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Role of Calcium during Lipopolysaccharide Stimulation of Neutrophils

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This study investigated the role of intracellular calcium concentration ($[Ca]_i$) as a possible intermediate in the lipopolysaccharide (LPS) second messenger pathway for the activation of neutrophils (polymorphonuclear leukocytes [PMNs]). Isolated PMNs were loaded with the calcium-sensitive fluorescent dye fura-2. The PMNs were stimulated with either LPS or the positive control formyl-Met-Leu-Phe (fMLP). As expected, PMN exposure to fMLP increased $[Ca]_i$. However, LPS stimulation did not induce any detectable changes. Depletion of intracellular Ca stores with thapsigargin, or extracellular Ca with EGTA, significantly inhibited the upregulation of the CD11b/CD18 integrin in response to fMLP but not LPS. We conclude that $[Ca]_i$ is not an early intermediate in the second-messenger pathway for the activation of PMNs by LPS.

Lipopolysaccharide (LPS) is responsible for the pathophysiologic phenomena associated with infection by gram-negative organisms (29). LPS interacts with the acute-phase protein, LPS-binding protein. The LPS–LPS-binding protein complex then binds to the CD14 receptor on polymorphonuclear leukocytes (PMNs), resulting in cellular activation (36). Neutrophil activation results in increased adherence, phagocytosis, oxidative burst, and enzyme release, as well as microbicidal and cytotoxic activity (6, 16).

The second-messenger system of LPS activation has remained elusive (3, 5, 6, 8, 13, 24–26, 28, 32, 35, 38, 39). The role of intracellular calcium concentration ($[Ca]_i$) in LPS stimulation of macrophages has been studied extensively, and the consensus has been that LPS does not cause an increase in $[Ca]_i$; however, a baseline calcium concentration is required for proper cell activation in response to LPS (4, 5, 9, 20, 21, 27).

The role of $[Ca]_i$ in LPS stimulation of PMNs has been studied, with many conflicting results. It was proposed initially that LPS priming of PMNs resulted in an increase in $[Ca]_i$ (10, 12, 40), although this has been refuted by others (33). It has been shown that many of the LPS-induced signs of activation could be blocked by calcium chelators such as quin-2 (14, 40). However, in a study by Klein et al. (14), LPS stimulation in and of itself did not increase $[Ca]_i$ nor did calcium ionophores produce PMN activation. The authors postulated that, as in the model proposed for macrophages, a baseline $[Ca]_i$ was needed for activation but changes in $[Ca]_i$ were not involved directly in LPS signal transduction.

In contradiction to this model, results with macrophages demonstrated that LPS stimulation increases phosphatidylinositol turnover (28), that cross-linking CD14 increases $[Ca]_i$ (17, 19), and that in individually observed rat peritoneal macrophages, LPS exposure causes a heterogeneous increase in $[Ca]_i$ in 47% of cells (16). As in the case of macrophages, the cross-linking of CD14 results in an increase in PMN $[Ca]_i$ (17, 19). However, this response was found to be very epitope specific and did not occur for all clones or for Fab′ fragments. Also, Yee et al. (40), using Fluo-3 dye and flow cytometry, observed a small heterogeneous increase in the $[Ca]_i$ of individual PMNs after LPS stimulation.

We decided to further investigate the role of $[Ca]_i$ in LPS signal transduction in PMNs. This study is unique in that a population of PMNs, as well as individual PMNs, was evaluated. Results demonstrated that formyl-Met-Leu-Phe (fMLP) stimulation appropriately increased $[Ca]_i$, but no changes in $[Ca]_i$ were observed after exposure of these PMNs to LPS. Activation of PMNs by LPS or fMLP increases the expression of the integrin CD11b/CD18 (18). However, depletion of available intracellular and extracellular calcium by thapsigargin and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], respectively, did not inhibit the upregulation of CD11b/CD18 expression on PMNs after LPS stimulation but did reduce the upregulation of CD11b/CD18 after fMLP stimulation. These results further support the hypothesis that changes in $[Ca]_i$ are not involved in the second-messenger pathway for LPS activation of PMNs.

All chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, Mo.). The Hanks balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), and the RPMI 1640 culture medium were obtained from Gibco (Grand Island, N.Y.). The calcium fluorescent dye fura-2 was purchased from Molecular Probes, Inc. (Eugene, Oreg.).

