Lipopolysaccharide Priming of Human Neutrophils for an Enhanced Respiratory Burst

Role of Intracellular Free Calcium

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Abstract

Lipopolysaccharide (LPS) pretreatment "primes" neutrophils to release increased amounts of superoxide anion (O_2^-) when stimulated. We investigated the molecular basis of this enhanced activity. Comparison of kinetic parameters of the respiratory burst NADPH oxidase in unstimulated LPS-primed and control neutrophils disclosed a similar K_m for NADPH and no difference was seen in the content of cytochrome b. Pertussis toxin, which inhibits some G proteins, did not prevent priming. Change in membrane potential ($\Delta\psi$) was five-fold greater in LPS-primed cells and paralleled the increased O_2^- release. Cytofluorographic analysis indicated that the increased change in $\Delta\psi$ was due to the creation of a new population of active cells. Changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are believed to antecede changes in $\Delta\psi$. There was a consistent increment (67±8%, n = 12) in resting $[Ca^{2+}]_i$ in cells preincubated with LPS compared with control. When stimulated, the peak $[Ca^{2+}]_i$ was significantly higher in LPS-primed cells. Ca^{2+}-dependent protein kinase C activity was unaltered in resting and FMLP-stimulated neutrophils preexposed to LPS. Addition to cells of the intracellular Ca^{2+} chelator MAPTAM before preincubation with LPS blocked the changes in $[Ca^{2+}]_i$ and the enhanced respiratory burst that characterize LPS priming. The increased resting $[Ca^{2+}]_i$ in LPS-primed cells may enhance stimulus-induced cellular activity by modifying a Ca^{2+}-dependent step in signal transduction.

Introduction

Stimulated human neutrophils respond with a characteristic "respiratory burst" in which oxygen is consumed and reduced to toxic metabolites, including superoxide anion (O_2^-)[1] and hydrogen peroxide (1). The in vitro (2) and possibly in vivo (3) exposure of neutrophils to endotoxin (lipopolysaccharide, LPS) primes the cells for enhanced release of these microbicidal oxygen metabolites during stimulation. Thus, priming might increase resistance to infection yet predispose the host to increased oxidative tissue damage during inflammation (2, 4).

The precise mechanism of LPS priming is unknown. A change in binding characteristics of the stimuli might play a part, but the number of receptors and binding affinity for an effective stimulus, the chemotactic peptide FMLP, were not found to be increased in LPS-primed cells (2). However, O_2^- release occurred sooner (diminished lag time after exposure to stimulus) and at a faster rate, suggesting that the increased activity of LPS-pretreated neutrophils results from modification of one or more components of the cellular signal transduction mechanism, or from the increased catalytic activity of the enzyme responsible for the respiratory burst, the NADPH oxidase, or from both (2).

Changes in the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$), alteration in $\Delta\psi$, and translocation of cytochrome *b* or of protein kinase C (PKC) activity from cytosol to plasma membrane have all been implicated in linking receptor-ligand interaction with NADPH oxidase activation (5–8). Alteration of one or more of these or other signal transduction components or a change in NADPH oxidase kinetics by LPS could result in enhanced NADPH oxidase activity. We monitored the kinetics of NADPH oxidase activity and steps in signal transduction after LPS priming of neutrophils, before and after subsequent stimulation, in an attempt to gain insight into the molecular basis of the induction of an enhanced respiratory burst by LPS.

Methods

Preparation of neutrophils. Neutrophils from healthy donors were prepared as previously described (2). All reagents (Mallinckrodt Chemical Co., St. Louis, MO) and plastic (Falcon Labware, Oxnard, CA) used in the preparation of cells were demonstrated to be LPS-free (2). Neutrophils were isolated, washed, and resuspended in Krebs-Ringer phosphate buffer with 0.2% dextrose (KRP-D), pH 7.34. In some experiments, Ca^{2+} was omitted from and 5 mM EGTA (Sigma Chemical Co., St. Louis, MO) was added to the KRP-D.

Priming of neutrophils with LPS. After isolation, neutrophils were preincubated at 37°C in polypropylene tubes for 60 min with KRP-D containing LPS, 50 ng/ml, or with KRP-D alone (control). The LPS was a phenol-extracted product from *Escherichia coli* K235 (a gift from Dr. Floyd McIntire, University of Colorado School of Dentistry, Denver, CO). There was no loss of cell viability nor aggregation resulting from LPS treatment (2). In some experiments, neutrophils, before being incubated with LPS, were exposed to buffer or to the nonfluorescent intracellular Ca^{2+} chelator bis-(2-amino-5-methylphenoxy)-ethane-*N,N,N',N'*tetraacetic acid tetraacetoxymethyl ester (MAP-

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium; $\Delta\psi$, transmembrane potential; KRP-D, Krebs Ringer phosphate buffer with dextrose; O_2^-, superoxide anion; PKC, protein kinase C; PLA_2, phospholipase A; PT, *Bordatella pertussis* toxin.

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TAM; Calbiochem-Behring, La Jolla, CA), 105 μM for 30 min at 37°C (9). The cells were centrifuged at 500 for 5 min and resuspended in KRP-D to remove excess MAPTAM.

Measurement of O_2^- production by neutrophils. O_2^- was measured as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* (type III; Sigma) (2). Buffer- or LPS-preincubated neutrophils, 2.5×10^6, were added to paired cuvettes containing 80 μM cytochrome *c* in KRP-D with a final assay volume of 1.5 ml. In some experiments KRP-D was replaced with a high K^+ buffer containing 137 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM K_2HPO_4, 1.3 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 5.5 mM glucose. SOD, 50 μg/ml (DDI Pharmaceuticals, Mountain View, CA) was added to the reference cuvette. The reaction was initiated with the addition of 1 μM FMLP (Vega Biochemicals, Tucson, AZ) or 100 ng/ml phorbol myristate acetate (PMA; Consolidated Midland, Brewster, NY). In some experiments 1 μM ionomycin (Calbiochem-Behring Corp., La Jolla, CA) was added 60 s before the initiation of the reaction with PMA or FMLP (10). The reduction of cytochrome *c* was measured continuously in a double beam spectrophotometer with a heated cuvette holder (37°C), magnetic stirrer and recorder.

With each of the experiments exploring the mechanism of priming reported in this paper, confirmation that the cells were fully primed was obtained by measurement of O_2^- release in control and LPS-exposed cells.

