Bacterial lipopolysaccharide primes human neutrophils for enhanced release of arachidonic acid and causes phosphorylation of an 85-kD cytosolic phospholipase A2.

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Research Article

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Bacterial Lipopolysaccharide Primes Human Neutrophils for Enhanced Release of Arachidonic Acid and Causes Phosphorylation of an 85-kD Cytosolic Phospholipase A_2

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Abstract

Production of leukotriene B_4 (LTB_4) by human neutrophils (PMN) in response to different stimuli is increased after pretreatment with lipopolysaccharides (LPS). We have analyzed the steps in arachidonic acid (AA) metabolism affected by LPS by examining release of AA and its metabolites from [^{3}H]AA prelabeled PMN. Pretreatment of PMN for 60 min with up to 1 μg/ml of LPS alone had no effect, but release of [^{3}H]AA was stimulated up to fivefold during subsequent stimulation with a second agent. In the absence of LPS-binding protein (LBP), priming was maximal after pretreatment of PMN with 10 ng of LPS/ml for 60 min; in the presence of LBP maximal priming occurred within 45 min at 0.1 ng of LPS/ml and within 15 min at 100 ng of LPS/ml. Treatment of PMN with 10 ng of LPS/ml also increased uptake of opsonized zymosan by up to 60%. Phospholipids are the source of released [^{3}H]AA. No release was observed from [^{14}C]oleic acid (OA)-labeled PMN suggesting that phospholipolysis may be specific for [^{3}H]AA-labeled phospholipid pools. Cytosol from PMN primed with LPS contains two to three times the phospholipase A_2 (PLA_2) activity of control PMN, against 1-palmitoyl-[2-^{14}C]-arachidonoyl-phosphatidylcholine. This activity is Ca^{2+} dependent and dithiothreitol resistant. LPS priming is accompanied by reduced migration during SDS-PAGE of an 85-kD protein, identified as a cytosolic PLA_2. The extent and kinetics of this effect of LPS on $cPLA_2$ parallel the priming of [^{3}H]AA release, both depending on LPS concentration either with or without LBP. These findings suggest that priming by LPS of AA metabolism by PMN includes phosphorylation of an AA-phospholipid-selective cytosolic PLA_2 that is dissociated from activation until a second stimulus is applied. (*J. Clin. Invest.* 1994. 93:1583–1591.) Key words: CD14 • leukotriene B_4 • lipopolysaccharide-binding protein • mitogen-activated protein kinase

Introduction

Infections with Gram-negative organisms and septic shock remain a major cause of morbidity and mortality of hospitalized patients (1). Outer membrane LPS plays a central role in alerting the host to the presence of Gram-negative bacteria. The host responses can be beneficial, by optimizing defensive reactions, or may become excessive and self-destructive as in septic shock. In monocytes, macrophages, and PMN a variety of cell responses to LPS are mediated by CD14, a glycosyl-phosphatidylinositol-linked membrane protein. These responses are, in turn greatly amplified by first complexing of LPS to an extracellular LPS-binding protein (LBP)[1] of hepatic origin (2, 3).

The PMN is a major target for the action of bacterial LPS and plays an essential role in host defense to bacterial infection. LPS in concentrations as low as 0.1 ng/ml has been shown to "prime" PMN for enhanced production of leukotriene B_4 (LTB_4) in response to both particulate (opsonized zymosan; OZ) and soluble (A23187, PMA) stimuli (4). LTB_4 is a potent chemo-attractant (5) and promotes fluid extravasation into the interstitium by PMN-mediated increases in vascular permeability (6). The initial step in eicosanoid production is the release of arachidonic acid from the *sn*-2 position of phospholipase (PL). The enzyme(s) involved in hydrolysis have not yet been definitively identified. An 85-kD cytosolic phospholipase A_2 ($cPLA_2$) with preference for AA containing PL has been described in rabbit PMN and HL-60 cells (7, 8) and similar enzymes have been purified from macrophage-like cell lines (9–11). Several granule-associated PLA_2 have also been characterized which have an uncertain role in AA metabolism (12, 13). PLC followed by diacylglycerol-lipase with or without monoacylglycerol-lipase digestion of PL is probably of limited significance in AA metabolism by PMN (14). LTB_4 produced by 5-lipoxygenase (5LO) is the major PMN metabolite of released AA. Upregulation by LPS of either a lipolytic step and/or 5LO activity could account for the enhanced LTB_4 production found after a second stimulus.

LPS triggers increased surface expression of CR1 and CR3 (15, 16) and enhanced phagocytosis of opsonized bacteria (17) and yeast (18). Thus, increased phagocytic activity might also contribute to increased AA metabolism (LTB_4 production) triggered by a particulate stimulus.

In this report we show that priming of PMN by LPS results in preferential hydrolysis of AA containing phospholipids when a second stimulus is applied. LPS priming is both amplified and accelerated by the presence of LBP. LPS also increases phagocytosis of OZ by PMN, which might contribute to improved host defense, but does not account for the increase in AA release. Priming of PMN is accompanied by phosphorylation of an arachidonoyl-selective 85-kD $cPLA_2$ and greater DTT-resistant, Ca^{2+}-dependent, arachidonoyl-selective PLA_2

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1. *Abbreviations used in this paper:* CE, cholesterol ester, cPLA, cytosolic phospholipase A_2; LBP, LPS-binding protein; LT, leukotriene; MG, monoglyceride; OZ, opsonized zymosan; PE, phosphatidyl ethanol-amine; PI, phosphatidylinositol; PKC, phosphokinase C; PL, phospholipase; PS, phosphatidyl serine; TG, triglyceride.

activity in cytosolic fractions, suggesting that phosphorylation of this $cPLA_2$ may contribute to the enhanced release of AA and production of LTB_4 by primed PMN.

Methods

Materials. Special reagents used are as follows: sterile pyrogen- and, preservative-free heparin (porcine intestine), (Squibb-Marsam, Cherry Hill, NJ), Pyrogen-free dextran, mol wt range 200,000–300,000 (United States Biochemical Corp., Cleveland, OH), HBSS (GIBCO, Grand Island, NY), [^{3}H]AA 60–100 Ci/mmol, L-α-1-palmitoyl-[2-^{14}C]arachidonoyl-phosphatidylcholine 40–60 mCi/mmol, [^{14}C]oleic acid (OA) 40–60 mCi/mmol (Dupont-New England Nuclear, Boston, MA), Ficoll-Hypaque, (lymphocyte separation media; Organon Teknika Corp., Durham, NC), HSA (Armour Pharmaceutical Co., Kanakee, IL), LPS from *Salmonella minnesota* (Re595) (List Biological Laboratories, Campbell, CA), zymosan, FITC, FMLP, ionophore A23187, PMA, α-phorbol-diacetate, PGB_2, L-α-1-palmitoyl-2-arachidonoyl-phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, aprotonin, chymostatin, pepstatin, PMSF, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), iodoacetamide and fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO), HPLC grade ethanol (Aldrich Chemical Co., Milwaukee, WI), HPLC water (J. T. Baker Inc., Phillipsburg, NJ), methyl-formate redistilled (19) (Eastman-Kodak Co., Rochester, NY), HPLC grade methanol and acetonitrile (Burdick-Jackson, Muskegon, MI), LTB_4, LTB_4-OH, and LTB_4-COOH standards (BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-CD14 mAb (MY4) and isotype control mAb (IgG_{2B}) (Coulter Corp., Hialeah, FL), protein-A-sepharose (Pharmacia, Uppsala Sweden), alkaline phosphatase (Boerhinger Manheim Biochemicals, Indianapolis, IN). mAb 1,1,1 against $cPLA_2$ and rabbit polyclonal antiserum against $cPLA_2$ were obtained as previously described (20). LBP was provided by Drs. Peter Tobias and Richard Ulevitch (Scripps Institute, LaJolla, CA).

