells, NO accounts for the biological activity of the endothelium-derived relaxing factor (EDRF) (Palmer *et al.*, 1987). In the latter cells, NO is synthesized in response to several stimuli which elevate the intracellular free calcium concentration ($[Ca^{2+}]_i$) (Lückhoff & Busse, 1986; Lückhoff *et al.* 1988). In previous studies we have shown that production and release of EDRF depends on sustained increases in $[Ca^{2+}]_i$ and on the presence of extracellular Ca^{2+} (Lückhoff *et al.*, 1988). These findings may be explained, on a molecular basis, by the strict Ca^{2+}-dependency of the cytosolic NO synthase in endothelial cells, an enzyme system which exhibits a 3-fold increase in activity when the Ca^{2+} concentration is increased from 0.01 to 1 μM (Mülsch *et al.*, 1989). Ca^{2+}-dependent cytosolic NO formation has, furthermore, been demonstrated in cells from the forebrain (Knowles *et al.*, 1989), cerebellum (Bredt & Snyder 1990), and adrenal gland (Palacios *et al.*, 1989).

The cellular regulation of NO synthesis in macrophages is different in that NO release does not occur immediately after stimulation, but requires a latency period of several hours, during which a cytosolic NO synthase system is induced (Marletta, 1989). Thereafter, long-lasting release of NO is observed, which can be assessed by the accumulation of nitrite in the incubation medium (Hauschildt *et al.*, 1990*a*; Marletta *et al.*, 1988).

It is not known whether Ca^{2+} plays a role in the regulation of NO synthesis in bone-marrow-derived macrophages. Such a role may be tentatively hypothesized from the fact that a synthetic analogue of the biologically active part of bacterial lipoprotein {[*N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylalanylglycine (Pam_3-Cys-Ala-Gly)} induces increases in $[Ca^{2+}]_i$ as well as nitrite production in bone-marrow-derived macrophages (Hauschildt *et al.*, 1990*a*,*c*). However, it is not clear from these experiments whether a causative link exists between both these effects. The aim of the present study was to analyse in detail whether rises in $[Ca^{2+}]_i$ are involved in the cellular processes eventually leading to NO production in macrophages. Therefore we compared the dose-dependent effects of various synthetic analogues of bacterial lipoprotein and of lipopolysaccharide (LPS) on $[Ca^{2+}]_i$ and on nitrite production. Furthermore, we investigated whether Ca^{2+} had any direct effect on NO synthase in cytosolic preparations from stimulated macrophages.

Abbreviations used: LPS, lipopolysaccharide; Indo-1 (/AM), 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*NNN′N′*-tetra-acetic acid (penta-acetoxymethyl ester); Pam_3-Cys-Ala-Gly, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylalanylglycine; Pam_3-Cys-OH, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine; Pam_3-Cys-Ser-Lys_4, *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylseryl-lysyl-lysyl-lysyl-lysine; $Pam(C_{16})_2$-Ser-Lys_4, (*S*)-(1,2-dicarboxyhexadecyl)ethyl-*N*-palmitoylcysteinyl-seryl-lysyl-lysyl-lysyl-lysine; EDRF, endothelium-derived relaxing factor; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; GC, guanylate cyclase.

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EXPERIMENTAL

Cell culture of bone-marrow-derived macrophages

Cells were obtained from the femur bone marrow of Balb/c mice (age 8–12 weeks) and were cultured in Teflon bags in the presence of L-cell conditioned medium as described in detail previously (Hoffmann *et al.*, 1989).

Determination of nitrite production in macrophages

Macrophages cultured for 7 days were suspended in 2 ml of RPMI 1640 (free of Phenol Red) with 5% (v/v) fetal-calf serum at a density of 5×10^6/ml in 6-well culture plates (Falcon 3046; Becton–Dickinson, Heidelberg, Germany). They were incubated at 37 °C for 24 h. Thereafter, the supernatants were collected, centrifuged (350 *g*, 10 min) and stored at −70 °C. The nitrite concentration was determined by diazotization (Green *et al.*, 1982) in a volume of 0.28 ml in 92-well microtitre plates (Falcon 3072) and corrected for the background levels of nitrite in the cell-free medium.