O_2^- production by subcellular fractions of neutrophils. After preincubation with KRP-D or LPS, cells were centrifuged at 500 *g* and resuspended ($0.5–1.0 \times 10^8$ cells/ml) in ice-cold 0.34 M sucrose (Sigma Chemical Co.) and disrupted by sonication (2) (20 W for three bursts of 15 s each separated by 1-min intervals) in 12×75 mm glass tubes in ice water. To remove whole cells and debris, the sonicate was centrifuged at 800 *g* for 10 min at 4°C. The supernatant was centrifuged at 12,800 *g* in a microfuge for 5 min to reduce granule content, and the membrane-enriched supernatant ("cell sonicate") was kept at 0°C until assayed promptly for O_2^--producing activity. Part of the 12,800 *g* supernatant was centrifuged at 48,000 *g* for 45 min at 4°C. The 48,000 *g* pellet was resuspended by sonication (20 W, two 5-s bursts separated by 1 min) in the original volume of ice-cold 0.34 M sucrose.

NADPH-dependent O_2^- production by subcellular fractions was measured as the SOD-inhibitable reduction of cytochrome *c*, using a method modified from that of Bromberg and Pick (11). Fresh sample (0.1 ml) was mixed thoroughly with 1.9 ml of 65 mM Na,K-phosphate buffer (pH 6.5) containing 100 μM ferricytochrome *c*, and 170 mM sucrose, 2 mM sodium azide (Difco Laboratories, Detroit, MI), 1 mM EGTA, 100 μM sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Richmond, CA) and 10 μM flavin adenine dinucleotide (Sigma) and maintained at 0°C. Aliquots, 0.1 ml, were added to paired cuvettes containing either 50 μM SOD (reference) or buffer (sample), and the assay mixture was warmed to 37°C. The reaction was started by adding 200 μM NADPH (Boehringer Mannheim, Indianapolis, IN). The NADPH was freshly prepared before each experiment and the concentration was verified spectrophotometrically (12). Protein content was determined with BSA as standard (12). The rate of O_2^- production was determined from the initial slope of the change in absorbance at 550 nm.

Determination of cytochrome b. After preincubation with buffer or LPS, the cytochrome *b* content of neutrophils or of plasma membrane-enriched fractions was determined as described (13, 14). Briefly, 15 million intact cells or the 48,000 *g* pellet (described above) from 15 million cells were extracted with Triton X-100 in 100 mM KPO_4, pH 7.3 for 20 min on ice. The extracts were centrifuged for 30 min at 48,000 *g* and treated with haptoglobin-Sepharose to remove hemoglobin. The absorbance spectra of the supernatants were measured before and after reduction with sodium dithionite, and the content of cytochrome *b* was calculated (13).

Pertussis toxin treatment of neutrophils. In some experiments LPS preincubation was preceded by the addition of *Bordatella pertussis* toxin (PT; List Biological Laboratories, Campbell, CA), 100 ng/ml, or buffer (control) for 120 min at 37°C.

PKC activity. Neutrophils, 5×10^6, in KRP-D were incubated with buffer or LPS for 60 min at 37°C. Preparation of subcellular fractions and assay of PKC were done with modifications of the methods of Wolfson et al. (8) as previously described (10). Control or LPS-primed cells were resuspended and disrupted by sonication in an ice-cold extraction buffer (10) containing 2 mM EGTA. The sonicate was centrifuged at 100,000 *g* for 60 min. The resultant supernatant (cytosolic fraction) and pellet fractions were stored at 4°C and assayed within 48 h. Activity was measured as the incorporation of ^{32}P into TCA-precipitated histone protein (10).

Measurement of membrane potential change. Change in $\Delta\psi$ was measured using the fluorescent dye 3,3'-dipentyloxacarbocyanine (Di-O-C_5(3); Molecular Probes, Junction City, OR) as previously described (15). A fluorescence spectrophotometer with a heated cuvette holder (37°C) and magnetic stirrer was used.

The Di-O-C_5(3) assay was begun by adding 3×10^6 neutrophils, preincubated with buffer or LPS, to a cuvette containing the prewarmed (37°C) reaction mixture, which contained 0.25 μM Di-O-C_5(3) in KRP-D (final assay volume of 3 ml). In some experiments KRP-D was replaced with a high K^+ buffer (see above). The mixture was allowed to equilibrate until no further change in fluorescence was noted (about 5 min) before the addition of 1 μM FMLP. The excitation and emission wavelengths were set at 460 and 510 nm, respectively.

Alterations in $\Delta\psi$ were also monitored using the fluorescent dye bis-(3-phenyl-5-oxoisoxazol-4-yl)pentamethine oxonol (Oxonol V; Molecular Probes) (16). For measurement of changes in $\Delta\psi$ using Oxonol V, 5×10^6 cells (preincubated with buffer or LPS) were placed in a quartz cuvette with 1 μM Oxonol V in KRP-D preheated to 37°C (final assay volume, 2 ml). After equilibration, the reaction was initiated by the addition of 1 μM FMLP, and the change in fluorescence was recorded continuously using an excitation wavelength of 577 nm and an emission wavelength of 610 nm.

Cytofluorographic analysis of membrane potential change. Cytofluorographic measurement of the change in $\Delta\psi$ of individual neutrophils, using Di-O-C_5(3)-loaded cells, was performed using an Ortho System 50-H cytofluorograph and Ortho 2150 Data Handler software (Ortho Diagnostic Systems, Westwood, MA) (17). An excitation wavelength of 480 nm and an emission wavelength of 520 nm were used. Control or LPS-primed cells in dye-buffer solution were first evaluated for baseline membrane potential by recording the relative fluorescence of 10^4 cells before stimulation. Counting began 3 s after the addition of 1 μM FMLP, and the change in fluorescence was measured over 1 min. Total cells in each fluorescence channel were counted and the results reported as the percentage of cells with a change in $\Delta\psi$. In each experiment, lymphocytes and dead cells were excluded from analysis by gating based upon forward light scatter (18).

Determination of the concentration of intracellular free Ca^{2+}. $[Ca^{2+}]_i$ in neutrophils was derived from the fluorescence emitted by the Ca^{2+}-sensitive dye, fura-2 (Molecular Probes) (19). Cells were first exposed to LPS or buffer for 60 min at 37°C, centrifuged at 500 *g* for 5 min, and resuspended at room temperature with the membrane-permeable, acetoxymethyl derivative, fura-2AM, 20 μM, in KRP-D, for 4 min followed by an additional 20 min incubation in 2 μM fura-2. Once inside the cell, the compound is hydrolyzed to impermeable fura-2 (19). In some experiments cells were preloaded with MAPTAM for 20 min before exposure to LPS or buffer and then were incubated with fura-2 AM in an identical manner.