Laboratory glassware and sonicator micro-tips were baked in dry heat for 4 h at 180°C to eliminate contaminating LPS. All reagents and buffers were determined to be LPS free by a chromogenic limulus lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD) sensitive to 10 pg/ml of United States standard endotoxin.

Preparation of neutrophils. Neutrophils were isolated from heparinized venous blood of normal volunteers by standard techniques as previously described (4, 21). The blood was mixed with 3% dextran at a 1:2 ratio and allowed to stand for 20 min followed by Ficoll-Hypaque centrifugation at 250 *g* (model IEC Centra 7R; International Equipment Co., Needham Hts., MA) for 30 min at 18°C. The cell pellet was subjected to hypotonic lysis to remove contaminating red cells then hypertonic saline to achieve isotonicity and was resuspended in HBSS (−) at the desired concentration. The final suspensions consisted of > 98% neutrophils, > 98% of which excluded trypan blue. Platelet contamination was < 0.5%.

Labeling of PMN with [^{3}H]AA or [^{14}C]OA. [^{3}H]AA or [^{14}C]OA, 4 × 10^4 cpm/10^6 PMN in ethanol were dried under N_2. PMN were added and incubated in a shaking bath at 37°C for 30 min. The cells were then washed three times with HBSS(−) containing 1.5% HSA and incubated for an additional 10 min to chase unincorporated labeled-FFA into ester positions. HSA was added to a final concentration of 1.5%.

Preparation of endotoxin. Highly purified protein-free LPS extracted from *S. minnesota* Re595 mutant was prepared as a stock solution (1 mg/ml) in sterile, pyrogen-free water and sonicated as previously described (4).

Neutrophil priming by LPS. 250 μl of a neutrophil suspension (2.0 × 10^7/ml) in HBSS(−) with or without LPS was incubated in a shaking water bath at 37°C for 45–60 min in most experiments or for varied times as indicated (4, 21). After the priming incubation > 95% of the neutrophils excluded trypan blue.

Preparation of stimulants. A stock solution of OZ was prepared by incubating zymosan with fresh human serum at 37°C for 20 min and resuspending in 0.9% NaCl at 50 mg/ml after four washes. FITC-labeled OZ was prepared by incubating zymosan in FITC (1 mg/ml) in 0.05 M sodium carbonate buffer for 10 min at 20°C and washing three times before opsonization. Stock solutions of calcium ionophore A23187 were stored in ethanol, PMA, and α-phorbol diacetate in DMSO and FMLP in PBS at −70°C. The stimulants were diluted in HBSS(−) on the day of use with the maximal final concentration of ethanol or DMSO not exceeding 0.05%.

Stimulation of primed cells for LTB_4 generation. After incubation of the cell suspension with LPS, calcium and magnesium salts were added to a final concentration equivalent to that present in HBSS(+). The stimulant was then added, and the cell suspension incubated for the desired time. Incubations were terminated with the addition of 1.5 vol of iced HPLC grade ethanol containing PGB_2 (200 ng) as an internal standard and then refrigerated at −20°C. LTB_4 and metabolites were extracted using the method of Shak (19) and quantitated by HPLC as previously described (4, 19). A Waters chromatography system (Millipore Corp., Milford, MA) was used with dual pumps (model 510), an autosampler (WISP model 712), a variable wavelength UV detector (model 481) at 270 nm, an Ultrasphere C18 column (4.7 mm × 250 mm; Beckman Instruments, Inc., Palo Alto, CA) with 5-μm particles, and a (4.7 × 45 mm) precartridge. Chromatogram plots and integrations for peak areas were generated using a Waters model 746 Data module. Concentrations of LTB_4 and metabolites were obtained from the integrated areas by comparison to standards run under identical conditions. The limit of detection of the assay is 1.0 ng of LTB_4.

Determination of the release of [^{3}H]AA or [^{14}C]OA. After incubation the cells were either sedimented by centrifugation at 25,000 *g* for 2 min and a portion of the supernatant removed for counting in a liquid scintillation counter (Beckman LS500TD) or the whole-cell suspension was extracted and the FFA were separated from other lipid species by column chromatography as described below. The identical results were obtained by the two methods and therefore experiments were routinely analyzed by the first method. 2–3% of total radiolabeled FFA remained in the supernatant following the labeling procedure described above and an additional 0.5–1.0% radiolabeled FFA accumulated in the supernatant during subsequent incubations of unprimed, unstimulated PMN in the presence of 1.5% HSA. The percentage of radiolabeled FFA released from esterified lipids, in response to LPS and or subsequent stimulation, was determined as [cpm (supernatant *x*) − cpm (supernatant of unstimulated cells)/(cpm (total) − cpm (supernatant of unstimulated cells))] × 100.

Determination of the [^{3}H]AA or [^{14}C]OA content of PMN phospholipids. Total lipids in the PMN suspensions were extracted according to the method of Bligh and Dyer (22). Lipid classes were separated using the solid-phase method of Kaluzny et al. (23) and Bond Elut aminopropyl cartridges (Analytichem International, Harbor City, CA). The extract was fractionated into FFA, phospholipid, cholesterol ester (CE), triglyceride (TG), diglyceride (DG), and monoglygeride (MG) and each fraction was counted. The CE fractions contained no radioactivity. 95% of total counts were recovered with this technique. To confirm selectivity of this separation, extracts were also fractionated by TLC on a silica gel G plate (Analtech, Newark, DE) with ether/petroleum ether/acetic acid (20:80:1). Parallel samples were resolved by two-dimensional chromatography on silica gel G HPTLC plates (10 × 10 cm, E. Merck, Gibbstown, NJ) using the method of Rouser et al. (24) to determine whether label was released from specific phospholipid classes. After drying of the plates the spots were visualized in I_2 vapor and scraped for counting. This method gives good separation of phospholipid classes including phosphatidylinositol (PI) from phosphatidylserine (PS). In preliminary experiments PS, phosphatidic acid and phosphatidyl glycerol combined contained < 2% of the incorporated label, and released none. In subsequent analyses these PL fractions were ignored.

