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Involvement of Cytosolic Phospholipase A<sub>2</sub> and Secretory Phospholipase A<sub>2</sub> in Arachidonic Acid Release from Human Neutrophils

John Marshall,* Eric Krump,† Thomas Lindsay,* Gregory Downey,† David A. Ford,* Peihong Zhu,* Paul Walker,* and Barry Rubin**

The purpose of this study was to define the role of secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>), calcium-independent PLA<sub>2</sub>, and cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) in arachidonic acid (AA) release from fMLP-stimulated human neutrophils. While fMLP induced the release of extracellular sPLA<sub>2</sub> activity and AA, 70% of sPLA<sub>2</sub> activity remained associated with the cell. Treatment with the cell-impermeable sPLA<sub>2</sub> inhibitors DTT or LY311-727, or the anti-sPLA<sub>2</sub> Ab 3F10 all inactivated extracellular sPLA<sub>2</sub> activity, but had minimal effect on neutrophil AA mass release. In contrast, coinubcation of streptolysin-O toxin-permeabilized neutrophils with DTT, LY311-727, or 3F10 all decreased [³H]AA release from [³H]AA-labeled, fMLP-stimulated cells. Exposure to fMLP resulted in a decrease in the electrophoretic mobility of cPLA<sub>2</sub>, a finding consistent with cPLA<sub>2</sub> phosphorylation, and stimulated the translocation of cPLA<sub>2</sub> from cytosolic to microsomal and nuclear compartments. The role of cPLA<sub>2</sub> was further evaluated with the cPLA<sub>2</sub> inhibitor methyl arachidonoyl fluorophosphonate, which attenuated cPLA<sub>2</sub> activity in vitro and decreased fMLP-stimulated AA mass release by intact neutrophils, but had no effect on neutrophil sPLA<sub>2</sub> activity. Inhibition of calcium-independent PLA<sub>2</sub> with haloenol lactone suicide substrate had no effect on neutrophil cPLA<sub>2</sub> activity or AA mass release. These results indicate a role for cPLA<sub>2</sub> and an intracellular or cell-associated sPLA<sub>2</sub> in the release of AA from fMLP-stimulated human neutrophils. The Journal of Immunology, 2000, 164: 2084–2091.

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) hydrizes arachidonic acid (AA) from the sn-2 position of glycerophospholipids (1). The hydrolysis of AA by PLA<sub>2</sub> is a critical regulatory step in the development of an inflammatory response. Neutrophils (polymorphonuclear leukocytes (PMN)) release the proinflammatory mediator AA via the function of PLA<sub>2</sub> when activated by bacterial peptides, phorbol esters, calcium ionophores, and other agonists. Released AA may then be converted to biologically active metabolites such as prostaglandins, leukotrienes, lipoxins, and thromboxanes, which are thought to directly modulate the inflammatory response (2).

Multiple PLA<sub>2</sub> isoforms have been described in human neutrophils. An 85-kDa cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) that selectively hydrolyzes glycerophospholipids with AA in the sn-2 position and translocates to the nucleus in a Ca<sup>2+</sup>-dependent manner in response to agonists (3, 4) has been identified in PMN (5). Neutrophils also have Ca<sup>2+</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) activity (6). iPLA<sub>2</sub> has been identified in murine macrophages, and is thought to function in membrane remodeling in these cells (7, 8). In addition, neutrophils contain low m.w., secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) that are stored in granules (9–11). sPLA<sub>2</sub> isoforms have six to eight disulfide bridges, require micromolar to millimolar levels of Ca<sup>2+</sup> for catalytic activity (12), and do not exhibit specificity for phospholipids with AA in the sn-2 position (13–15). The catalytic activity of the low m.w. sPLA<sub>2</sub> isoforms, in contrast to cPLA<sub>2</sub> and iPLA<sub>2</sub>, is inhibited by the reducing agent DTT (15) and by the substituted indole, LY311-727 (16, 17).