All culture media and solutions used were made with LPS-free sterile water (Baxter, McGaw Park, Ill.) and were filtered with a Falcon 7105 sterile 0.22-μm-pore-size filter (Becton Dickinson, Oxnard, Calif.). LPS contamination was <2 pg/ml as determined by a chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, Md.). PMNs were separated from whole blood within 30 min of being drawn. PMN separation was accomplished by means of a Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) and neutrophil isolation medium (containing 85% Hypaque) centrifuge gradient at 500 × *g* for 30 min at room temperature as previously described (1). All subsequent procedures were carried out at 4°C.

PMNs were collected and washed with DPBS plus calcium (DPBS+), and any remaining erythrocytes were removed by hypotonic lysis. Cell suspensions were >95% PMNs by morphology after staining with Turk's stain. PMNs were resuspended in RPMI 1640 culture medium containing 4% fetal calf serum (HyClone, Logan, Utah) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) at pH 7.4. Incubations were performed with approximately 10^7 PMNs in 3 ml of culture medium for 30 min at 37°C in an incubator with

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5% CO_2. Parallel cultures were prepared containing *Escherichia coli* O55:B5 LPS (Difco, Detroit, Mich.) and either 1% type AB pooled sera or fMLP (Calbiochem, La Jolla, Calif.). The AB serum was used to provide LPS-binding protein and any other serum components needed for LPS binding and activity. At the end of incubation the cells were agitated, removed from the plate, and washed with DPBS+. PMN recovery was between 80 and 90% and viability was >95% as determined by trypan blue dye exclusion.

The ratio of fluorescence intensity at 340 and 380 nm (340/380 nm ratio) is proportional to the $[Ca]_i$. PMNs were loaded with fura-2 by a variation of the method described by Forehand et al. (12). Briefly, a 0.5-μg vial of fura-2AM was reconstituted in 50 μl of dimethyl sulfoxide and added to 2.5 ml of RPMI 1640 culture medium. To load isolated PMNs with fura-2, 2×10^7 PMNs were incubated at 37°C with 1 ml of the RPMI 1640–fura-2 stock solution (final fura-2 concentration, 10 μM). After 10 min, 9 ml of RPMI 1640 was added to the PMNs (final fura-2 concentration, 1 μM), which were then incubated for an additional 20 min at 37°C. After fura-2 loading, the cells were washed with HBSS–1 mM EGTA and centrifuged three times. The PMNs subsequently were resuspended in HBSS–1 mM EGTA and placed on ice until use.

PMN population fluorescence was determined with a dual excitation-emission spectrofluorimeter (DM3000CM Spex; Spex Industries Inc., Edison, N.J.). The excitation wavelengths of 340 and 380 nm were used with an emission wavelength of 510 nm. The balance and intensity of the light emission were calibrated by using distilled water and a population of untreated PMNs. An aliquot of 2×10^6 PMNs were suspended in 2 ml of HBSS–1 mM EGTA at 37°C and placed in an acrylic cuvette (Sarstedt Inc., Newton, N.C.) containing a magnetic flea stir bar.

The fluorescence of individual PMNs was evaluated by the method described by McLeish et al. (21) with a Nikon Diaphot inverted microscope equipped with epifluorescence optics. Briefly, fura-2-loaded PMNs were allowed to adhere to a glass coverslip for 2 h at 4°C. The adherent cells were exposed first to HBSS, then to 10 or 100 ng of LPS per ml, and finally to 10^{-3} M fMLP at room temperature. The excitation and emission wavelengths were set as previously described. By means of the 340/380 nm ratio and a previously established standard curve, $[Ca]_i$ was determined.

To evaluate the expression of CD11b/CD18, 10^6 PMNs were incubated in DPBS–0.1% gelatin–0.1% sodium azide (DPBS++) with the optimal quantity or phycoerythrin-conjugated anti-CD11b monoclonal (clone 2LPM19c) antibody (DAKO, Carpinteria, Calif.) for 30 min at 4°C in a final volume of 200 μl (15). The optimal amount of antibody was approximately 1 μg/10^6 cells. Nonspecific antibody binding to the Fc receptor was blocked by preincubating the cells in 10 μg of human gamma globulin. Isotype-matched, phycoerythrin-labeled monoclonal antibodies with no known specificity for human cells were used as negative controls. After incubation, cells were washed with DPBS++ and then pelleted by centrifugation at 500 × *g* for 5 min. The PMNs were fixed in 500 μl of 1% paraformaldehyde solution and stored at 4°C in the dark.