Determination of PMN phagocytosis of OZ. After incubation of PMN with OZ the reaction was stopped by immersion in an ice bath and 10 μl of the cell suspension was removed for cytospin preparation of standard microscope slides (cytospin 2, Shandon Inc., Pittsburgh,

PA) which were Wrights' stained and examined under high power. At least two samples of 100 PMN were counted and if the counts were not within 5% of each other an additional sample of 100–200 cells counted. Random groups were independently examined by a second observer unfamiliar with the results of the first. FITC-labeled OZ was used in some experiments and examined by fluorescence microscopy after trypan blue staining to distinguish adherent from phagocytosed particles.

Cytosolic PLA_2 assays. PMN were pelleted and resuspended in cavitation buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and an antiprotease cocktail of leupeptin 100 μg/ml, pepstatin 100 ng/ml, aprotinin 100 μg/ml, TPCK 100 μg/ml, PMSF 2 mM, benzamidine 100 μM, iodoacetamide 1 mM, and chymostatin 100 μg/ml. The cell mixture was pressurized to 1,000 psi in a cell disruption bomb (Parr Instruments, Moline, IL) for 10 min and collected through the release valve. Cytosol was collected after centrifugation at 100,000 *g* for 30 min.

1-palmitoyl-[2-^{14}C]arachidonoyl-phosphatidylcholine and purified [^{3}H]OA-labeled phosphatidylethanolamine (PE) was dried under N_2. The labeled PE was extracted from *Escherichia coli* grown in the presence of [^{3}H]OA, which results in near-quantitative incorporation of unsaturated fatty acids into the *sn*-2 position of phospholipids (25). The phospholipid mixture was resuspended to a final concentration of 20 μM [^{14}C]AA-PC (2×10^4 cpm) and 20 μM [^{3}H]OA-PE (2×10^4 cpm) in 150 μl of 20 mM Hepes (pH 7.4) containing 200 μM Triton X-100, 250 μg/ml fatty acid-free BSA, and 70% (vol/vol) glycerol, and then sonicated to form mixed micelles (9, 26). 100 μl of PMN cytosol (4×10^6 PMN), 2 mM DTT, and 5 mM $CaCl_2$ was added and the mixture incubated at 37°C for 30 min in a shaker. The reaction was stopped by the addition of 500 μl methanol containing 25 μg FFA, and lipids were extracted by the method of Bligh and Dyer (22). The FFA fractions were recovered after TLC on silica-gel G plates (Analtech) and ether/petroleum ether/acetic acid, 20:80:0.1. The spots were visualized in I_2 vapor, scraped, and counted in a scintillation counter.

Immunoprecipitation and immunoblot of $cPLA_2$. PMN cytosol was prepared as above and incubated overnight at 4°C with mAb 1,1,1 raised against $cPLA_2$ from U937 cells (20). Protein A-sepharose was added for an additional 2 h, centrifuged, and washed three times followed by addition of SDS sample buffer for SDS-PAGE. Samples were electrophoresed on a SDS/7.5% polyacrylamide gel for 2 h longer than the time required for the solvent front to elute (27). Western transfer to nitrocellulose was accomplished overnight at 40 V at 4°C followed by immunoblot using polyclonal rabbit serum raised against $cPLA_2$ from U937 cells (20) and the ECL detection system (Amersham Corp., Arlington Hts., IL). Granule-free membrane fractions were prepared as previously described (28).

Results

Pretreatment of PMN with LPS increases AA release stimulated by OZ. To examine the relationship between the release of AA and the LPS-mediated increased production of LTB_4, PMN prelabeled with [^{3}H]AA were incubated with or without LPS before addition of OZ. In response to LPS alone, PMN released neither [^{3}H]AA nor LTB_4 (4). However, both release of [^{3}H]AA and production of LTB_4 were stimulated upon subsequent activation of the PMN with OZ (Fig. 1). Preincubation with as little as 1 ng/ml LPS resulted in increased [^{3}H]AA release with further increases at higher LPS concentrations, reaching a maximum at 10 ng/ml (Fig. 1 *A*). The priming effect of LPS was evident over a broad range of OZ concentrations (not shown). Maximal priming effects of LPS on both [^{3}H]AA release and LTB_4 production required 45–60 min of preincubation of PMN with LPS before addition of OZ (Fig. 1 *B*). In these (and all subsequent) assays, albumin (1.5%) was added to maximize recovery of released AA. Net recovery of [^{3}H]AA and ^{3}H-metabolites was 200–300% greater in the presence of albumin although albumin partially inhibited (20–45%) metabolic conversion of AA to LTB_4. Whereas AA release peaked within 10 min of addition of OZ, leukotrienes (measured radiochemically and by mass) were not detected before 10 min of incubation and continued to accumulate until 30 min (Fig. 1 *C*). PMN labeled with [^{14}C]OA instead of [^{3}H]AA and then challenged with OZ did not release ^{14}C-products from either LPS-primed or control cells but did produce LTB_4 as assessed by UV absorption-mass detection. Thus, LPS