The enzyme(s) that mediates AA release from activated PMN has not been completely characterized. Evidence that cPLA<sub>2</sub> directly mediates AA release includes the demonstration that cPLA<sub>2</sub> is activated in response to bacterial agonists (18), that pharmacological inhibitors of cPLA<sub>2</sub> attenuate PMN AA release (19), and that macrophages from mice in which the gene for cPLA<sub>2</sub> was disrupted exhibited decreased AA release in response to PMA and calcium ionophores (20). Evidence that sPLA<sub>2</sub> directly mediates AA release includes the observation that exogenous addition of group V sPLA<sub>2</sub> to PMN (21) or recombinant synovial PLA<sub>2</sub> to murine macrophages (21, 22) directly results in AA release. In addition, treatment with the cell-permeable sPLA<sub>2</sub> inhibitors SB203347 and Scalarinal prevented Ca<sup>2+</sup>-ionophore-stimulated AA mass release (23, 24). While these studies support a role for sPLA<sub>2</sub> in PMN AA release (23, 24), they are complicated by the...
potential inhibitory effects of SB203347 and Scalaradial on cPLA2 activity or Ca2+ metabolism (25). SPLA2 and cPLA2 both appear to function in AA release from PAF- and LPS-stimulated PMN, macrophages, and evidence has been presented in support of the concept that activation of SPLA2 at the plasma membrane is dependent on previous activation of cPLA2 (22, 26). The role of SPLA2 plays in AA release from neutrophils has not been completely defined.

In this study, we have systematically evaluated the roles of cPLA2, SPLA2, and iPLA2 in fMLP-stimulated AA release from human PMN. We present some evidence that cPLA2 and an intracellular or cell-associated sPLA2 are both involved in fMLP-stimulated AA release by human PMN.

Materials and Methods

Materials

Butylated hydroxytoluene, leupeptin, pepstatin, PMSF, fMLP, and [3H]AA were from Sigma (St. Louis, MO). Reagents for Krebs-Ringer-phosphate (KRPD) buffer (KRPD), including NaCl, KCl, NaH2PO4, K2HPO4, MgCl2, and CaCl2, were obtained from Mallinckrodt (Paris, KY). [3H]AA (sp. act., 180 Ci/mmol) was from New England Nuclear (Mississauga, ON, Canada). Organic solvents were from Fisher (Toronto, ON, Canada), and were HPLC grade or better. N-Methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide was from Pierce (Rockford, IL). Aminopropyl columns were obtained from Burdick and Jackson (Muskegon, MI), and Sep-Pak silica columns were obtained from Waters (Toronto, ON, Canada). The DB-23 cyanopropyl gas chromatography column was purchased from J&W Scientific (Folsom, CA). The PL2 inhibitors HEUSS and MAFF were obtained from Calbiochem (San Diego, CA), and the anti-cPLA2 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). SLO toxin was obtained from Scintibodies (South San Francisco, CA). The sPLA2 inhibitor 3-(3-acetamidophenyl)-1-benzyl-2-ethylindolyl-5-oxy-propanophosphonic acid (LY311-727) was the kind gift of Dr. E. Mihelich (Lilly Research Laboratories, Indianapolis, IN), and the lysosomal-type Ca2+-independent PL2 inhibitor 1-hexadecyl-3-trifluoroacryloxy-2-phosphophenolmethane (MJ33) (27) was kindly provided by Dr. M. K. Jain (Department of Chemistry and Biochemistry, University of Delaware, Newark, DE). The neutralizing anti-sPLA2 Ab 3F10 (26) and sPLA2 inhibitor SB203347 (29) were kindly provided by Dr. Lisa Marshall, Smith Kline Beecham Pharmaceuticals (King of Prussia, PA). pldA Escherichia coli was the kind gift of Dr. Peter Elsbach, New York University (New York, NY).

Isolation of neutrophils

Neutrophils from whole blood were isolated by dextran sedimentation and discontinuous plasma Percoll gradient centrifugation and resuspended in KRPD buffer without Ca2+ at 2 × 107 cells/ml (30, 31). This procedure yielded >95% PMN with >98% viability, as judged by trypan blue exclusion. PMN were treated with 1 mM DFP for 10 min on ice before experimental treatments, radiolabeling, cell lysis, subcellular fractionation, or other experimental procedures. We found that DFP, which prevents degradation of PMN proteins (32, 33), decreased nonspecific background activation and prevented cell clumping, and markedly enhanced cell viability over extended treatment and experimental regimes.