CD11b immunofluorescence studies were performed on an Epics 753 flow cytometer (Coulter, Hialeah, Fla.) by using the 488-nm line of an argon ion laser (2). Electronic gates (bit maps) were set to include granulocytes but exclude mononuclear cells, platelets, debris, dead cells, and any remaining erythrocytes. At least 10,000 events were counted within the gated region. The orange fluorescence from phycoerythrin was quantified after it passed through a 575 ± 15-nm band-pass filter with linear amplification on a 256-channel scale. Microbead reference standards (Flow Cytometry Standards Corporation, Research Triangle Park, N.C.) were used to adjust photomultiplier tube voltages by using predetermined benchmark channels for the fluorochrome (31). Histograms were analyzed with Easy 2 software programs (Coulter Electronics).

Population studies. PMNs, stimulated with LPS, are activated over a wide range of concentrations (36). At low concentrations of LPS (<100 ng/ml), PMN activation occurs through the CD14 receptor. LPS concentrations greater than 100 ng/ml activate PMNs via mechanisms independent of the CD14 receptor. PMNs in suspension were exposed to LPS at concentrations of 1 ng/ml, 10 ng/ml, 100 ng/ml, or 1 μg/ml. These LPS concentrations were used to differentiate between $[Ca]_i$ changes associated with CD14-mediated activation and other, non-CD14-mediated pathways. At each concentration tested, the addition of LPS to fura-2-loaded PMNs did not result in an increase in the 340/380 nm ratio (Fig. 1).

These results suggested that LPS did not cause an increase in $[Ca]_i$ through the release of intracellular calcium stores; however, the role of plasma membrane calcium channels still required evaluation. To investigate these channels, 2.5 mM $CaCl_2$ was added to the extracellular buffer at least 3 min prior to stimulation. The calcium-treated PMNs subsequently were stimulated with LPS, but again no detectable change in the 340/380 nm ratio was observed (Fig. 2A).

The positive control was fMLP, a known chemotactic and activating peptide for leukocytes that induces an increase in PMN $[Ca]_i$ after interaction with its receptor (23, 30). Upon stimulation of fura-2-loaded PMNs with 1 μM fMLP, there was an increase in the 340/380 nm ratio within seconds, reflecting an increase in $[Ca]_i$ (Fig. 1E). The increase in $[Ca]_i$ was potentiated with the addition of 2.5 mM $CaCl_2$ to the extracellular buffer (Fig. 2B). These results are consistent with those of previously published studies.

The addition of manganese (Mn) to the extracellular medium quenches extracellular fura-2 fluorescence as well as the intracellular fluorescence associated with the influx of calcium from the extracellular medium (22). In this study the addition of 1 mM Mn to the extracellular media after LPS stimulation had little effect on the fura-2 fluorescence (Fig. 3). The addition of ionomycin (5 μM), a calcium and Mn ionophore, to the media caused a significant decrease in the fluorescence observed, indicating that the fluorescence present in the system was due specifically to intracellular calcium. These results indicate that there was minimal fluorescence due to extracellular fura-2 and calcium and that there was no influx of calcium into the cells after LPS stimulation.

Individual PMNs. It has been proposed that, in monocytes and PMNs, LPS induces a heterogeneous increase in $[Ca]_i$ when the cells are measured individually (16, 40). Both groups hypothesized that the changes in $[Ca]_i$ are not detected in population studies because the $[Ca]_i$ changes are heterogeneous and therefore appear to be random variations in background fluorescence.

The $[Ca]_i$ of individual fura-2-loaded PMNs was determined by fluorescent microscopy after stimulation of the cells with LPS and fMLP. Over 40 PMNs were individually observed in this fashion. A representative sample of six PMNs is presented (Fig. 4). The study PMNs initially were exposed to HBSS-EGTA buffer as a negative control, and PMNs were then exposed to LPS at a concentration of either 10 ng/ml or 1 μg/ml. Again, there was no change in the 340/380 nm ratio. A subsequent addition of fMLP induced an increase in $[Ca]_i$.