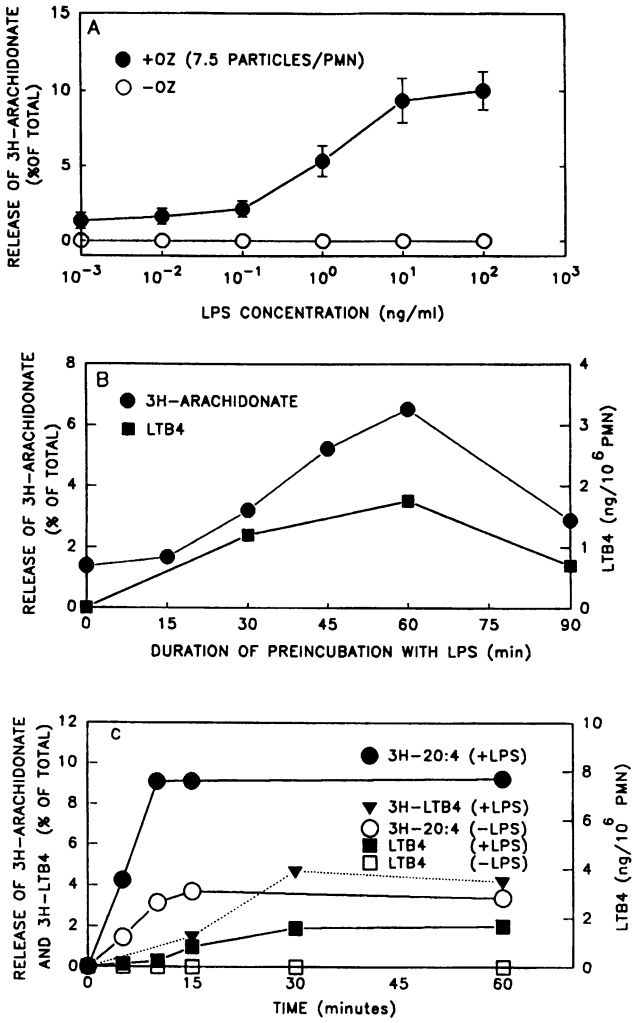

Figure 1. LPS priming of PMN results in enhanced [^{3}H]AA release in response to opsonized zymosan. (*A*) Effect of preincubating PMN with varying concentrations of LPS. (*B*) Effect of the duration of preincubation of PMN with LPS (10 ng/ml). (*C*) Kinetics of [^{3}H]AA release and LTB_4 production by control and LPS-primed (10 ng/ml) PMN stimulated with OZ. [^{3}H]AA-labeled Human PMN were incubated with *S. minnesota* Re595-LPS for 60 minutes (*A* and *C*) or varied times (*B*) and then stimulated with opsonized zymosan (4.5 particles/PMN) for 15 min (*A* and *B*) or for varying times (*C*) in the presence of 1.5% albumin. The reaction was stopped by placing the tubes on ice and the cells were pelleted by centrifugation at 25,000 *g*. The supernatant is collected and the percentage of total [^{3}H]AA released was quantitated as described in Methods. LTB_4 was determined by HPLC-A270 after extraction on C18 cartridges (4, 19). [^{3}H]LTB_4 was determined as the ^{3}H fractions coeluting with LTB_4, LTB_4-OH, or LTB_4-COOH. n = 8, in duplicate; Error bars indicate+SEM.

primes in PMN, a hydrolytic event that preferentially targets lipids labeled with [^{3}H]AA.

Role of LBP and CD-14 in LPS priming of PMN. Since LBP amplifies the LPS signal to cells (3, 15, 29), the effect of LBP on LPS priming of PMN release of [^{3}H]AA and production of LTB_4 was examined. Addition of LBP to the cell suspension before LPS, elicited a maximal priming response at levels of LPS as low as 0.1 ng/ml, i.e., a 100–1000-fold increase in the sensitivity of the PMN to LPS priming (Fig. 2). LBP alone did not prime PMN. With increasing LPS concentrations, LBP progressively reduced the time of preincubation needed to achieve maximal stimulation of [^{3}H]AA release after addition of OZ (from 60 min without LBP to ≤ 15 min with LBP, 0.75 μg/ml and 100 ng LPS/ml; Fig. 2). The transient nature of LPS-priming was observed at all doses of LPS (with and without LBP) (Figs. 1 and 2). The effects of LBP on LPS-priming of LTB_4 production parallel those on [^{3}H]AA release (not shown). LPS priming (with and without LBP) was completely inhibited when a neutralizing monoclonal antibody to CD14 was added before LPS but not when this antibody was added 5 min after LPS (with and without LBP) (Fig. 3). Unrelated, isotype-matched control monoclonal antibody had no inhibitory effect. Mabs altered the effect of OZ on PMN and PMA was therefore used as the second stimulant for these studies.

LPS stimulates uptake of OZ by PMN. Ingestion of bacteria by PMN is increased by pretreatment with LPS (17). The stimulated [^{3}H]AA metabolism triggered by addition of OZ stimulation as a second stimulus after LPS priming might therefore be secondary to enhanced phagocytic activity.

PMN pretreated with LPS (10 ng/ml) ingested more OZ than untreated PMN over a wide range of OZ concentrations (Fig. 4). Uptake of OZ both in the presence and absence of LPS was complete within 5 min (not shown). Uptake of unopsonized zymosan is < 25% of OZ and was not increased by pretreatment with LPS (not shown). That increased uptake represented ingestion rather than adherence was verified as described in Methods by fluorescent microscopy of LPS-pretreated and control PMN after incubation with FITC-labeled OZ.

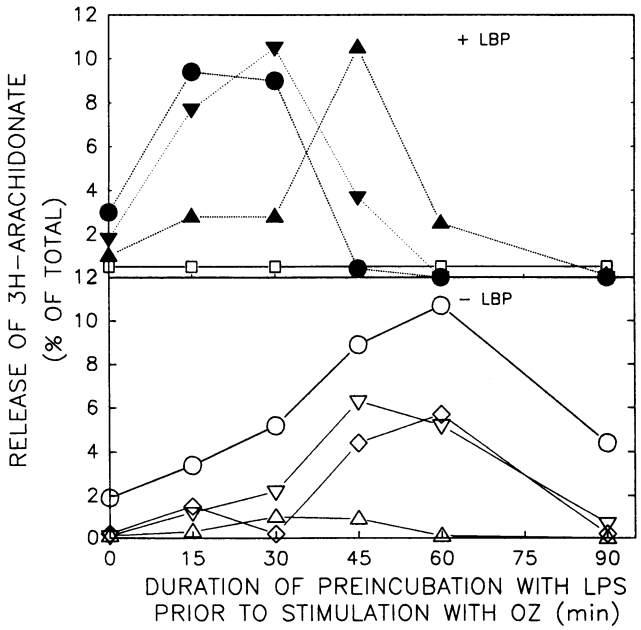

Figure 2. LBP enhances and accelerates the effect of LPS-priming on PMN metabolism of [^{3}H]AA. [^{3}H]AA-labeled PMN were incubated in the presence of LPS as in Fig. 1, for the time shown. The closed symbols represent LBP = 0.75 μg/ml + LPS and open symbols represent LPS alone. LPS = 0 (■), LPS = 0.1 ng/ml (▲), LPS = 1 ng/ml (♦), LPS = 10 ng/ml (▼), LPS = 100 ng/ml (●). PMN were then stimulated with OZ (4.5 particles/PMN) and the release of [^{3}H]AA was determined as in Fig. 1. LBP alone does not effect PMN release of [^{3}H]AA or the response to OZ.

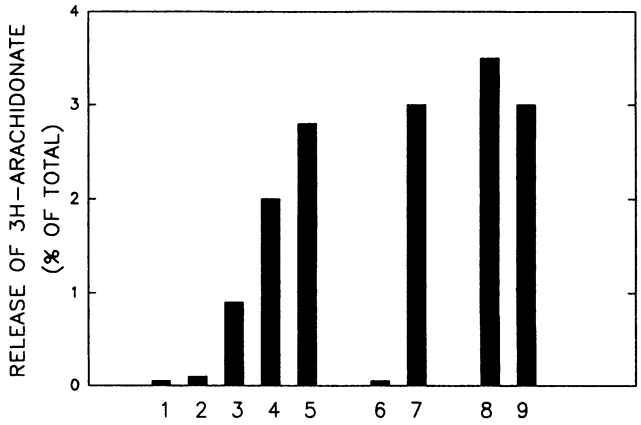

Figure 3. Anti-CD14 mAbs block LPS priming of PMN only if added before LPS. [^{3}H]AA labeled PMN were incubated with LPS for 45 min at 37°C as in Fig. 1. Column *1*, LPS = 0; *2*, LPS = 0.1 ng/ml; *3*, LPS = 1.0 ng/ml; *4*, LPS = 10 ng/ml; and *5–9*, LPS = 100 ng/ml. Anti-CD14 mAbs (MY4, 2.5 μg/ml) were added to PMN before LPS (*6*) or 5 min after LPS (*7*). Isotype control Mabs (IgG_{2B}, 2.5 μg/ml) were added prior to LPS (*8*) or 5 min after LPS (*9*). PMN were then incubated with PMA 0.03 μg/ml for 15 min and the release of [^{3}H]AA quantitated as in Methods. The results of a single experiment, in duplicate, representative of three separate experiments is shown.