Experimental protocol

PMN were treated with 0.1% DMSO, 10 μM MAP, 16 μM HELSS, 10 μM SB203347, 10 μM Scalaradial, 10 μM LY311-727, or 1 mM DTT for 0.5 h at 37°C, as indicated in the figure legends. PMN were then pelleted and resuspended at 2 × 107 cells/ml in KRPD with 0.25% fatty acid-free human albumin and 1 mM CaCl2. Following readdition of 10 μM SB203347, 10 μM Scalaradial, 10 μM LY311-727, or 1 mM DTT, which do not bind covalently and could therefore be washed out when cells were resuspended in KRPD with CaCl2 and albumin, neutrophils were equilibrated for 5 min at 37°C. Cells were subsequently treated with either 0.1% DMSO or 5 μM cytochalasin B for 2 min and 100 nM fMLP for 3 min at 37°C. Experiments were terminated by centrifugation for 10 s at 14,000 × g.

Acid extraction of sPLA2 from neutrophils

PMN (2 × 107/ml in KRPD) were mixed with an equal volume of water, brought to pH 1.6 with concentrated H2SO4, and stirred overnight at 4°C. Following centrifugation at 14,000 × g for 15 min, the supernatant was dialyzed against 500 vol of 10 mM sodium acetate buffer, pH 4.5, for 24 h. The dialysate was collected and centrifuged at 14,000 × g for 15 min, and the supernatant was used as a source of sPLA2.

Determination of extracellular and cell-associated sPLA2 activity: collection of extracellular and cell-associated sPLA2

PMN (2 × 107) were stimulated with fMLP, and the extracellular sPLA2 was collected in the supernatant by brief centrifugation at 14,000 × g. The supernatant was concentrated over a Centricon 3000 NMWL ultrafilter and diluted to a total of 270 μl in assay buffer A (150 mM NaCl, 10 mM CaCl2, 25 mM HEPES, pH 7.4, and 0.25% fatty acid-free human albumin). The cell pellet was resuspended in 270 μl sPLA2 assay buffer and disrupted on ice water by three 15-s bursts of a 50-W probe sonicator set to 20% amplitude (Sonics and Materials, Danbury, CT).

Radiolabeling of E. coli membranes

The PL2-deficient strain of E. coli (pldA) was radiolabeled during the log growth phase with [3H]AA, washed three times in KRPD with 0.25% fatty acid-free BSA, autoclaved and rewashed exactly as described (34), and then used as a substrate in the sPLA2 assay and cPLA2 assays.

sPLA2 assay

A total of 60 μl of assay buffer A was combined with 10 μl of [3H]AA-labeled E. coli membranes (~50,000 dpm/assay) and 5 μl of DMSO or MAP (10 μM), HELSS (16 μM), LY311-727 (10 μM), or 3F10 (1 μg/ml) on ice. sPLA2 reactions were initiated by the addition of 25 μl of cell lysates, cell supernatants, or sPLA2 from acid-extracted neutrophils. After 30 min at 37°C, reactions were terminated by the addition of 900 μl of tetrahydrofuran, followed by centrifugation at 14,000 × g for 15 min at 4°C. The reaction was then applied to an aminopropyl column, and the fatty acid fraction was selectively eluted with tetrahydrofuran:acetic acid (49:1), followed by liquid scintillation counting (28). Results are expressed as the percentage of free fatty acid hydrolyzed (dpm generated – nonspecific hydrolysis)/total dpm added.