Measurements of the intracellular free calcium concentration

Macrophages (10^7/ml) were suspended in Hepes buffer (concentration in mM: Na^+ 132, K^+ 4, Ca^{2+} 1, Mg^{2+} 0.5, Cl^- 139, Hepes 9.5, glucose 5; pH 7.3) and loaded with the fluorescent Ca^{2+} indicator 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*NNN′N′*-tetra-acetic acid (indo-1) by incubation with the penta-acetoxymethyl ester of indo-1 (indo-1/AM) for 60 min at 37 °C. The resulting intracellular indo-1 concentration was in the range of 20–60 μM, as calculated in some preparations after lysis of the cells and assuming an intracellular volume of 2 pl. The cells were washed twice and resuspended at a density of 10^6/ml. Fluorescence of these cells was monitored in a spectrofluorimeter (Schoeffel RRS 1000; Schoeffel Instruments, Westwood, NJ, U.S.A.) at 25 °C. The excitation wavelength was set to 350 nm, and emission was simultaneously recorded at 400 nm (yielding F_{400}) and 450 nm (isobestic wavelength, yielding F_{450}). The signals were digitized and stored in a computer at a rate of 4/s. After correction for the background signal, the $[Ca^{2+}]_i$ was calculated from the ratio ($R = F_{400}/F_{450}$) of both fluorescence emissions as:

$$[Ca^{2+}]_i = K_d\,(R - R_{min.})/(R_{max.} - R)$$

$R_{max.}$ and $R_{min.}$ are the values when indo-1 is Ca^{2+}-bound or Ca^{2+}-free respectively, and were determined in a calibration solution. K_d is the dissociation constant and was taken as 250 nM. The background signal was obtained after lysis of the cells with Triton-X (0.05%) and addition of Mn^{2+} (2 mM), a quencher of indo-1. Additionally, the fluorescence derived from extracellular indo-1 was taken as background. This extracellular indo-1 was calculated from the decline in fluorescence after addition of 100 μM-Mn^{2+} to samples of unstimulated cells and amounted to 0–15% of the intracellular indo-1.

Peak $[Ca^{2+}]_i$ values after stimulation were calculated with a computer program determining the 10 s period with the highest mean $[Ca^{2+}]_i$.

Preparation of cytosol from macrophages

Macrophages were scraped off the culture dishes with a rubber policeman and kept suspended in ice-cold buffer solution (composition in mM: Hepes, 10; Na^+, 130; K^+, 4; Ca^{2+}, 1.6; Mg^{2+}, 2; Cl^-, 140; HCO_3^-, 12; $H_2PO_4^-$, 0.36; glucose, 11; calcium disodium EDTA, 0.025; pH 7.5). Cells were washed twice (1000 *g*, 5 min, 4 °C) in 15 mM-Hepes, pH 7.5, and homogenized by sonication (three times; 10 s bursts; 100 W setting). The cytosolic fraction was then prepared by centrifugation (1 h/100000 *g* supernatant). Aliquots were stored at −30 °C. Protein was determined as described by Bradford (1975).