In contrast to the requirement for preincubation with LPS for the priming of [^{3}H]AA and LTB_4 release, no preincubation was necessary for the LPS effect on uptake of OZ, nor was stimulation diminished by longer preincubation with LPS (Fig. 4 *B*). The differences in the magnitude (Fig. 4, *A* and *C*) and time dependence of these two effects of LPS suggest that priming of AA metabolism includes effects on the cellular AA metabolic machinery that are distinct from effects on phagocytosis. Moreover, LPS priming of [^{3}H]AA release was also observed with non-phagocytic (soluble) stimuli including the Ca^{2+} ionophore A23187 and PMA (Table I). In contrast to OZ and A23187 as stimuli, PMA triggered [^{3}H]AA release but no production of [^{3}H]LTB_4. Mass determination of AA release by gas chromatography of control and PMA stimulated PMN, with and without LPS priming, confirmed the results shown with [^{3}H]AA, i.e., LPS alone had no effect on AA release and priming resulted in a two- to threefold increase in AA release from PMA (1 μg/ml) stimulated PMN. α-phorbol-diacetate (1 μg/ml) treatment of PMN results in no release of [^{3}H]AA from control or LPS-primed PMN (not shown). Another stimulus of many PMN responses, FMLP, stimulated little or no [^{3}H]AA release (and no production of LTB_4) from either control or LPS-primed PMN (Table I).

Released AA stems from phospholipid. The source of [^{3}H]AA released from LPS-primed and unprimed PMN was determined by separating extracted lipids, before and after stimulation with OZ or PMA. Table II shows the distribution of

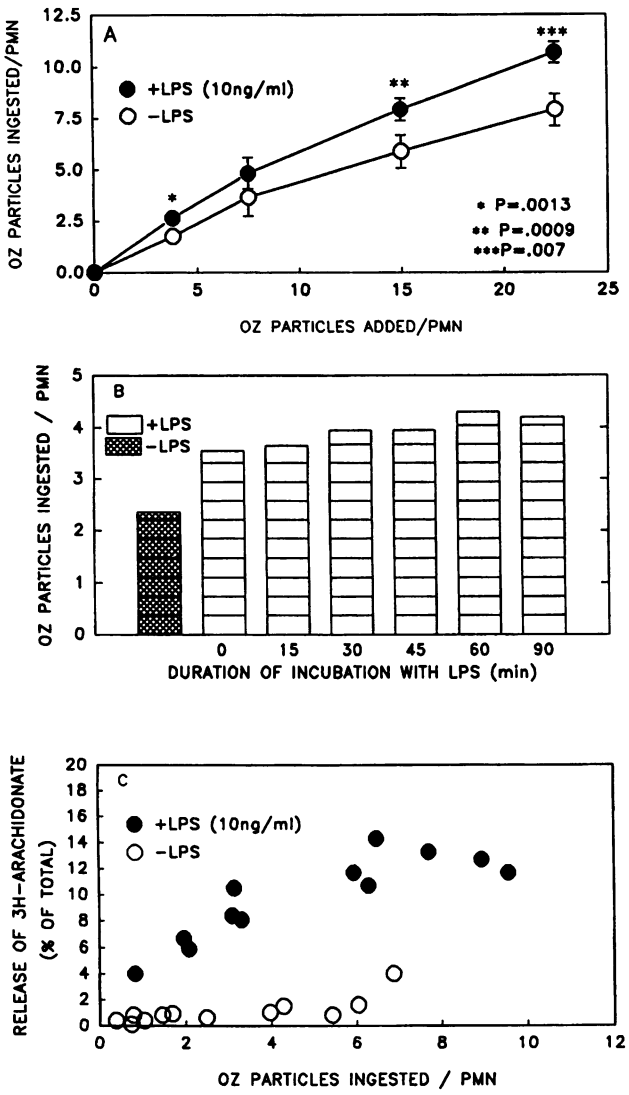

Figure 4. PMN exposed to LPS ingest greater amounts of OZ particles. Uptake of OZ was determined by light microscopy after cytospin and staining with a modified Wright's stain (Dif-Quik). Cell-associated OZ was confirmed as intracellular by fluorescent microscopy of fluoroscein-isothiocyanate labeled OZ in the presence of trypan blue dye which quenches the fluorescence of extracellular OZ. Uptake of OZ by both LPS-primed and control PMN reaches a maximum within 5 min (not shown). PMN were incubated with LPS for 60 min (*A* and *C*) and then stimulated with opsonized zymosan (4.5 particles/PMN; *B* and *C*) for 15 min. The release of [^{3}H]AA (*C*) was determined as in Fig. 1. n = 8 in duplicate, for *A* and *B*, Error bars =±SEM. Panel *C* is a single experiment in duplicate, representative of three separate experiments.

incorporated [^{3}H]AA among the main lipid classes. LPS, alone, did not affect this distribution. Approximately 50% of the incorporated [^{3}H]AA was in phospholipids, 60% of which was in PI, 25% in PC, and 15% in PE. Loss of label from primed and unprimed PMN after stimulation with OZ (or PMA, not shown) was restricted to these three phospholipid species in proportion to their relative radioactivities (and was accompanied by a corresponding accumulation of [^{3}H]AA and metabolites). Thus, the lipolytic step involved in AA release is most likely attributable to a phospholipase A_2 with preference for [^{3}H]AA vs [^{14}C]OA labeled phospholipids.

Table I. [^{3}H]AA Release by LPS-primed PMN in Response to Particulate and Soluble Stimuli

Stimulant	Percent release of total [^{3}H]AA ([^{3}H]LTB$_4$)		LTB$_4$ Production (ng/10^6 PMN)	
	−LPS	+LPS	−LPS	+LPS
No stimulant	0	0	0	0
OZ (750 μg/ml)	2.9 (0)	7.9 (2.8)	0	1.9
PMA (1 μg/ml)	16.6 (0)	26.0 (0.5)	0	0.2
A23187 (0.1 μg/ml)	11.8 (4.2)	24.4 (8.8)	8	14.6
FMLP (10^{-6} M)	0	1.7 (0)	0	0

[^{3}H]arachidonate labeled PMN were incubated±LPS (10 ng/ml) in the presence of 1.5% albumin for 60 min then stimulated for 15 min with various stimuli as shown. The release of [^{3}H]AA is determined as in Fig. 1. The means of five experiments, in duplicate, ±SEM are shown. Stimulation of [^{3}H]AA release by PMN (with or without LPS-priming) does not require extracellular calcium when PMA is used as an activating agent.