[3H]AA release from [3H]AA-labeled, SLO-permeabilized neutrophils

Neutrophils were labeled with [3H]AA for 2 h at 37°C, washed three times in KRPD with 0.25% BSA, resuspended in KRPD with 1 mM CaCl2, and 0.25% BSA, and incubated with 0.1% DMSO, 10 μM LY311-727, or 1 μg 3F10/ml for 5 min at 37°C. Cells were then treated with either SLO toxin (approximately 0.1 μg/ml per vehicle) (KRPD) for 2.5 min in the presence of 5 μM cytochalasin B before stimulation with IMPL. The quantity of SLO added was optimized for each experiment. Incubations were stopped after 10 min by centrifugation, and the supernatant was collected and counted for [3H]AA release using a liquid scintillation counter (Beckmann 6500) after the method of Mira et al. (35).

AA mass release

Following cellular stimulation and centrifugation for 15 s at 14,000 × g, 1.5 ml of supernatant from 3 × 107 PMN was collected and immediately transferred to siliconized borosilicate test tubes with 6 ml of chloriform-methanol (2:1, v/v) and 0.01% butylated hydroxytoluene. A total of 8 pmol of the internal standard deuterated AA ([2D8]AA) was then added to each sample. After vortexing and addition of 1.5 ml NaCl solutions, samples were centrifuged at 1000 × g for 5 min, and the lower phase was collected. The upper phase was then reextracted twice with chloriform-methanol-0.58% NaCl (86:14:1) (36). Lipids from the pooled lower phase were then dried under a stream of N2, reconstituted in hexane-methyl tert-butyl ether (200:3), and applied to a silicid acid column. Fatty acids were selectively eluted from the column with hexane-methyl tert-butyl ether-acetic acid (100:2:0.2) (37), dried under N2, and derivatized with N-methyl-N-(tert-butylidimethylsilyl)trifluoroacetamide (38, 39). The tert-butylidimethylsilyl ether of AA was then separated from other fatty acids by gas chromatography (model 5890; Hewlett Packard, Palo Alto, CA) on a DB-23 0.2 mm (25) with a quadrupole mass spectrometer (model 5971; Hewlett Packard).

Cell fractionation

A total of 6 × 107 cells were lysed in 600 μl 20 mM HEPES, pH 7.4, 150 mM K+5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM sodium orthovanadate, 2 mM DFP, 1 mM PMSF, 1 mM benzamidine hydrochloride, 50 μM Leupeptin, 50 μM pepstatin, and 50 μM chymostatin by brief pulses of probe sonication on ice water. The insoluble cellular
components, including nuclei, were removed by centrifugation at 14,000 × g for 5 min at 4°C. The supernatant was then separated into microsomal and cytosolic fractions by centrifugation at 150,000 × g for 20 min at 4°C.

Determination of PLA₂ activity in PMN cytosol

**cPLA₂ assay.** PLA₂ activity in PMN cytosol was measured by combining 75 μl of assay buffer B (150 mM NaCl, 2 mM 2-ME, 25 mM HEPES, pH 7.4, 5 mM EDTA, and 0.25% fatty acid-free human albumin) and 10 μl of [³H]AA-labeled *E. coli* membranes to a final concentration 5 nmol lipid phosphorus (or ~50,000 dpm/assay), and was initiated by the addition of 20 μl of cytosol. A total of 10 μM MAFP, 16 μM HELSS, or 10 μM LY311-727, or their diluents (DMSO or NaCl) was added to the cytosol 5 min before the initiation of the assay. After a 30-min incubation at 37°C, the reaction was terminated by addition of tetrohydrofuran and centrifugation at 14,000 × g for 15 min at 4°C. Fatty acids were eluted from an aminopropyl solid-phase silica column, as described for the sPLA₂ assay (28). Results are expressed as the percentage of free fatty acid hydrolyzed (dpm generated – nonspecific hydrolysis)/total dpm added.

**SDS-PAGE**

Cytosolic, microsomal, or nuclear fractions (200 μl) were combined with 50 μl of 5 × sample buffer (0.312 M Tris, pH 6.8, 50% sucrose (w/v), 25 mM DTT, 10% SDS, and 0.5% bromphenol blue) to a total volume of 250 μl. The samples were boiled for 5 min, and 25-μl aliquots were resolved on 12% 0.75-mm gels in a Bio-Rad (Richmond, CA) mini protean II vertical electrophoresis apparatus at 100 V.