Quantification of nitric oxide by activation of soluble guanylate cyclase

Soluble guanylate cyclase (GC) was purified to apparent homogeneity from bovine lung as described by Mülsch & Gerzer (1990). Cytosol (0.31 mg of protein/ml) was incubated for 30 min at 37 °C with L-arginine (1 mM) and NADPH (0.1 mM) and the complete test mixture for formation of cyclic [^{32}P]GMP from [α-^{32}P]GTP in a total volume of 50 μl. The mixture contained purified GC (1 μg/ml), [α-^{32}P]GTP (0.1 mM; sp. radioactivity 0.2 μCi/nmol), cyclic GMP (0.1 mM), glutathione (2 mM), Hepes (15 mM), $MgCl_2$ (4 mM), 3-isobutyl-1-methylxanthine (1 mM), phosphocreatine (3.5 mM), creatine phosphokinase (4.8 units) and bovine γ-globulin (0.1 mg/ml), pH 7.5. $[Ca^{2+}]_i$ was adjusted with EGTA and $CaCl_2$ and verified by fluorescence measurements with indo-1. The enzymic formation of cyclic GMP was stopped by the addition of 450 μl of zinc acetate (120 mM) and 500 μl of sodium carbonate (120 mM). Cyclic [^{32}P]GMP was isolated by chromatography on acid alumina, and GC activity (nmol of cyclic GMP·min^{-1}·mg^{-1} of purified GC) was calculated as described by Schultz & Böhme (1984). The endogenous GC activity present in the macrophage cytosol contributed less than 1% to the total cyclic GMP formation in the presence of purified GC and was therefore not considered. The enhancement of GC activity by cytosol was calculated from the difference of the GC activity in the presence or absence of cytosol.

Materials

The synthetic lipopeptide analogues used in this study were *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine (Pam_3Cys-OH; Wiesmüller *et al.*, 1983), Pam_3Cys-Ala-Gly (Reitermann *et al.*, 1989), *N*-palmitoyl-(*S*)-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteinylseryl-lysyl-lysyl-lysyl-lysine (Pam_3Cys-Ser-Lys_4; Reitermann *et al.*, 1989), and an analogue of Pam_3Cys-Ser-Lys_4, namely (*S*)-(1,2-dicarboxyhexadecyl)ethyl-*N*-palmitoylcysteinylseryl-lysyl-lysyl-lysyl-lysine [Pam$(C_{16})_2$-Ser-$(Lys)_4$; W. G. Bessler, B. Müller, P. Hoffmann, M. Strecker, J. Metzger, K.-H. Wiesmüller & G. Jung, unpublished work). These compounds, obtained from Dr. K.-H. Wiesmüller and Dr. G. Jung, Institut für Organische Chemie, Universität Tübingen, Tübingen, Germany, were prepared by chemical synthesis and were characterized by analytical methods including m.s., ^{13}C n.m.r. spectroscopy and chiral-phase amino acid analysis. Indo-1 and indo-1/AM were from Boehringer (Mannheim, Germany). Ionomycin was from Calbiochem (Frankfurt a.M., Germany). All other substances, including lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 were from Sigma (Deisenhofen, Germany).

Data analysis and statistics

Data are presented as means ± S.E.M. The Wilcoxon matched pair signed-rank test (Sachs, 1978) was used for evaluation of differences between groups. A probability of error of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of LPS and lipopeptides on $[Ca^{2+}]_i$ and nitrite production in bone-marrow-derived macrophages

The immediate effects of LPS on $[Ca^{2+}]_i$ in bone-marrow-derived macrophages and the induction of nitrite production after incubation for 24 h are compared in Fig. 1. LPS elicited small increases in $[Ca^{2+}]_i$ that exhibited only a weak dose-

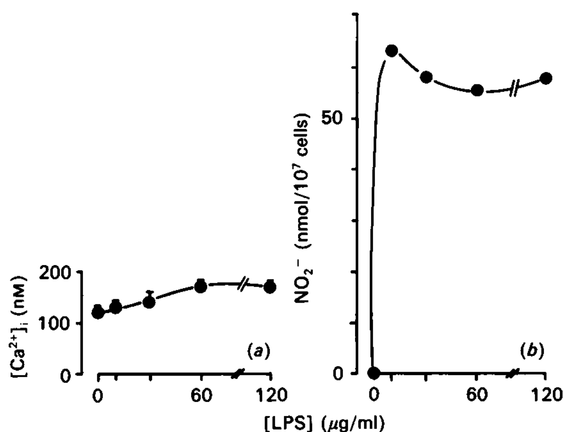

Fig. 1. Effects of lipopolysaccharide on bone-marrow-derived macrophages

(*a*) Effect on $[Ca^{2+}]_i$. Each symbol represents peak $[Ca^{2+}]_i$ values of three to five cell preparations under stimulation with LPS at different concentrations. (*b*) Effect on NO_2^- formation. Each symbol represents NO_2^- production after stimulation with LPS for 24 h from three cell preparations all from the same culture.