AA-selective PLA_2 activity in cytosol of PMN is increased after LPS priming. Many cells, including PMN, contain a cytosolic AA-selective PLA_2 activity (9, 30). To determine the effect of LPS priming on this activity, cytosolic fractions were isolated from PMN before and after treatment with LPS. Table III shows that cytosolic fractions of LPS-primed PMN hydrolyzed two- to threefold more 1-palmitoyl-[2-^{14}C]arachidonoyl PC than did similar fractions from unprimed cells. This activity was resistant to DTT and strictly dependent on added Ca^{2+}. No PLA_2 activity was detected against [^{3}H]OA labeled phosphatidylethanolamine, a substrate that is readily hydrolyzed by type II PLA_2s from various sources, including PMN and inflammatory fluids (13, 31–33).

Phosphorylation of cPLA$_2$ during LPS priming. These findings suggest that a $cPLA_2$ with preference for AA-containing phospholipids (27, 30) is effected during LPS-priming of PMN.

To explore this possibility further, we examined the electrophoretic mobility during SDS-PAGE of an 85-kD $cPLA_2$ recov-

Table II. Distribution of [^{3}H]AA in PMN Lipids Before and After LPS Priming and Stimulation with OZ

	Percentage of [^{3}H]AA present by lipid class			
	Treatment of PMN			
	Control	LPS	OZ	LPS;OZ
FFA	3.1±0.6	3.0±0.5	7.0±0.6*	20.3±2.0‡
PL	52.2±2.0	51.1±2.6	42.2±1.6*	30.4±1.9‡
TG	37.0±3.0	37.2±1.7	38.6±3.2	37.9±3.7
DG	2.6±0.3	2.2±0.2	3.1±0.5	3.4±0.3
MG	6.0±1.5	7.4±1.6	9.0±1.4	8.0±1.1

[^{3}H]AA-labeled PMN were incubated with or without LPS = 10 ng/ml, for 60 min and then with or without OZ for 15 min in the presence of 1.5% albumin. $CHCl_3$/MeOH extracts were prepared and the lipids separated on amino-propyl cartridges (23). The results represent the means±SEM of five experiments. * Differs significantly from column 1 ($P < 0.05$, student's paired t-test). ‡ Differs significantly from columns 1, 2, and 3.

Table III. $cPLA_2$ Activity Is Increased in Cytosolic Fractions of LPS-treated PMN

	[^{14}C]AA released
	cpm
Control PMN	402±43
PMN primed with LPS 100 ng/ml for 45 min	872±117
PMN primed with LPS 100 ng/ml + LBP for 45 min	1047±32

Cytosolic fractions of LPS treated and control PMN were collected as described in Methods. After addition of DTT (2 mM) the mixture was incubated for 15 min with 1-palmitoyl-[^{14}C]arachidonoyl-PC and [^{3}H]OA-PE in 20 mM Hepes, pH 7.4, containing 200 μM Triton X-100, 250 μg/ml BSA, and 70% glycerol (vol/vol) which had been sonicated to form mixed micelles (9). The reaction was stopped by addition of MeOH and free fatty acids were quantitated by scintillation counting after lipid extraction and TLC as described in Methods. (No ^{3}H release was detected, data not shown). Means±SEM are shown, $n = 5$ in duplicate for columns 1 and 2 and $n = 3$ in duplicate for column 3. $P < 0.002$ by students paired t-test for comparison of column 1 with column 2 or 3. Comparison of column 2 with 3, p = NS.

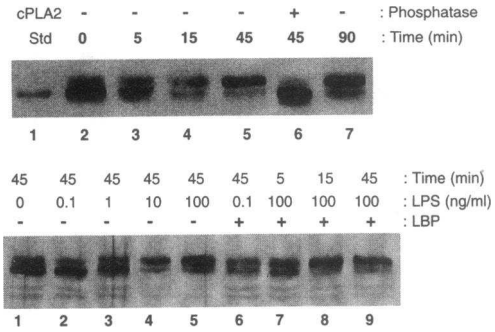

Figure 5. PMN $cPLA_2$ is phosphorylated in response to LPS. 2×10^7 PMN were incubated with or without LPS then disrupted by N_2 cavitation. PMN $cPLA_2$ was immunoprecipitated from cytosolic fractions (100,000 *g* supernatant) with Mab directed against $cPLA_2$ from U937 cells and run on 20 × 20-cm gels containing 7.5% acrylamide; 0.1% bis-acrylamide. Gels were run at 125 V through the stacking gel then 250 *v* for 5 h using the Laemmli buffer system (54). Proteins were transferred from gel to nitrocellulose by conventional procedure and reacted subsequently with polyclonal antiserum directed against U937 $cPLA_2$ and detected with the Amersham ECL detection system. The results of a single experiment are shown which are representative of four separate experiments. *Top:* Lane *1* is purified $cPLA_2$ from U937 cells (40 ng); lane *2* is the cytosolic fraction from control PMN, lanes *3–7* are cytosolic fractions from PMN treated with 100 ng LPS/ml for times as shown. Lane *6* protein was incubated for 30 min at 37°C pH 9.0 with alkaline phosphatase prior to SDS-PAGE. *Bottom:* Lane *1* is the cytosolic fraction from control PMN, lanes *2–9* are cytosolic fractions from PMN treated with varied concentrations of LPS as indicated above each lane for the times shown. The presence or absence of LBP 0.75 μg/ml (lanes *6–9*) is indicated by±above each lane (+ lanes *6–9*).