Calcium and CD11b/CD18 expression. LPS and fMLP stimulation of PMNs normally causes an increase in the expression of the integrin CD11b/CD18 (18, 34, 37). To investigate the

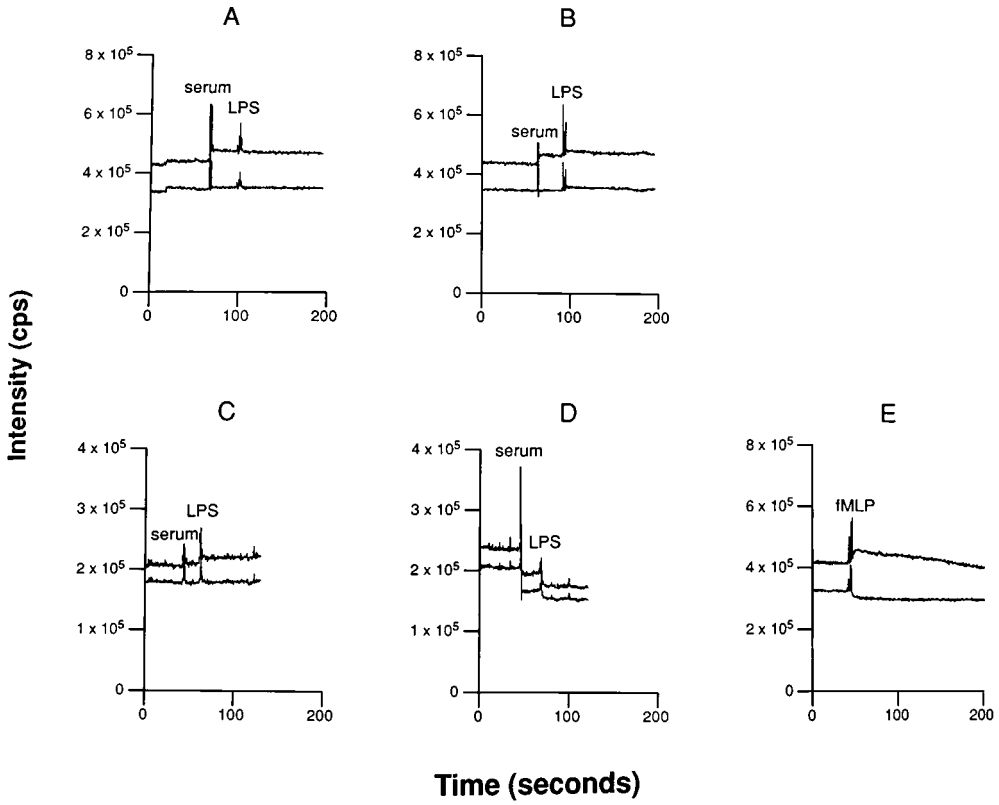

FIG. 1. Changes in 340- and 380-nm fluorescent emission from a population of fura-2 loaded PMNs after stimulation with LPS and fMLP. LPS was added at 1 ng/ml (A), 10 ng/ml (B), 100 ng/ml (C), or 1 μg/ml (D) or 10^{-6} M fMLP (E) was added to 2×10^6 PMNs in 2 ml of HBSS–1 mM EGTA with 1% AB serum at 37°C. Each record is representative of a triplicate experiment.

role of extracellular calcium in CD11b/CD18 upregulation, PMNs in whole blood were incubated with LPS (1 ng/ml to 10 μg/ml; $n = 5$) in the presence and absence of normal serum calcium. The increase in PMN CD11b receptor expression after LPS stimulation was not dependent upon free extracellular calcium, since the addition of EGTA (1.5 mg/ml) had no effect upon CD11b levels (Fig. 5).

To test the possible role of $[Ca]_i$, thapsigargin, a calcium pump inhibitor (11), was used to deplete PMN intracellular calcium stores. It has previously been reported that thapsigargin can inhibit the increase in PMN $[Ca]_i$ secondary to fMLP stimulation (7). PMNs were stimulated with maximal doses of fMLP (10 nM) and LPS (10 μg/ml) after a 10-min incubation with thapsigargin (100 nM) and EGTA (2.5 mM). The expression of CD11b was quantified by flow cytometry. The thapsigargin dose selected did not stimulate the PMNs to express CD11b itself (CD11b mean channel fluorescence [MCF] ± standard error of the mean, 410 ± 24 for untreated versus 322 ± 25 for thapsigargin-treated PMNs) but was sufficient to significantly inhibit the fMLP-induced change in CD11b expression (CD11b MCF, 1,319 ± 61 for fMLP-treated versus 744 ± 16 for fMLP-thapsigargin-treated PMNs; $P < 0.05$ by