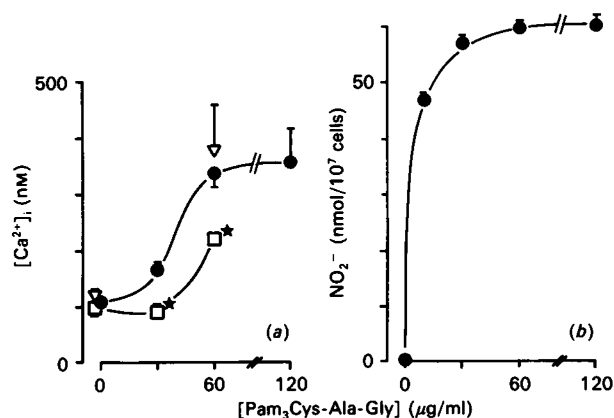

Fig. 2. Effects of the lipopeptide Pam_3Cys-Ala-Gly on bone-marrow-derived macrophages

(*a*) Effect on $[Ca^{2+}]_i$. Each symbol represents three to eight experiments performed either in control buffer (●), or in the presence of verapamil (1 µM; preincubation 2 min; ▽), or in buffer made Ca^{2+}-free with 1.2 mM-EGTA (□). A star indicates a result significantly different ($P < 0.05$) from control. (*b*) Effects on nitrite formation. Each symbol represents nitrite production after stimulation with Pam_3Cys-Ala-Gly for 24 h from six cell preparations from two different cultures.

Table 1. Effect of EGTA on NO_2^- formation induced by LPS and Pam_3Cys-Ala-Gly in bone-marrow-derived macrophages

Experiments were performed with macrophages from the same cell culture stimulated with LPS or Pam_3Cys-Ala-Gly for 24 h in the presence or absence of EGTA ($n = 3$ for each concentration). Similar results were obtained in two further cultures.

	NO_2^- formation (nmol/10^7 cells) in response to:	
[EGTA] (mM)	LPS (0.1 µg/ml)	Pam_3Cys-Ala-Gly (10 µg/ml)
0 (Control)	61.5±1.1	47.6±0.8
1	59.5±0.5	51.3±0.6
5	68.2±0.9	57.5±0.8

dependency. In contrast, large amounts of nitrite were produced in response to already low concentrations of LPS. This effect was completely preserved when the extracellular Ca^{2+} was reduced by addition of EGTA (1 and 5 mM; Table 1).

Fig. 2 shows that Pam_3Cys-Ala-Gly elicited increases in $[Ca^{2+}]_i$ as well as in nitrite production in a dose-dependent manner. However, almost maximal nitrite production was induced by a concentration of 30 µg/ml, whereas the effects on $[Ca^{2+}]_i$ were moderate at this concentration. Furthermore, Table 1 demonstrates that reduction of extracellular Ca^{2+} had no inhibitory effects on nitrite production. In contrast, there was a significant inhibition of increases in $[Ca^{2+}]_i$ by EGTA [1.2 mM in the presence of $CaCl_2$ (1 mM); Fig. 2].

The Ca^{2+}-channel blockers verapamil and nifedipine had virtually no effect on nitrite production induced either by LPS or by Pam_3Cys-Ala-Gly (Table 2). Verapamil (1 µM; preincubation 3 min) did not inhibit the $[Ca^{2+}]_i$ response to Pam_3Cys-Ala-Gly (60 µg/ml), either (Fig. 2). Similarly, increases in $[Ca^{2+}]_i$ were not markedly attenuated by preincubation with La^{3+} (0.1 mM, 3 min, $n = 2$; or 1 mM, 5 min, $n = 4$, results not shown).