ered by immunoprecipitation from N_2 cavitates of LPS-primed and control PMN. The electrophoretic mobility of this $cPLA_2$ transfected into CHO cells is reduced when the cells are stimulated and AA release is triggered (27). This "gel-shift" is linked to phosphorylation and reversed by phosphatase treatment (27). Fig. 5 shows that LPS treatment of PMN also causes a "gel-shift" of an 85-kD protein species reactive with anti-$cPLA_2$ antiserum. This gel-shift does not occur in PMN incubated with anti-CD14 Mabs before LPS treatment (not shown). Treatment of the immunoprecipitated protein with alkaline phosphatase before electrophoresis converts the protein back to a faster migrating form (Fig. 5, *top;* lanes *5* and *6*). The extent of apparent phosphorylation of the $cPLA_2$ depends on the dose of LPS and time of LPS treatment (with or without LBP) in a manner that parallels the initial phase of priming of [^{3}H]AA release by LPS (compare Figs. 1, 2, and 5). However, in contrast to the transient effect of LPS-priming of AA-metabolism in intact PMN, the altered $cPLA_2$-gel mobility (Fig. 5, *top,* lane *7,* and *bottom,* lane *9*) and the enhanced cytosolic PLA_2 activity persist (Table III). Both $cPLA_2$ species were recovered only from cytosolic fractions (100,000-*g* supernatant) when sedimentation of PMN cavitates was carried out in 1 mM EGTA (Fig. 6; lanes *1, 2, 5,* and *6*). By contrast, both species were lost from the cytosolic fractions and recovered in purified PMN granule-free membrane fractions when these two fractions were mixed and reseparated by centrifugation in the presence of 10 μM free Ca^{2+} (lanes *3, 4, 7,* and *8*) (20, 34). At 100 nM free Ca^{2+} essentially no $cPLA_2$ associates with the membranes and at 300 nM free Ca^{2+} 80–90% is membrane associated (not shown) similar to what has been previously described with U937 cell derived $cPLA_2$ (20).

Effect of staurosporine on PMA-mediated $cPLA_2$ phosphorylation and activation of AA (with and without LPS priming) in PMN. Table I shows that PMA activates [^{3}H]AA release in PMN. In contrast, PMA primes but causes little or no direct activation of [^{3}H]AA release in CHO cells overexpressing $cPLA_2$ (35). To further explore the apparent differences in regulation of [^{3}H]AA release in these two cell types, the effects of staurosporine, a putative protein kinase C inhibitor were tested in PMN. In CHO cells, staurosporine fully inhibits the effects of PMA on both [^{3}H]AA release and phosphorylation (e.g., gel shift) (27, 35). In PMN, by contrast, staurosporine

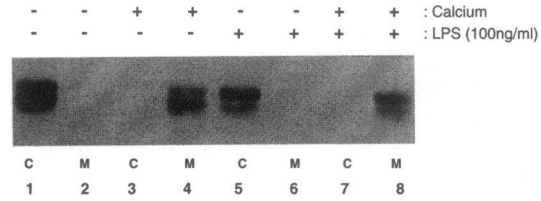

Figure 6. PMN $cPLA_2$ associates with the membrane fraction in the presence of calcium. LPS-primed and control PMN were disrupted by N_2 cavitation in the presence of 1 mM EGTA and centrifuged on a cushion of 40% sucrose at 100,000 *g* for 1 h. The cytosol was removed and the membranes (the fraction layered on the sucrose cushion) were collected, rinsed and resuspended in 50 mM Tris/HCl, pH 7.4. Membranes and cytosol were recombined and calcium was added to one-half of each sample. After centrifugation the pellet and supernatant were again separated and $cPLA_2$ was immunoprecipitated and run on SDS-PAGE as described in Methods. Lanes *1–4* are from control PMN and lanes *5–8* are from LPS-primed PMN. The presence or absence of added calcium (calculated free calcium 10 μM) is indicated with±above each lane. *C* and *M* below each lane indicate whether the sample is the cytosol or membrane fraction. No $cPLA_2$ was detected in crude membrane fractions (unfractionated cell pellet after N_2 cavitation).

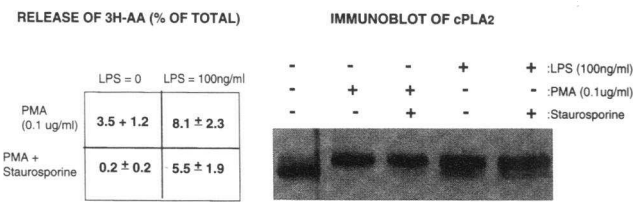

Figure 7. The effects of PMA on PMN, with or without LPS, involve staurosporine sensitive and insensitive pathways. PMN were incubated at 37°C in the presence or absence of staurosporine (1 μg/ml) for 15 min and then in the presence or absence of LPS 100 ng/ml for 45 min followed by stimulation with PMA (0.1 μg/ml) for 15 min. [^{3}H]AA release was determined as [cpm (supernatant x) − cpm (supernatant of unstimulated cells)/(CPM (total) − CPM (supernatant of unstimulated cells))] × 100 and is shown in the left panel and immunoblots of $cPLA_2$ are shown in the right panel. Lane *1* represents control PMN. PMN treated with LPS, PMA, and staurosporine are indicated with±over each lane.

inhibits PMA stimulated release of [^{3}H]AA by PMN but not the gel shift of $cPLA_2$ (Fig. 7). Moreover, even in the presence of staurosporine, pretreatment of PMN with LPS permits (near) maximal activation of [^{3}H]AA release upon addition of PMA (Fig. 7). α-phorbol-diacetate (1 μg/ml) treatment of PMN results in neither [^{3}H]AA release or the gel shift of $cPLA_2$ (not shown).

Discussion

Incubation of PMN in vitro with minute quantities of LPS provokes a range of immediate and delayed responses that reflect the primary involvement of this cell in vivo in the defense mounted by the host against invading Gram-negative bacteria and/or its envelope LPS. Among the many PMN functions elicited or amplified by exposure to LPS are generation of reactive oxygen derivatives (21) and LTB_4 (4), expression on the cell surface of receptors for components of complement and other signals (15, 16), and enhanced phagocytosis of bacteria (17). In this study we have focussed on the further analysis of the process that provides AA for the generation of the potent inflammatory mediator LTB_4.

We show that treatment of PMN with LPS alone causes neither appreciable release of [^{3}H]AA from esterified lipid pools (Fig. 1) nor production of LTB_4 or LTB_4 metabolites (4). Although the distribution in esterified lipid pools of [^{3}H]AA derived from extracellular sources differs from that of endogenous pools of esterified AA (36, 37), no release of AA from endogenous pools in PMN has been described without concomitant measurable deacylation of radiolabeled AA pools (36, 37). This was confirmed in our investigations by gas chromatography of supernatants from LPS-primed and control cells with and without PMA (not shown). The increased production by LPS-pretreated PMN of LTB_4 that is triggered by addition of OZ parallels increased release of [^{3}H]AA (Fig. 1). Under all conditions studied, the requirements for formation of LTB_4 were the same whether measured radiochemically or by mass suggesting that metabolism of [^{3}H]AA and endogenous (unlabeled) AA was similar. Dose and time requirements for LPS priming of [^{3}H]AA release and LTB_4 formation (triggered by OZ) were virtually identical suggesting that under these experimental conditions the lipolytic step governs LTB_4 formation. Apparently this is also the case in FMLP-treated PMN that do not release [^{3}H]AA and do not generate LTB_4 from endogenous AA (labeled or unlabeled) stores (Table I) but can metabolize exogenously added AA to LTB_4 (38). In contrast, PMA-treated PMN (with and without LPS priming) actively release [^{3}H]AA but little or no [^{3}H]LTB_4 (Table I), indicating that under these conditions biochemical mechanisms mediating AA release and metabolism to leukotrienes are dissociated.