**Western blots**

Gels were placed against nitrocellulose membranes in 25 mM Tris and 192 mM glycine with 20% (v/v) methanol and transferred at 100 V for 1 h. The blots were stained with 0.1% Ponceau Red to ensure equal protein loading before destaining in water. Blots were blocked for 2 h in TBST (20 mM Tris, pH 7.3, 0.24 M NaCl, 2.6 mM KCl, and 0.05% Tween 20) containing 5% (w/v) skin milk powder and 1% (v/v) goat normal serum. The blots were then washed 2 × 5 min in 10 ml TBST and incubated with 1/1000 anti-cPLA₂ overnight at 4°C before washing 1 × 5 min in 10 ml TBST and incubation with a 1/20,000 dilution of goat anti-mouse secondary Ab conjugated to HRP for 1 h. Blots were then washed 3 × 5 min in TBST before detection with 1.24 μM 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), 0.65 μM 4-hydroxycinnamic acid (p-coumaric acid), and 0.0001% hydrogen peroxide against KODAK ECL blue film.

**Statistical analysis**

Data were analyzed by ANOVA, followed by comparison of all means with the Tukey-Kramer honestly significant difference (HSD) test using SAS software (SAS Institute, Carey, NC). Results were considered to be significantly different when p < 0.05 was observed.

**Results**

**A minor role for extracellular sPLA₂ in PMN AA release**

We examined the kinetics of sPLA₂ release by PMN in response to fMLP and found that approximately 30% of the apparently cell-associated sPLA₂ activity was lost within the first 30 s after stimulation (Fig. 1A). Concurrently, the sPLA₂ activity observed in the supernatant rapidly increased in the first 30 s after treatment with fMLP and then remained constant (Fig. 1B). Under these assay conditions, cell-associated and extracellular PLA₂ activity were each inhibited more than 98% by coincubation with 10 μM LY311-727 (Fig. 2A), which selectively inhibits group IIA (16) and group V sPLA₂ activity (17), but has no effect on cPLA₂ or iPLA₂ activity (17). In a similar pattern to sPLA₂ activity release, the greatest change in [³H]AA release from neutrophils occurred within 30 s after fMLP stimulation, with a decline in the rate of release over the next several minutes (Fig. 1C). Hence, there was a strong temporal correlation between the secretion of sPLA₂ and the release of [³H]AA from fMLP-stimulated PMN.

As the release of sPLA₂ activity correlated with extracellular [³H]AA release, we sought to determine whether extracellular sPLA₂ mediated AA mass release. Stimulating PMN with fMLP in the presence of either DTT or the cell-impermeable sPLA₂ inhibitor LY311-727 (16) significantly inhibited extracellular sPLA₂ activity (Fig. 2A). In contrast, incubation with either DTT or LY311-727 only decreased fMLP-stimulated AA mass release by approximately 15%, a difference that failed to reach statistical significance (Fig. 2B). Therefore, sPLA₂-mediated hydrolysis of AA from glycerophospholipids on the extracellular face of the plasma membrane of PMN does not appear to be a major source of released AA under these conditions.

**A central role for cPLA₂ in PMN AA release**

cPLA₂ has been implicated in AA release from human neutrophils (40). Using immunoblot analysis, we found that most of the cPLA₂ in unstimulated neutrophils is present in the cytosolic fraction (Fig. 3). The cPLA₂ in the cytosolic fraction was the more rapidly migrating form, indicating that most of the cPLA₂ in this fraction was not phosphorylated (41). Comparatively little cPLA₂ was detected in the nuclear or microsomal fraction of unstimulated PMN. In fMLP-stimulated cells, cPLA₂ was detected in both nuclear and microsomal fractions, and a concomitant decrease in the amount of cPLA₂ in the cytosolic fraction was observed. In addition, the majority of cPLA₂ detected in fMLP-stimulated cells migrated to a relatively higher m.w., demonstrating that most of the cPLA₂...
that translocated to nuclear and microsomal fractions was phosphorylated (41).