The lipopeptide Pam_3Cys-OH proved to be a strong stimulus for increases in $[Ca^{2+}]_i$ in macrophages (Fig. 3). Representative tracings are shown in Fig. 4. At an extracellular Ca^{2+} concentration of 1 mM there was a rapid rise in $[Ca^{2+}]_i$ which

Table 2. Effects of Ca^{2+} antagonists on NO_2^- formation induced by LPS and Pam_3Cys-Ala-Gly in bone-marrow-derived macrophages

Experiments were performed with macrophages from the same cell culture, stimulated with LPS or Pam_3Cys-Ala-Gly for 24 h in the presence of Ca^{2+} antagonists ($n = 3$ for each concentration). Similar results were obtained in two further cultures.

	NO_2^- formation (nmol/10^7 cells) in response to:	
Ca^{2+} antagonist Concn. (M)	LPS (0.1 µg/ml)	Pam_3Cys-Ala-Gly (10 µg/ml)
Verapamil		
0 (Control)	41.7±1.3	83.8±0.9
10^{-9}	42.9±0.4	81.9±1.5
10^{-8}	40.6±2.0	79.4±1.1
10^{-7}	37.7±0.7	81.9±2.2
10^{-6}	36.6±0.7	78.1±4.7
10^{-5}	41.4±0.3	80.0±0.7
Nifedipine		
10^{-9}	39.1±0.3	81.9±0.1
10^{-8}	38.7±0.9	79.4±2.0
10^{-7}	39.6±0.5	83.8±2.0
10^{-6}	39.0±0.5	82.5±0.2
10^{-5}	40.3±0.8	83.2±2.4

remained elevated over a prolonged time. In buffer made Ca^{2+}-poor with EGTA (1.2 mM), peak $[Ca^{2+}]_i$ levels were significantly reduced (Fig. 3) and the response was more transient (Fig. 4). These kinetics of $[Ca^{2+}]_i$ were similar to those obtained with Pam_3Cys-Ala-Gly as stimulus (not shown), although Pam_3Cys-OH induced somewhat higher increases in $[Ca^{2+}]_i$ than did Pam_3Cys-Ala-Gly and evoked maximal effects already at lower concentrations (Figs. 2 and 3).

In contrast, nitrite production induced by Pam_3Cys-OH (Fig. 3) was less than that induced by Pam_3Cys-Ala-Gly (Fig. 2) at all concentrations tested, and the dose–response relationship

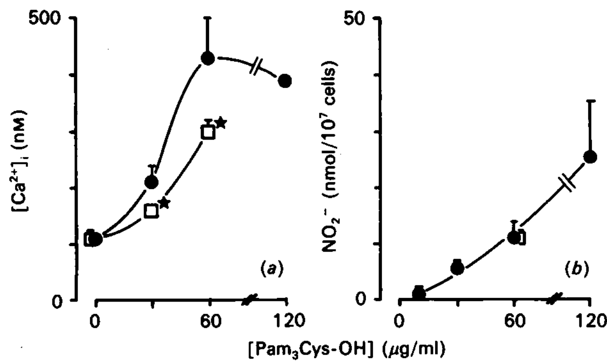

Fig. 3. Effects of the lipo-amino acid $Pam_3Cys\text{-}OH$ on bone-marrow-derived macrophages

(*a*) Effect on $[Ca^{2+}]_i$. Each symbol represents three to six experiments performed either in control buffer (●) or in buffer made Ca^{2+}-free with 1.2 mM-EGTA (□). A star indicates a result significantly different ($P < 0.05$) from control. (*b*) Effect on NO_2^- formation. Each symbol represents NO_2^- production after stimulation with $Pam_3Cys\text{-}OH$ for 24 h from six cell preparations from two different cultures, either in control medium (●) or in medium made Ca^{2+}-free with 5 mM-EGTA (□).