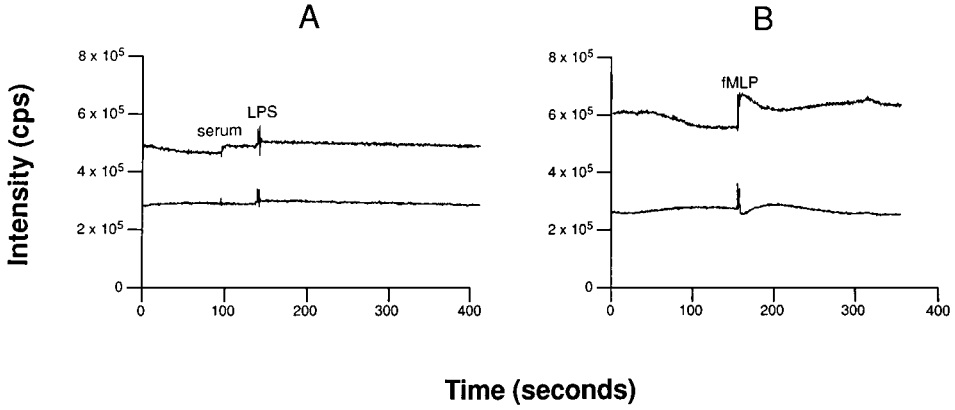

FIG. 2. Effect of extracellular calcium on fura-2 response to LPS and fMLP. LPS (10 ng/ml) (A) or fMLP (10^{-6} M) (B) was added to 2×10^6 PMNs in 2 ml of HBSS–1 mM EGTA with 1% AB serum and 2.5 mM $CaCl_2$. Each record is representative of a triplicate experiment.

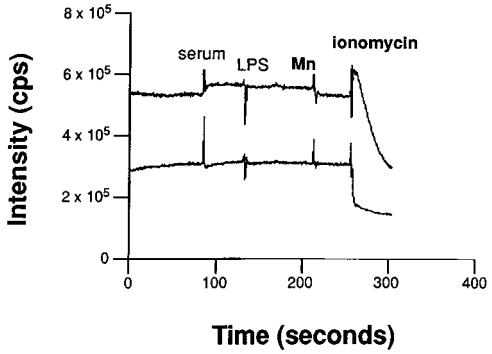

FIG. 3. Quenching of PMN 340- and 380-nm fura-2 fluorescence with manganese (Mn^{2+}). The addition of 2.5 mM $CaCl_2$ to 2×10^6 PMNs in 2 ml of HBSS–1 mM EGTA was followed by the sequential additions of 1% AB serum and 10 ng of LPS per ml, 5 μM ionomycin, and finally 1 mM Mn^{2+}.

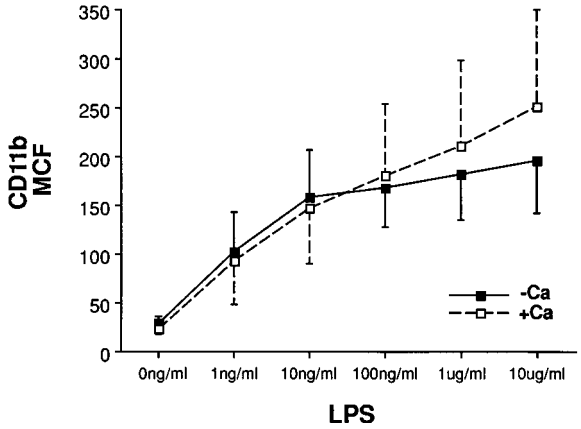

FIG. 5. Effect of extracellular calcium on PMN CD11b/CD18 expression in response to LPS stimulation. Two hundred units of heparin (20 U/ml) or 15 mg of EGTA (1.5 mg/ml) were added to 10 ml of venous blood from healthy individuals to provide anticoagulated whole blood which did or did not contain free extracellular calcium. LPS (1 ng/ml to 10 μg/ml) was added to 900 μl of whole blood and incubated at 37°C for 30 min. CD11b receptors were labelled with phycoerythrin-conjugated anti-CD11b monoclonal antibodies and measured by flow cytometry. The data are presented as means ± standard errors of the means ($n = 5$).

repeated measures analysis of variance; $n = 4$). However, for PMNs stimulated with LPS after treatment with thapsigargin, the increase in CD11b expression was equivalent to that of PMNs not treated with thapsigargin (CD11b MCF, 1,226 ± 66 versus 1,068 ± 45 SEM). Together these data suggest that changes in neither intra- nor extracellular calcium concentration are involved in LPS-mediated upregulation of PMN CD11b/CD18 expression.