That the Ca^{2+}-insensitive species fura-2AM was converted completely to the Ca^{2+}-sensitive fura-2 was verified in buffer- and LPS-preincubated cells and in cells preloaded with MAPTAM using the method of Scanlon et al. (20). Briefly, excitation spectra from 300 to 400 nm were obtained from samples of cells in the presence of 1 μM ionomycin or 10 mM EGTA (pH 8.3) and compared to excitation spectra obtained in the same manner using a solution of fura-2 free acid (Molecular Probes). In every instance the excitation spectra obtained from cell-associated fura-2 was identical to that obtained from the fura-2 solution, indicating the complete deesterification of fura-2AM to a Ca^{2+}-sensitive species. The intracellular concentration

of fura-2 in each sample was determined by lysing the cells in 0.05% Triton X-100 in KRP-D and comparing the fluorescence intensity at 340 nm to that derived from known amounts of fura-2 free acid in the presence of 0.05% Triton X-100 and lysates of unloaded neutrophils.

Immediately before each determination aliquots of cell suspensions were centrifuged at 500 *g* for 5 min to remove excess fura-2AM or released fura-2, resuspended in KRP-D, and transferred to quartz cuvettes. In some experiments Ca^{2+}-free KRP-D, to which 5 mM EGTA was added, was used. The baseline fluorescence was determined using a recording fluorescence spectrophotometer with a heated cuvette holder and magnetic stirrer. After the addition of 1 μM FMLP, the change in fluorescence was measured and all data were stored for subsequent analysis.

$[Ca^{2+}]_i$ was calculated at the completion of each experiment by the ratio method using the formula $[Ca^{2+}] = K_d(R - R_{min})/(R_{max} - R)(S_{f2}/S_{b2})$ as described by Grynkiewicz et al. (19). K_d was 224 nM, and R_{max} and R_{min} were derived in the presence of ionomycin and EGTA, respectively. Autofluorescence from cells and/or added reagents was monitored during each experiment and found not to be a significant factor when neutrophils were loaded with 2 μM fura-2.

Expression of data. Results are expressed as mean±SEM. Statistical comparison was made with Student's *t* test, except as noted.

Results

O_2^- release in control and LPS-primed neutrophils. As previously reported (2), preincubation with LPS for 60 min resulted in the enhanced release of O_2^-. In our experiments, using 1 μM FMLP as stimulus, LPS-primed neutrophils released 10.6±4 times (range 3 to 27 times, $n = 25$) more O_2^- than did similarly prepared control cells (Fig. 1, *top*). When preincubated with LPS, PMA-stimulated cells had a shorter lag time, and both FMLP- and PMA-stimulated cells had a greater rate of O_2^- release (Fig. 1). In the absence of FMLP or PMA, LPS exposure did not stimulate the release of O_2^- (data not shown).

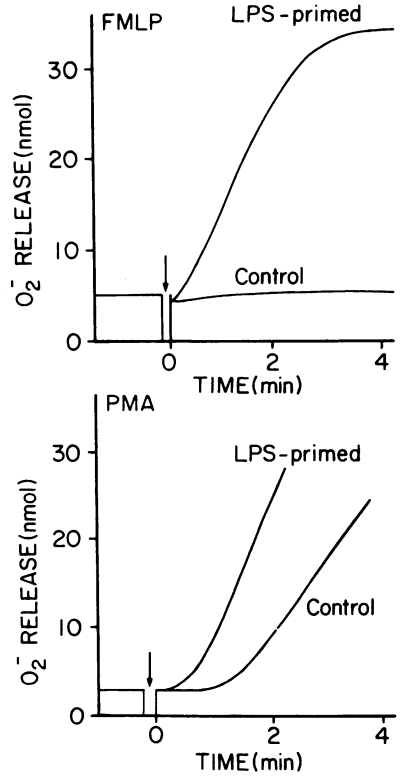

Figure 1. The time course of O_2^- release from control and LPS-primed human neutrophils stimulated with FMLP (1 μM) (*top*), or PMA (100 ng/ml) (*bottom*). The tracings were drawn from a representative experiment of 25 performed in which O_2^- release from control and LPS-primed neutrophils was compared. Arrows indicate the addition of stimulus.

*Table I. NADPH Oxidase Activity in Subcellular Fractions from Control and LPS-primed Human Neutrophils**

Cellular fraction	Generation of O_2^-	
	Control	LPS-primed
	nmol/min per mg protein	
Unfractionated cell sonicate	26.3±8.0	21.6±8.9
48,000 *g* supernatant	2.1±0.8	2.5±1.9
48,000 *g* pellet	6.5±0.5	7.2±1.4
48,000 *g* pellet + supernatant	28.5±7.6	25.0±10

* Values are the result of five paired experiments using sonicates of control or LPS-primed neutrophils in the presence of SDS, 100 μM.

O_2^- production by subcellular fractions of neutrophils. The addition of arachidonic acid (21, 22) or SDS (11) to sonicates of unstimulated neutrophils permits study of the NADPH-dependent respiratory burst oxidase in resting, unstimulated cells. In this system, NADPH-dependent O_2^- release requires the presence of both the 48,000 *g* particulate and cytosolic fractions from the cell sonicate for optimal activity. Preexposing intact cells to suboptimal concentrations of soluble stimuli partially removes the requirement for the cytosolic component (11, 22). We explored the possibility that LPS might exert a similar effect. Cell sonicates, membrane-enriched particulate fractions, or cytosolic fractions of cells preincubated with KRP-D or LPS were compared for NADPH-dependent O_2^- production in the presence of SDS, as shown in Table I. The cells studied were primed for a mean of 17 times more O_2^- release. Cell sonicates and subcellular fractions of cells preincubated with LPS did not demonstrate the enhanced production of O_2^- observed in whole cells. LPS priming did not enhance activity of the 48,000 *g* particulate fraction or eliminate the requirement for the cytosolic component. In some experiments, SDS was omitted to assess the possibility that LPS might have activated the NADPH oxidase during preincubation. However, NADPH oxidase activity was barely detectable in the absence of SDS in preparations from LPS-primed and control cells ($n = 3$, data not shown).

We also investigated the possibility that LPS priming might alter the kinetic properties of the NADPH oxidase, using the SDS-activated cell-free system with fractions from unstimulated cells (11). NADPH oxidase activity of the cell sonicate from control and LPS-pretreated cells was measured over a range of NADPH concentrations. As shown in Fig. 2, the NADPH oxidase activity of fractions from LPS-treated cells never exceeded that of control cells at any NADPH concentration. By generating the hyperbolic curve of the Michaelis-Menten equation from a nonlinear regression fit of the data, the kinetic parameters, K_m and V_{max}, of the two groups were calculated and compared (23). The computed K_ms of membrane-enriched fractions from control cells and LPS-pretreated cells were similar (19.6±3.3 and 16.8±2.0 μM, respectively, $n = 3$), whereas the V_{max} of fractions from LPS-primed cells (12.2±8.6 nmol O_2^-/min per mg) was decreased compared to that from controls (20.2±8.2 nmol O_2^-/min per mg, $P < 0.05$, $n = 3$). Thus, despite a 17-fold increase in O_2^- release of the LPS-primed cells before disruption, LPS priming appeared not to enhance directly the function of the NADPH oxidase.