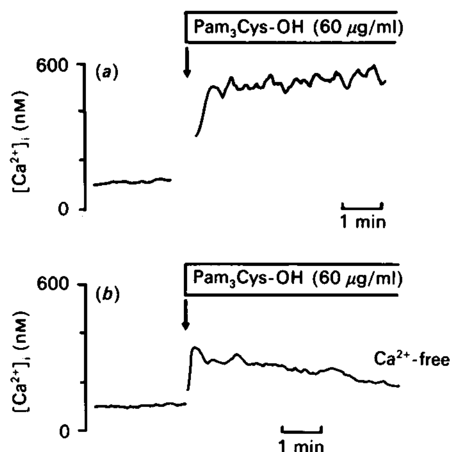

Fig. 4. Effects of the lipo-amino acid $Pam_3Cys\text{-}OH$ on $[Ca^{2+}]_i$ in bone-marrow-derived macrophages (original tracings)

(*a*) An experiment in control buffer. $Pam_3Cys\text{-}OH$ was present during the time indicated by the bar. (*b*) An experiment performed with cells from the same culture in medium made Ca^{2+}-free with 1.2 mM-EGTA.

was shifted to the right (Fig. 3). Again, reducing Ca^{2+} in the culture medium had no effect on nitrite production (Fig. 3).

The effects of the lipopeptide $Pam_3Cys\text{-}Ser\text{-}Lys_4$ and its analogue $Pam(C_{16})_2\text{-}Ser\text{-}Lys_4$ on $[Ca^{2+}]_i$ in macrophages are shown in Fig. 5. Both lipopeptides induced similar increases in $[Ca^{2+}]_i$. However, they evoked markedly different effects on nitrite production (Fig. 5).

Application of $AlCl_3$ (20 μM) together with NaF (10 mM) led to a slow, but steady, elevation in $[Ca^{2+}]_i$, as shown in Fig. 6. After 7 min, values of 440 ± 50 nM ($n = 4$) were reached. At this time, addition of $Pam_3Cys\text{-}Ala\text{-}Gly$ (60 μg/ml) elicited further increases in $[Ca^{2+}]_i$ to 650 ± 130 nM (Fig. 6). Stimulating effects on $[Ca^{2+}]_i$ by $AlCl_3$ and NaF were also observed in the presence of EGTA (1.2 mM; preincubation 2 min). Mean $[Ca^{2+}]_i$ after 7 min was 280 ± 50 mM ($n = 3$). However, no significant amounts of nitrite were produced when incubating macrophages with $AlCl_3$ and NaF for 24 h (results not shown).

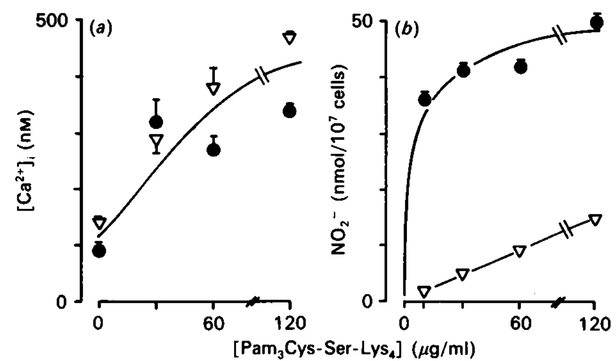

Fig. 5. Effects of the lipopeptide $Pam_3Cys\text{-}Ser\text{-}Lys_4$ and of its analogue $Pam(C_{16})_2\text{-}Ser\text{-}Lys_4$ on bone-marrow-derived macrophages

(*a*) Effect on $[Ca^{2+}]_i$. Each symbol represents three or four experiments. (*b*) Effect on NO_2^- formation after an incubation for 24 h. ●, Experiments with $Pam_3Cys\text{-}Ser\text{-}Lys_4$; ▽, with $Pam(C_{16})_2\text{-}Ser\text{-}Lys_4$.

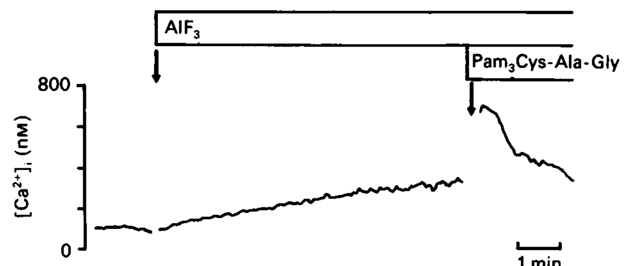

Fig. 6. Effect of AlF_3 on $[Ca^{2+}]_i$ in bone-marrow-derived macrophages (original tracing)

AlF_3 [as $AlCl_3$ (20 μM) and NaF (10 mM)] and $Pam_3Cys\text{-}Ala\text{-}Gly$ (60 μg/ml) were present during the time indicated by the bars.