PMN have long been known to contain various PLA_2 activities (12, 30, 39). One activity that is primarily associated with the cytoplasmic granules and subcellular membrane fractions (12), has been best characterized in rabbit PMN, where it has been shown to be a 14-kD type-II (nonpancreatic) PLA_2 (13). This enzyme participates in the digestion of bacterial phospholipids during phagocytosis (33), a role consistent with its presence in the granules of the PMN. In contrast, no role of this PLA_2 in the turnover of endogenous phospholipids and specifically AA-containing phospholipids has yet been established.

On the other hand, several of our findings now strongly suggest that a cytosolic PLA_2 preferring [^{3}H]AA-labeled phospholipids participates in the lipolytic event that is primed by LPS: (*a*) the apparent specificity of the lipolysis for AA-containing phospholipids; (*b*) the remarkable synchrony of the phosphorylation (gel shift) of the $cPLA_2$ and the priming for AA release; and (*c*) the similar dependence of these two sequelae of LPS pretreatment on LPS concentration both in the presence and absence of LBP (Figs. 2 and 5). The fact that LPS treatment does not activate AA release in intact PMN is consistent with recent studies of Lin et al. who have shown a similar separation of phosphorylation and activation of the $cPLA_2$ overexpressed in CHO cells (27). These investigators have proposed that while phosphorylation of $cPLA_2$ is necessary for maximal activation, hydrolytic activity is expressed only if the enzyme associates with its substrate in an additional, Ca^{2+}-dependent, step (35). As in that study, we have shown that phosphorylation of the $cPLA_2$ in PMN is associated with a two- to threefold increase in calcium-requiring hydrolytic activity of cytosolic fractions (containing all recovered $cPLA_2$) toward 1-palmitoyl-[^{14}C]arachidonoyl PC (Table III). Because LPS treatment of PMN causes little (40) or no (4) increase in Ca_i^{2+}, we speculate that activation of AA release awaits a second stimulus (e.g., OZ, A23187) that triggers increased Ca_i^{2+} (4) and, hence, Ca^{2+}-dependent translocation of the $cPLA_2$ to a membrane (substrate) site where hydrolysis occurs. Such a scheme is consistent with the inability of FMLP to activate [^{3}H]AA release (with or without LPS priming) apparently because the increase in Ca_i^{2+} triggered by FMLP is insufficient to meet the requirements for $cPLA_2$ activation (41, 42).

However, this scheme does not readily explain the potent activating effects of PMA in PMN (Table I) and the ability of LPS to further sensitize PMN to PMA. PMA treatment of CHO cells overexpressing $cPLA_2$ and LPS treatment of PMN produce similar effects: phosphorylation leading to a gel shift of $cPLA_2$ but no change in intracellular calcium; and priming but little or no activation of AA release (27). All effects of PMA on $cPLA_2$ and AA release in CHO cells are blocked by staurosporine, an inhibitor of protein kinase C (PKC), indicating that, as expected, this enzyme is the primary target of PMA action and upstream of a mitogen-activated protein kinase 2 in CHO cells (35). A different picture emerges when PMN are treated with staurosporine. In these cells this agent prevents the activation by PMA of AA release but does not block the gel-shift of

$cPLA_2$. Staurosporine also does not block the gel-shift in LPS-treated PMN. However, in contrast to the blocking by staurosporine of AA release when PMN are treated with PMA alone, PMA added subsequent to LPS treatment allows full activation of AA release (Fig. 7). These findings suggest the following: (*a*) Because PMA treatment does not mobilize Ca_i^{2+} in PMN (43, 44) the ability of PMA to activate AA release in PMN (with or without LPS priming) suggests an alternative mechanism of activation of AA release in PMN, primed by LPS, either lowering the Ca^{2+} requirement of $cPLA_2$ or perhaps involving a different lipolytic system. (*b*) The gel shift of $cPLA_2$ induced by PMA in the presence of staurosporine implies the presence of a PMA-sensitive, staurosporine-insensitive kinase in PMN but not in CHO cells. The fact that staurosporine blocks activation by PMA of AA release but not the $cPLA_2$ gel shift in PMN further suggests that PMA action in PMN involves both staurosporine-sensitive (?PKC) and staurosporine-resistant effects. The $cPLA_2$ contains multiple potential phosphorylation sites including consensus sites for PKC (20, 44) and several potential mitogen-activated protein kinase 2 sites including ser-505 (20, 35). The gel shift of $cPLA_2$ in CHO cells requires phosphorylation of ser-505 (35). Hence, the different effects of PMA on AA metabolism by CHO cells and by PMN could reflect differences in the sites of phosphorylation of $cPLA_2$ induced by PMA in these different cell types. Further, although the similar $cPLA_2$ gel shifts produced in LPS- or PMA-treated PMN and PMA-treated CHO cells strongly suggest that in PMN ser-505 of $cPLA_2$ is also phosphorylated by mitogen-activated protein kinase 2, the different effects of staurosporine on the actions of LPS and PMA alone and in combination imply additional and as yet undefined biochemical events underlying LPS (PMA-triggered) regulation of phospholipolysis.

The complexity of LPS action is widely appreciated (1). Nevertheless, recent findings in many laboratories have revealed that the diverse effects of LPS on certain host cells including PMN appear to emanate from interactions of LPS with a single membrane protein, CD14, particularly after interaction of LPS in the extracellular medium with LBP (3, 15, 46). We provide further evidence of the primary role of CD14 in LPS signaling with or without LBP. The initial engagement of LPS with CD14 is very rapid ($\leq$ 5 min even in the absence of LBP; Fig. 3) and hence the time dependence of LPS priming of AA metabolism reflects mainly post-CD14 signal transduction events presumably involving a cascade of kinase activation and phosphorylation steps. CD14 is a GPI-linked membrane protein (47). How such membrane proteins transduce extracellular signals is still a mystery but may involve associated tyrosine kinases (48). LPS action in macrophages includes priming for enhanced [^{3}H]AA release (49–51) and activation of protein tyrosine phosphorylation (52). How LBP accelerates LPS priming is not known but apparently involves increasing the rate of post-CD14 events. How LPS almost immediately increases cellular uptake of OZ is also unknown but suggests a very different mechanism of action. Finally, how the priming effects of LPS on AA metabolism are reversed (see Figs. 1 and 2) is unknown but reversal is apparently distinct from the phosphorylation event(s) that cause the gel shift and increased cell-free activity of $cPLA_2$. With tumor necrosis factor as the priming agent (53), the transient nature of priming of PMN AA metabolism is also observed with effects on $cPLA_2$ phosphorylation (gel shift) similar to LPS-priming (unpublished observations) suggesting that reversible priming is an inherent feature of complex mechanisms regulating AA metabolism ($cPLA_2$) activity in PMN.

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