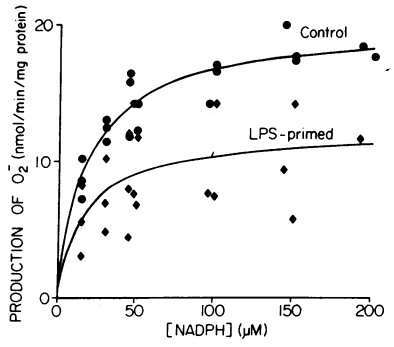

Figure 2. The NADPH-dependent production of O_2^- by SDS-activated, membrane-enriched fractions prepared from neutrophils preincubated with buffer (●) or LPS (50 ng/ml) (♦) for 60 min at 37°C, as described in Methods. Each line represents the nonlinear regression best fit to the hyperbolic Michaelis-Menten equation of all the values from three experiments (24).

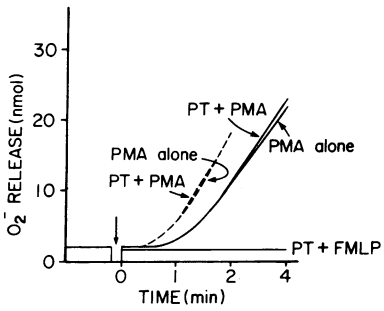

Figure 3. The time course of O_2^- release from pertussis toxin (PT)-treated human neutrophils stimulated with PMA (100 ng/ml) or FMLP (1 μM). Cells were incubated with buffer (*solid line*) or LPS (50 ng/ml) (*dashed line*), with or without prior preincubation with PT. The tracings were drawn from a representative experiment of four performed. Lag times in cells incubated in buffer were −PT, 76.5±4.1 s; +PT, 78.6±4.0 s; and in LPS −PT, 38.9±1.2 s; +PT, 45.3±1.0 s (n = 4). Rates of O_2^- release by cells incubated in buffer were, −PT, 17.6 nmol/min; +PT, 18.3±1.3 nmol/min; and in LPS −PT, 24.0±1.4 nmol/min; +PT, 22.8±0.8 nmol/min (n = 4).

Concentration and distribution of cytochrome b in primed neutrophils. After incubation of the neutrophils with buffer or LPS, the concentration of cytochrome *b* in Triton X-100 extracts was determined spectrophotometrically (13). Intact LPS-treated neutrophils from the same preparations produced a mean of 4.2 times more O_2^- than did control neutrophils (n = 5). The total cytochrome *b* present in control neutrophils was 6.2±0.5 pmol/10^6 cells; LPS-primed neutrophils contained 6.5±0.8 pmol/10^6 cells. Thus, a 60-min incubation with LPS, which caused substantial priming for an enhanced respiratory burst, had no significant effect on the total amount of cytochrome *b* in the neutrophils.

To explore the possibility that LPS priming might induce increased translocation of cytochrome *b* to the plasma membrane, believed to be the site of the active NADPH oxidase (1, 7, 24), we measured cytochrome *b* activity in membrane-enriched fractions made from control or LPS-primed cells. Equivalent concentrations of cytochrome *b* were present in such fractions from control (2.1±0.2 pmol/10^6 cells) and LPS-primed cells (2.4±0.5 pmol/10^6 cells, n = 5).

Effect of pertussis toxin on O_2^- release. We investigated the possibility that a purified component of PT, which reportedly inhibits LPS priming in B cell and macrophage cell lines (25), might exert a similar effect in neutrophils. PT, an inhibitor of some G proteins, one of which links FMLP-receptor binding to triggering of the NADPH oxidase (26), was added to suspensions of cells before LPS in an effort to inhibit priming. The addition of PT predictably blocked the FMLP-induced signal, and, therefore, prevented the demonstration of priming in FMLP-stimulated cells (Fig. 3). Using PMA as a stimulus, we demonstrated the characteristic effects of LPS priming in PT-pretreated cells by noting the decreased lag time and increased rate of O_2^- production (Fig. 3). Thus, PT-sensitive G proteins did not appear to be a primary target of LPS.

PKC activity. The involvement of the Ca^{2+}- and phospholipid-dependent PKC in the activation of the neutrophil respiratory burst has been proposed by others (8, 27). In unstimulated neutrophils the localization of PKC activity, measured as phosphorylation of histone, is primarily cytosolic. However, after exposure of cells to direct activators of PKC (PMA or *rac*-1-*O*-myristoyl-2-*O*-acetylglycerol) or to FMLP in the presence of cytochalasin B, the enzyme becomes associated with the particulate fraction (8, 28).

We explored the possibility that LPS-priming might be achieved through a change in the subcellular distribution of PKC. Comparison of the distribution of PKC activity between the cytosolic and particulate fractions of unstimulated control cells and neutrophils treated for 60 min with 100 ng/ml LPS did not demonstrate any change in the total or subcellular localization of PKC activity (cytosolic PKC in 10^7 cell equivalents from unprimed cells = 578±115 pmol/min, from LPS-primed cells = 579±82 pmol/min; particulate PKC from unprimed cells = 39±3 pmol/min, from LPS-primed cells = 31±2 pmol/min, n = 3). LPS itself (100 ng/ml) added to cell extracts did not directly affect PKC. Treatment of control neutrophils with 1.6 μM PMA for 5 min did result in translocation of the enzyme from the cytosol to the particulate fraction, as previously described (data not shown) (8, 28). Stimulation of neutrophils with FMLP (1 μM) did not affect the total or apparent distribution of PKC activity in either control or LPS-treated neutrophils (n = 2, data not shown).

Due to the possibility that Ca^{2+} is required to maintain the association of PKC with cellular membranes (29, 30), the experiments were repeated using an EGTA-free extraction buffer. Although an increased association of PKC activity with the particulate fraction was noted in the absence of EGTA (particulate PKC in unprimed cells = 89±32 pmol/min, in primed cells = 80±21 pmol/min, n = 3), there remained no significant difference in total or subcellular localization of PKC activity between control and LPS-primed neutrophils.

Changes in membrane potential associated with priming and O_2^- release. Stimulated neutrophils undergo a change in $\Delta\psi$ that mirrors ionic events and is thought to precede O_2^- release (6, 31). The effects of LPS priming on the alteration of $\Delta\psi$ were explored. Control and LPS-preexposed cells were incubated with fluorescent probes of membrane potential to measure the alteration in $\Delta\psi$ in response to FMLP.