Table 3. Effects of ionomycin on NO_2^- formation in bone-marrow-derived macrophages

The upper part represents NO_2^- formation in macrophages from the same cell culture, incubated for 24 h in the presence of ionomycin and in the presence or absence of LPS ($n = 3$ for each concentration). The lower part shows the effects of 1 μM-ionomycin in two different cell preparations ($n = 6$ for each concentration). * Significantly different from control.

	NO_2^- formation (nmol/10^7 cells)	
[Ionomycin] (M)	−LPS	+LPS (0.1 μg/ml)
0 (Control)	0.3 ± 0	39.1 ± 0.3
10^{-8}	0.3 ± 0	39.4 ± 0.1
10^{-7}	0.4 ± 0.0	41.7 ± 1.1
10^{-6}	0.4 ± 0.1	68.5 ± 0.4
0 (Control)	0.3 ± 0	55.4 ± 6.2
10^{-6}	0.3 ± 0.1	73.9 ± 5.9*

The Ca^{2+} ionophore ionomycin (0.1–1 μM) induced large increases in $[Ca^{2+}]_i$, near to saturation with Ca^{2+} of intracellular indo-1. However, ionomycin alone (10 nM–1 μM, Table 3) failed to induce nitrite production. Only if ionomycin (1 μM) was applied in addition to LPS (0.1 μg/ml) was an enhancing effect on nitrite production found for the ionophore (Table 3).

Dependency on Ca^{2+} of cytosolic NO synthesis in bone-marrow-derived macrophages

Production of NO in the cytosolic fraction of bone-marrow-derived macrophages stimulated for 24 h with Pam_3Cys-Ala-Gly (60 μg/ml) was assessed by measuring the activation of purified soluble guanylate cyclase (GC) added to the cytosol from three different cultures. In nominally calcium-free buffer (EGTA, 0.1 mM; Ca^{2+}, 10 mM), cytosol (0.2–0.8 mg of protein/ml) enhanced the activity of soluble GC from 31 ± 9 to values ranging from 115 ± 2 up to 210 ± 4 nmol of cyclic GMP $\cdot$ min^{-1} $\cdot$ mg of GC^{-1}. For comparison, soluble GC was activated 5-fold by sodium nitroprusside at 1 μM and 30-fold at 100 μM. NO synthesis was not affected by the $[Ca^{2+}]_i$ values tested (120, 290, 880 and 2000 nM). The activities, related to the value at 10 nM-Ca^{2+}, were 87 ± 9, 109 ± 8, 105 ± 6 and 100 ± 12%.

DISCUSSION

The present study shows clear differences between the effects of LPS and various lipopeptides on $[Ca^{2+}]_i$ and on nitrite formation in bone-marrow-derived macrophages. Furthermore, NO production in the cytosolic fraction of homogenized macrophages was not modified by changes in the Ca^{2+} concentration from 10 nM to 2 μM. Thus increases in $[Ca^{2+}]_i$ are unlikely to play a role either as a signal mediating induction of NO synthase or as a regulator of cytosolic NO formation.