The purpose of this study was to further investigate the role of $[Ca]_i$ as a possible mechanism for LPS signal transduction in PMNs. Our results demonstrate that LPS stimulation does not increase $[Ca]_i$ in normal human PMNs. In both PMN population and single-cell experimental models, the PMNs were stimulated with either LPS or, as a positive control, fMLP. In both population and individual-cell studies, fMLP caused an increase in $[Ca]_i$, indicating that the cells were able to respond to an appropriate stimulus and that the systems for measuring $[Ca]_i$ were capable of monitoring $[Ca]_i$. The role of extracellular and intracellular calcium in LPS stimulation was also evaluated by using CD11b expression as a functional marker for PMN activation. In both of these experiments calcium was not required for the expression of CD11b after LPS stimulation.

Klein et al. (14) proposed a model in which intracellular calcium was necessary for LPS activation of PMNs but in which an increase in $[Ca]_i$ alone was not sufficient to induce activation. The results of our population studies are consistent with this model. However, after the reports by Letari et al. (16) and Yee et al. (40), we felt the need to address the possibility that individual PMNs were responding to LPS in a heterogeneous pattern that was being lost in the population studies. Our results showed that there was no change observed in $[Ca]_i$ for individual PMNs after LPS stimulation. The most likely reason for the discrepancy with the results obtained by Letari et al. would be that they were examining monocytes. Differences in the dose of LPS or the period of observation cannot be used as an explanation since both were comparable with those in this study. Yee et al., using Fluo-3 and flow cytometry, demonstrated that $[Ca]_i$ increased heterogeneously in PMNs after LPS stimulation. There is a possibility of a type 2 (β) error in this study as the MCF difference with LPS stimulation was only 7.7 ± 0.6 versus 9.7 ± 1.4 with an n of 17. Differences in the calcium dye and the method of fluorescence detection offer another explanation as to why these results conflict with the results of our own study.

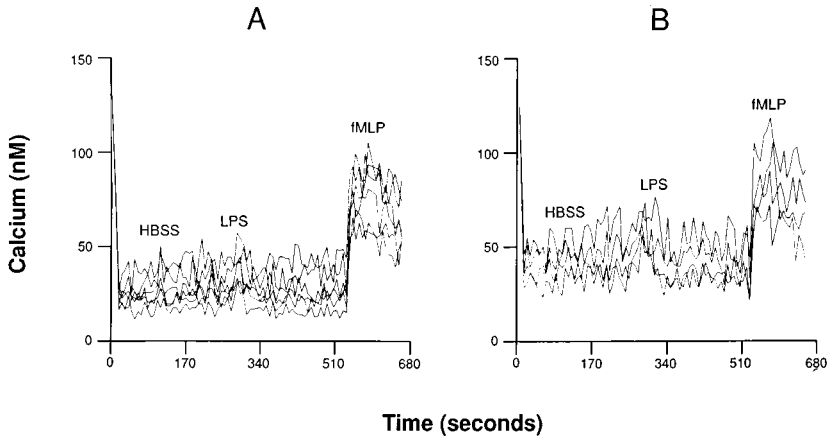

FIG. 4. Changes in 340- and 380-nm fluorescent emission from an individual fura-2-loaded PMN after stimulation with LPS and fMLP. Individual PMNs were sequentially exposed to HBSS, as a negative control, followed by LPS at 1 μg/ml (A) or 10 ng/ml (B) and finally, as a positive control, fMLP at 10^{-4} M.

We conclude that the second-messenger system for the LPS activation of PMNs does not include changes in $[Ca]_i$ but may involve other second-messenger pathways. It is possible that G proteins, protein kinase C, tyrosine kinase, mitogen-activated protein kinases, and the promoters AP-1 and NF-κB may all play roles in the LPS signal transduction pathway.

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