At the completion of equilibration between dye and cells, the baseline fluorescence was equivalent in control and LPS cells (data not shown). Our results are similar to those of Larsen et al. (32) who found that LPS does not induce an alteration of $\Delta\psi$ in unstimulated monocytes.

The FMLP (10^{-6} M)-induced shift in fluorescence of Di-O-C_5(3) exhibited a monophasic response that began within 5 s and peaked by 45 s (Fig. 4). The decrease in fluorescence of Di-O-C_5(3) represented an apparent depolarization, confirming the work of others (15, 33). Using a concentration of FMLP that does not stimulate the respiratory burst (10^{-10}

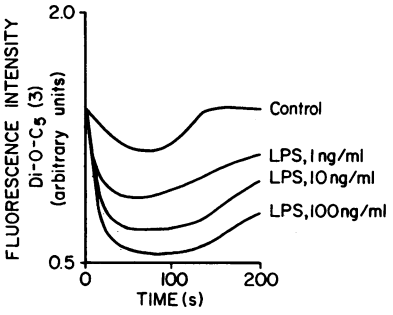

Figure 4. The time course of change in membrane potential ($\Delta\psi$) in control and LPS-primed human neutrophils, measured as change of fluorescence intensity of Di-O-C_5(3), expressed as arbitrary units (15). The graph shows superimposed tracings of $\Delta\psi$ for control and LPS-primed cells exposed to various concentrations of LPS then stimulated with FMLP (1 μM) at time 0. The curves are redrawn from actual tracings from a representative experiment of six performed and have been superimposed for ease of comparison.

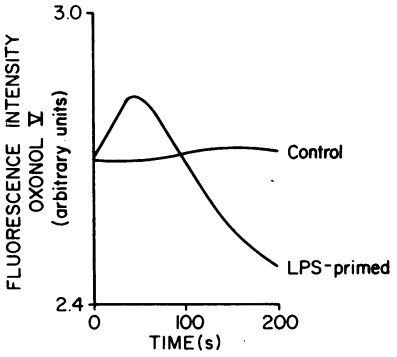

Figure 5. The time course of change in membrane potential ($\Delta\psi$) in control and LPS-primed human neutrophils, measured as change of fluorescence intensity of Oxonol V, expressed as arbitrary units (16). The graph shows superimposed tracings of the change in $\Delta\psi$ for control and LPS-primed cells stimulated with FMLP (1 μM) at time 0, which are redrawn from actual tracings from a representative experiment of four performed.

M), Lazzari et al. (34) have demonstrated a stimulus-induced initial increase in fluorescence of Di-O-C_5(3) (an apparent hyperpolarization), but they too report an initial loss of fluorescence of Di-O-C_5(3) in response to concentrations of FMLP $> 10^{-7}$ M. The magnitude of the change in $\Delta\psi$ 45 s after the addition of FMLP was increased in LPS-primed cells (Fig. 4). As seen with FMLP-triggered O_2^- release (2), the magnitude of the $\Delta\psi$ change was dependent upon the concentration of LPS (Fig. 4). Thus, Di-O-C_5(3) appears to be measuring an event linked to the activation of the respiratory burst.

The relationship between $\Delta\psi$ and O_2^- release was studied by attempting to dissociate the two events with buffer in which Na^+ was replaced by K^+ (137 mM) (15), which markedly reduces the net membrane potential (15, 35, 36). We demonstrated a 45±5% (n = 2) reduction in the resting fluorescence of Di-O-C_5(3) when either control or LPS-pretreated cells were tested in a high K^+ buffer. There was no release of O_2^- in the absence of FMLP when the cells were placed in high K^+ buffer. Although the change in $\Delta\psi$ in response to FMLP of both control and LPS-primed cells was markedly diminished (< 5% of the response in normal buffer in all experiments), parallel measurement of O_2^- in high K^+ buffer from FMLP-stimulated control (2.0±0.2 nmol) and LPS-primed cells (35±2 nmol) demonstrated the normal enhancement of LPS priming (n = 2). Thus, stimulation of the NADPH oxidase did not require a large alteration of $\Delta\psi$, and depolarized LPS-primed cells remained capable of an enhanced respiratory burst.

Fluorescence microscopy indicates that the carbocyanine dyes such as Di-O-C_5(3) move into negatively charged intracellular organelles such as the mitochondria, and stimulus-induced loss of fluorescence is blocked by inhibitors of mitochondrial respiration (37). Therefore, these probes may not accurately report changes in $\Delta\psi$ at the plasma membrane. Oxonol V is negatively charged and does not accumulate in the interior of the cell (38). Thus, it is a suitable probe for measuring alterations in $\Delta\psi$ at the plasma membrane. As shown in Fig. 5, cells preloaded with Oxonol V and stimulated with FMLP displayed a biphasic response with an initial increase in fluorescence reflecting an apparent hyperpolarization followed by a decrease in fluorescence reflecting an apparent depolarization (38). These responses were much more rapid and pronounced in LPS-primed cells. These results indicate that LPS affects a step in signal transduction that precedes FMLP-induced changes in $\Delta\psi$.

Cytofluorographic measurement of membrane potential change. FMLP-elicited alteration in $\Delta\psi$ was measured cytofluorographically in individual cells with Di-O-C_5(3) in control and LPS-pretreated neutrophils (Fig. 6). Unstimulated, control cells demonstrated a uniform fluorescence. A cell count of each fluorescence channel indicated that 92±3% (n = 3) of cells possessed the same membrane potential (Fig. 6 *A*). Control cells stimulated with FMLP exhibited a bimodal fluorescence pattern (Fig. 6 *B*). Depolarized cells appeared as a new population of cells with decreased fluorescence, although 69±5% of the cells did not show a change. As shown in Fig. 6 *C*, 83% of unstimulated, LPS-primed cells had fluorescence similar to that of unstimulated, unprimed cells. In spite of the presence of the small population of cells demonstrating the lower fluorescence, and thus, a change in $\Delta\psi$, a parallel measure of O_2^- production detected no release of O_2^- in unstimu-