The most striking dissociation between increases in $[Ca^{2+}]_i$ and nitrite formation became apparent when comparing the effects of lipopeptide Pam_3Cys-Ala-Gly and of the LPS. Whereas both substances were potent stimuli for nitrite production, only Pam_3Cys-Ala-Gly induced marked and dose-dependent elevations of $[Ca^{2+}]_i$. Effects opposite to those of LPS were evoked by the calcium ionophore ionomycin. Large increases in $[Ca^{2+}]_i$ were observed in the absence of any nitrite formation. Only when ionomycin was applied at the highest concentration tested (1 μM) in combination with LPS (0.1 μg/ml) (which at this concentration had no effect on $[Ca^{2+}]_i$), a moderate but significant enhancement of nitrite formation was found. Thus it appears possible that extremely high levels of $[Ca^{2+}]_i$ may facilitate cellular processes related to NO formation which are not regulated by $[Ca^{2+}]_i$ under physiological conditions. This phenomenon is well known in other biological systems, e.g. the control of degranulation in mast cells (Penner *et al.*, 1988).

Reduction of Ca^{2+} in the medium by addition of EGTA had no inhibitory effect on nitrite production, but partly inhibited increases in $[Ca^{2+}]_i$ in response to Pam_3Cys-Ala-Gly and Pam_3Cys-OH. This indicates, first, that extracellular Ca^{2+} is not required for the induction of NO synthase and, secondly, that transmembrane Ca^{2+} influx as well as mobilization of Ca^{2+} from intracellular stores contributed to the action of the lipopeptides. However, the molecular mechanisms involved in the lipopeptide-induced changes in $[Ca^{2+}]_i$ could not be further characterized in the present study. $[Ca^{2+}]_i$ responses were neither inhibited by the blocker of voltage-dependent Ca^{2+} channels verapamil nor by lanthanum, which prevents Ca^{2+} entry in various non-excitable cell types, e.g. endothelial cells (Colden-Stanfield *et al.*, 1987). On the other hand, the second messengers $InsP_3$ and cyclic AMP, as well as protein kinase C, were found to remain unaffected by Pam_3Cys-Ala-Gly (Hauschildt *et al.* 1990*c*). Hence Ca^{2+} mobilization by $InsP_3$ does not account for the actions of Pam_3Cys-Ala-Gly and, probably, of the other lipopeptides. However, this signalling pathway does seem to exist in bone-marrow-derived macrophages, since AlF_3 evoked increases in $[Ca^{2+}]_i$ and also enhanced production of phosphoinositolphosphates and translocation of protein kinase C in the same cells (Hauschildt *et al.*, 1988). At present, the stimulation of $[Ca^{2+}]_i$ by lipopeptides may best be explained by their possibly ionophore-like properties (due to their amphipathic structure).

Lipopeptides induce not only NO synthesis in macrophages, but also secretion of other important biological mediators such as interleukin 1, interleukin 6 and tumour-necrosis factor (Hoffmann *et al.*, 1989; Hauschildt *et al.*, 1990*b*). Thus the increases in $[Ca^{2+}]_i$ by the lipopeptides may well have a role as signals for cellular responses other than NO formation. In addition to the differences in NO synthesis between macrophages and several other cells which can be seen by comparing the time course of NO release after stimulation (Marletta, 1989), the present study reveals that there is a contrasting sensitivity to Ca^{2+}. In endothelial (Lückhoff *et al.*, 1988) and cerebellar (Garthwaite *et al.*, 1988) cells, NO release is initiated by increases in $[Ca^{2+}]_i$ and depends critically on the presence of extracellular Ca^{2+}. Furthermore, NO synthase in the cytosol of endothelial (Mülsch *et al.*, 1989), forebrain (Knowles *et al.*, 1989), cerebellar (Bredt & Snyder, 1990) and adrenal (Palacios *et al.*, 1989) cells is constitutively expressed and is activated by Ca^{2+}. In contrast, NO synthesis in the cytosolic preparations from stimulated macrophages, measured under assay conditions similar to endothelial cells (Mülsch *et al.*, 1989), was not affected by changes in Ca^{2+}. Removal of Ca^{2+} did not attenuate nitrite formation in response to any of the stimuli tested. Likewise, no increase in $[Ca^{2+}]_i$ was required for the induction of the NO synthase system by LPS. Therefore, although NO is considered an archaic, widely distributed principle of cellular communication (Vanhoutte, 1987; Böhme & Schmidt, 1989), major differences in the cellular regulation of NO formation exist between different mammalian cell types.

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