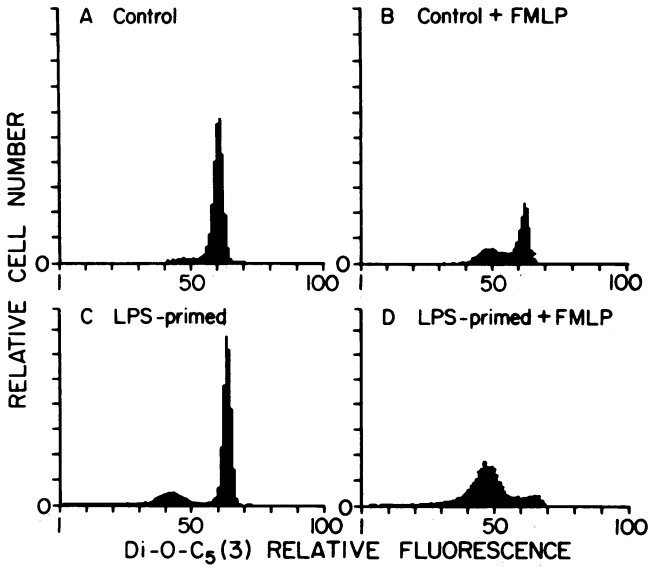

Figure 6. Cytofluorographic measurement of membrane potential (derived from the fluorescence of Di-O-C_5(3)) of individual neutrophils preincubated with buffer (*A, B*) or LPS, 50 ng/ml (*C, D*), before (*A, C*) and 30 s after (*B, D*) the addition of 1 μM FMLP. Fluorescence intensity of Di-O-C_5(3) is plotted on the abscissa and the number of cells per fluorescence channel is plotted on the ordinate. The results shown are representative of three experiments performed.

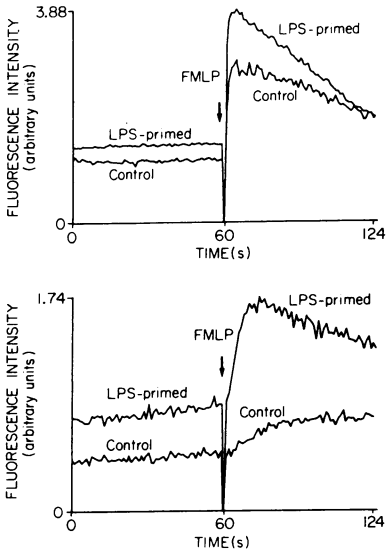

Figure 7. Fluorescence of fura-2, reflecting intracellular cytosolic free Ca^{2+} concentrations in control or LPS-primed neutrophils before and after stimulation with FMLP. Cells were stimulated with 1 μM FMLP (*top*) or 1 nM FMLP (*bottom*). The tracings are representative of the results of six experiments with 1 μM FMLP, and five with 1 nM FMLP.

lated cells. FMLP stimulation of LPS-treated cells again resulted in the bimodal pattern of Di-O-C_5(3) fluorescence (Fig. 6 *D*), but the distribution of cells suggested that most (83±4%) had depolarized. The bimodal pattern of Di-O-C_5(3) fluorescence of FMLP-stimulated cells suggested that each cell was responding in an all-or-none fashion. These results indicate that LPS priming may lead to the recruitment of a previously unresponsive population of neutrophils.

Cytosolic free Ca^{2+} in LPS-pretreated neutrophils. Using the fluorescent Ca^{2+}-sensitive dye, fura-2, we measured $[Ca^{2+}]_i$ in control and LPS-pretreated neutrophils both before and after FMLP stimulation. As shown in Fig. 7 and Table II, LPS pretreatment significantly elevated basal $[Ca^{2+}]_i$ in unstimulated cells.

Continuous measurement of $[Ca^{2+}]_i$ during 60 min of LPS priming was attempted, but the fluorescence created from leakage of cell-associated fura-2 into the Ca^{2+}-containing buffer (39) made it difficult to interpret the time course of changes in $[Ca^{2+}]_i$. However, cells were loaded with fura-2 and measurements of $[Ca^{2+}]_i$ were undertaken after incubation with LPS for various fixed times followed by removal of extracellular fura-2 by washing. By 20 min of incubation with LPS, the earliest time point that could be studied because of the time required for loading, baseline $[Ca^{2+}]_i$ was at maximal levels, and it remained there for at least 80 min (n = 3).

Intracellular concentrations of fura-2 were equivalent in LPS- and buffer-exposed cells (57±2 and 56±14 nmol/ml per 5×10^6 neutrophils, respectively, n = 3). Therefore, LPS did affect the uptake or deesterification of fura-2AM. Preloading with MAPTAM also did not affect the intracellular concentration of fura-2, in the presence or absence of LPS (57±3 and 56±1 nmol/ml per 5×10^6 neutrophils, respectively, n = 3). Thus, the presence of MAPTAM did not interfere with the intracellular conversion of fura-2AM to fura-2 free acid.

The $[Ca^{2+}]_i$ response of LPS-primed cells to both concentrations of FMLP tested (1 μM and 1 nM) was significantly greater than that of control cells (Fig. 7 and Table II). $[Ca^{2+}]_i$ in both control and LPS-primed cells increased rapidly in response to 1 μM FMLP, reaching a peak level in 4±1 s in LPS-primed cells, and 6±1 s in control cells, ($P < 0.05$, n = 6). Suboptimally stimulated cells (1 nM FMLP) were slower to attain a peak $[Ca^{2+}]_i$, but the difference between LPS-primed cells (13±2 s) and control cells (47±5 s) was even greater (n = 5).

In the absence of extracellular Ca^{2+} during the 60-min incubation with LPS (Ca^{2+}-free KRP-D with 5 mM EGTA), basal (unstimulated) levels were lower. However, the difference in $[Ca^{2+}]_i$ between control (25±3 nM) and LPS-primed cells (43±6 nM) remained significant ($P < 0.01$, n = 3).

Measurement of $[Ca^{2+}]_i$ with fura-2 depends upon the ratio of the fluorescence at two different wavelengths, so that $[Ca^{2+}]_i$ does not vary with the cellular content of fura-2 (19). Therefore, the results cannot be attributed to a higher concentration of fura-2 in LPS-primed cells.

Effects of chelation of intracellular Ca^{2+} on LPS priming. To explore the relationship in LPS-primed neutrophils between the increase in baseline and stimulated $[Ca^{2+}]_i$ and the accelerated respiratory burst, we preexposed cells to LPS or buffer in the presence or absence of the nonfluorescent, intracellular Ca^{2+} chelator MAPTAM. The basal level of $[Ca^{2+}]_i$ was reduced in both LPS-preincubated and in control neutrophils preloaded with MAPTAM so that the difference in basal $[Ca^{2+}]_i$ noted between LPS-primed and control cells was no longer evident (n = 3, data not shown). Basal $[Ca^{2+}]_i$ in control neutrophils was reduced by 17±1%, and the rise in $[Ca^{2+}]_i$ in response to FMLP was abolished (no response) in cells preloaded with MAPTAM (n = 3). Preloading control (LPS-free) neutrophils with MAPTAM had no significant effect on O_2^- release when cells were stimulated with FMLP or PMA (Fig. 8). Lag time in response to PMA was also not significantly altered (control neutrophils, 72.3±1.8 s; control neutrophils + MAPTAM, 80.0±3.5 s, NS, n = 3). However, the enhanced O_2^- release in response to stimulation with FMLP or PMA noted in LPS-primed neutrophils was no longer evident when LPS-primed cells were first preloaded with MAPTAM (Fig. 8). The shortened lag time in LPS-primed cells preloaded with MAPTAM and stimulated with PMA was similarly prevented (LPS-primed neutrophils, 47.3±5.4 s; LPS-primed neutrophils + MAPTAM, 69.0±3.2 s, $P < 0.05$, n = 3).

To test whether the inhibitory effect of MAPTAM was specific for buffering intracellular Ca^{2+}, cells preloaded with MAPTAM were incubated with 1 μM ionomycin for 60 s

*Table II. The Effect of LPS Priming on the Concentration of Intracellular Free Calcium ($[Ca^{2+}]_i$) and on the Rate of Increase in $[Ca^{2+}]_i$ in Response to FMLP Stimulation**

		FMLP-stimulated			
		1 nM		1 μM	
Unstimulated					
Cell type	$[Ca^{2+}]_i$	Peak $[Ca^{2+}]_i$	Rate	Peak $[Ca^{2+}]_i$	Rate
	nM	*nM*	*$\Delta nM/s$*‡	*nM*	*$\Delta nM/s$*‡
Control	42±3	87±22	1.0±0.5	268±66	37.3±9
LPS-primed	69±5§	140±29‖	5.5±1.1‖	329±68‖	65.0±13‖

* The values reported are the results of six experiments for 1 μM FMLP, 5 experiments for 1 nM FMLP, and 14 experiments unstimulated.

‡ The maximal change in $[Ca^{2+}]_i$ with time is shown.

§ $P < 0.001$.

‖ $P < 0.05$.

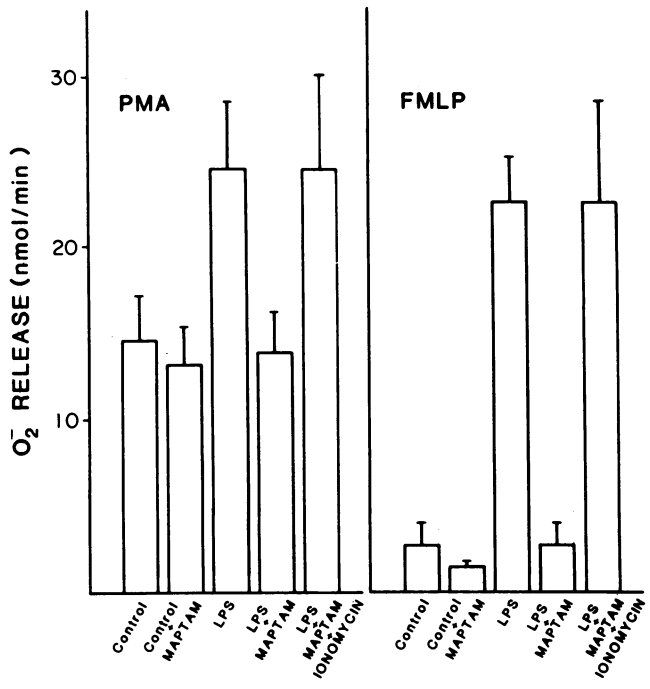

Figure 8. The effect of the intracellular Ca^{2+} chelator MAPTAM on LPS priming. Neutrophils, preincubated in the presence or absence of MAPTAM for 30 min and then exposed to buffer or LPS, were stimulated with 100 ng/ml PMA or 1 μM FMLP as described in Methods. The column at the right of the panel with each stimulus represents results obtained after adding ionomycin, 1 μM, 60 s before adding the stimulus. The results are expressed as the initial rate of O_2^- release and represent the mean±SEM of three experiments. Initial rate of O_2^- release and total O_2^- release in response to stimulation by FMLP are comparable parameters; $r = 0.98$, $P < 0.005$, $n = 6$ (Pearson's coefficient of correlation). The use of rates permits more rapid analysis of cells stimulated with PMA.

before stimulation with FMLP or PMA. At the end of this 60-s incubation, $[Ca^{2+}]_i$ was well above pre-MAPTAM levels (~ 700 nM). This stimulated no measurable release of O_2^- over 4 min of incubation (separate cuvettes), but addition of either FMLP or PMA 60 s after the ionomycin induced release of O_2^- to rates achieved in the absence of MAPTAM (Fig. 8). Thus, by buffering changes in $[Ca^{2+}]_i$, MAPTAM blocked the effects of LPS priming without affecting the baseline release of O_2^- in response to stimulation with FMLP or PMA.

Discussion

We have attempted to determine the biochemical mechanisms that might account for the way in which preexposure to LPS greatly enhances O_2^- release from stimulated human neutrophils. The fact that the O_2^- release occurred both sooner and at a faster rate suggested that LPS priming might have modified components of the signal transduction pathways or altered directly the NADPH oxidase. These possibilities have been previously explored in the LPS-primed macrophage (12, 15, 40, 41).

We reexamined the hypothesis that LPS priming resulted from a modification of the NADPH oxidase. However, the similar NADPH oxidase kinetics and the equivalent activities and content of components of the NADPH oxidase (cytosolic and particulate fractions and cytochrome *b*) from control and LPS-primed neutrophils would not explain the difference in the release of O_2^-. This suggested that the target of LPS priming preceded enzyme activation.

It has been reported that an FMLP-induced cellular response requires the presence of guanine nucleotide regulatory proteins (G proteins) to initiate the cell signal (26). Their apparent importance in signal transduction and close association with the plasma membrane made it reasonable to consider G proteins as a target of the effects of LPS. However, PT did not inhibit LPS priming, which implies that alteration of at least certain G proteins is not an essential feature of LPS priming.

A change of neutrophil $\Delta\psi$ is associated with the early steps of stimulus-response coupling (6), and its apparent absence in cells from patients with chronic granulomatous disease has led others to propose that a change in $\Delta\psi$ is a trigger of the respiratory burst (6, 31, 42). In our studies, measurements of $\Delta\psi$ demonstrated an enhanced change in $\Delta\psi$ in LPS-preincubated cells in parallel with the greater O_2^- response. However, the enhanced release of O_2^- was also measured in LPS-primed cells placed in a high K^+ buffer, which inhibits an alteration in $\Delta\psi$ in response to stimulation. The uncoupling of O_2^- release and a change in $\Delta\psi$ in a high K^+ buffer indicated that a change in $\Delta\psi$ was unlikely to be the trigger of NADPH oxidase activity. The enhanced alteration of $\Delta\psi$, seen in stimulated, LPS-primed cells, could reflect the modification of a step in signal transduction that precedes both the change in $\Delta\psi$ and NADPH oxidase activation.

An increase in $[Ca^{2+}]_i$ is one of the earliest measurable events in signal transduction and reportedly precedes changes in $\Delta\psi$ (6, 34, 43). Using the Ca^{2+}-sensitive fluorescent dye, fura-2, to monitor $[Ca^{2+}]_i$, neutrophils preincubated with LPS for 60 min consistently demonstrated a sustained 67±8% increment in basal $[Ca^{2+}]_i$ (Fig. 6, Table II). The sharp rise in $[Ca^{2+}]_i$ seen after stimulation with FMLP was also accentuated in LPS-primed cells.

To examine further the role of an increase in $[Ca^{2+}]_i$ as a basis of LPS priming in neutrophils, cells were first preloaded with the nonfluorescent Ca^{2+} chelator MAPTAM before exposing them to LPS (9). Neutrophils preloaded with MAPTAM before exposure to LPS did not demonstrate the increase in resting $[Ca^{2+}]_i$ nor the enhanced respiratory burst. The respiratory burst, with FMLP or PMA as stimulus, in these cells was equivalent to that in cells incubated in LPS-free buffer. Therefore, the results presented here indicate that an incremental change in resting $[Ca^{2+}]_i$ is necessary for the neutrophil to manifest LPS priming with an enhanced respiratory burst in response to FMLP or PMA.

An increase in the basal $[Ca^{2+}]_i$ may also be the basis of priming by other agents. FMLP (44), platelet-activating factor (45), and the Ca^{2+} ionophore, ionomycin (10), can prime neutrophils for an enhanced respiratory burst. A low concentration of each stimulus has been shown to induce an increase in basal $[Ca^{2+}]_i$ without stimulating O_2^- release, an effect similar to that which we report here with LPS. Therefore, an increase in $[Ca^{2+}]_i$ may be central to the molecular basis of priming by these agents.

The source of Ca^{2+} appeared to be an intracellular pool, as the increases in basal levels of $[Ca^{2+}]_i$ in LPS-primed cells could be demonstrated with or without Ca^{2+} in the buffer during LPS priming (absence or presence of EGTA). Priming

for enhanced release of O_2^- was also achieved in the absence of Ca^{2+} (data not shown, $n = 3$), in confirmation of previous experiments (2).

How LPS priming might mediate a change in cellular Ca^{2+} homeostasis is not clear. Although a unique plasma membrane-associated receptor for LPS has not been proved, LPS attaches quickly and avidly to neutrophils (2). This interaction could result in activation of phospholipase C (46, 47), thereby degrading membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP_2) (48, 49), with the creation of the intracellular messenger inositol 1,4,5-tris-phosphate (IP_3) capable of mobilizing Ca^{2+} from intracellular stores. Perhaps LPS itself or one of its breakdown products exert an IP_3-like effect on intracellular organelles, releasing sequestered Ca^{2+}; or LPS might act by modifying the efficiency by which intracellular signals release Ca^{2+} into the cytosol. LPS might be degraded to release free fatty acids (50, 51) that could modify the physicochemical properties of organelle membranes, and thereby modify Ca^{2+} channels.

LPS-induced increments in $[Ca^{2+}]_i$ in unstimulated neutrophils could arm the cell for enhanced signal transduction in several ways. Ca^{2+} serves a dual role as an intracellular second messenger in signal transduction by activating both the PKC and the calmodulin-dependent pathways (52, 53). PKC activity and its distribution is Ca^{2+}-dependent, and exposure to higher levels of $[Ca^{2+}]_i$ as a result of LPS priming might influence association of the enzyme with the plasma membrane and the NADPH oxidase (30). This could sufficiently modify a PKC-dependent signal to shorten the lag time and to heighten NADPH oxidase activity upon stimulation. Other Ca^{2+}-dependent protein kinases thought to be involved in signal transduction (52) could be similarly affected when exposed to an LPS-induced elevation in $[Ca^{2+}]_i$. A small elevation in $[Ca^{2+}]_i$, inadequate to trigger O_2^- release, might also activate calmodulin (52). LPS-induced increases in $[Ca^{2+}]_i$ could enhance signal transduction synergistically by affecting both pathways simultaneously.

Phospholipase A_2 (PLA_2) catalyzes the release from phospholipids of arachidonic acid, proposed to be an intracellular messenger in signal transduction (54). This phospholipase is Ca^{2+}-dependent (55, 56), and the additional availability of intracellular free Ca^{2+} in LPS-primed neutrophils might enhance such activity. Enhanced PLA_2 activity and release of arachidonic acid has been measured in neutrophils primed with granulocyte-macrophage colony-stimulating factor (57, 58). Ca^{2+} has also been shown to have a direct effect on NADPH oxidase kinetics in membrane-enriched fractions (59).

An increase in $[Ca^{2+}]_i$ might modify signal transduction by facilitating ionic movement (measured as a change in $\Delta\psi$), particularly K^+, through Ca^{2+}-dependent K^+ channels located in the plasma membrane (60–62). Our data, showing an enhanced change in fluorescence of the probe of $\Delta\psi$, oxonol V, in LPS-primed cells, is quite consistent with this hypothesis.

LPS may be exerting its effect in part through an intermediate cell such as platelets or mononuclear cells. Wright et al. (63) have recently described an LPS-inducible platelet-derived factor capable of partially restoring the enhanced O_2^--releasing activity of neutrophils believed to be platelet-free after separation through a plasma-Percoll gradient. We have found that neutrophils separated by plasma-Percoll can be actively primed by LPS, however (64). LPS induces the release of tumor necrosis factor α (TNFα) from mononuclear cells (65). Neutrophils exposed to TNFα undergo an enhanced secretion of lactoferrin (66) and are primed for an increase in O_2^- release in response to FMLP or PMA (67). It seems unlikely that contaminating monocytes might release sufficient TNFα to prime within the 60-min incubation, but this possibility is currently under investigation in our laboratory. In any event, this paper reports studies of the interrelationship of being primed for an enhanced respiratory burst and possible changes in the NADPH oxidase and stimulus-response coupling.

Study of the molecular basis of the LPS-mediated enhanced activity of neutrophils should be a useful tool to aid in better understanding cell signals and how they are regulated. A precise description of the events modified by LPS-priming might also furnish a clue to the defect in certain forms of chronic granulomatous disease. The ability to modulate $[Ca^{2+}]_i$ may provide a means of pharmacologically modifying signal-induced cellular responses. Such agents might enhance microbicidal activity during a serious infection, or reduce inflammation in disorders such as the adult respiratory distress syndrome or immune complex disease.

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