We then examined the effect of the cPLA₂ inhibitor MAFP on AA mass release from fMLP-stimulated cells. We controlled these pharmacological experiments with the putative sPLA2 inhibitors SB203347 and Scalaradial, which have previously been shown to inhibit AA release (23, 24), and with the iPLA₂ inhibitor HELSS. We observed that MAFP decreased fMLP-induced AA mass release from intact PMN to levels similar to those observed with the cell-permeable sPLA₂ inhibitor SB203347, while preincubation with Scalaradial resulted in a further decrease in AA mass release (Fig. 4). The iPLA₂ inhibitor HELSS had little effect on AA release at a concentration that has previously been shown to decrease cytosolic PMN iPLA₂ activity by 80% (42). Importantly, MAFP had no effect on the catalytic activity of sPLA₂ that had been acid extracted (43) from neutrophils (Fig. 5). As expected, neutrophil sPLA₂ activity was significantly decreased by LY311-727 and the neutralizing anti-sPLA₂ Ab 3F10, and was not affected by coinubcation with HELSS (Fig. 5).

We then evaluated the effect of MAFP on cPLA₂ activity in intact cells by incubating cells with MAFP and evaluating PLA₂ activity in the cytosolic fraction of these cells. We found that the

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**FIGURE 2.** Effect of LY311-727 or DTT on extracellular sPLA₂ activity and fMLP-stimulated neutrophil AA mass release. PMN were treated with vehicle (DMSO) or fMLP in the presence of DMSO, LY311-727, or DTT. The release of sPLA₂ activity (A) or AA mass (B) into the supernatant was then measured, as described in Materials and Methods. Results shown are the mean ± SE of four determinations. Different lower case letters indicate differences by the Tukey-Kramer HSD test, p < 0.05.

**FIGURE 3.** Effect of fMLP on cPLA₂ translocation and electrophoretic mobility. PMN were treated with DMSO or fMLP, disrupted by sonication, and separated into cytosolic, microsomal, and nuclear fractions by centrifugation. After SDS-PAGE, immunoblot analysis was performed with the putative sPLA₂ inhibitors SB203347 and Scalaradial, which have previously been shown to inhibit AA release (23, 24), and with the iPLA₂ inhibitor HELSS. We observed that MAFP decreased fMLP-induced AA mass release from intact PMN to levels similar to those observed with the

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**FIGURE 4.** Effect of cell-permeable PLA₂ inhibitors on neutrophil AA mass release. Cells were pretreated with DMSO, HELSS, MAFP, SB203347, or Scalaradial before treatment with DMSO or fMLP. AA mass release was then assessed by gas chromatography-selective ion monitoring mass spectrometry, as described in Materials and Methods. Results shown are the mean ± SE of three determinations. Different lower case letters indicate differences by the Tukey-Kramer HSD test, p < 0.05.

**FIGURE 5.** Effect of PLA₂ inhibitors on acid-extracted neutrophil sPLA₂ activity. sPLA₂ was partially purified from neutrophils by incubation with H₂SO₄. The effect of LY311-727, 3F10, HELSS, or MAFP on acid-extracted sPLA₂ activity was then assessed, as described in Materials and Methods. Results shown are the mean ± SE of four determinations. Different lower case letters indicate differences by the Tukey-Kramer HSD test, p < 0.05.
PLA₂ activity in PMN cytosol, using [³H]AA-labeled bacterial membranes as a substrate, was almost completely inhibited by MAFP (Fig. 6). To determine whether the PLA₂ activity in PMN cytosol was attributable to cPLA₂ activity, we controlled this assay against the presence of iPLA₂ activity using the iPLA₂-specific inhibitor HELSS and against sPLA₂ activity using the sPLA₂ inhibitor LY311-727 (16). We found that neither HELSS nor LY311-727 had any effect on AA hydrolysis from bacterial membranes under these conditions (Fig. 6). Two other iPLA₂ inhibitors (PACOCF₃ and MJ33) also failed to inhibit the PLA₂ activity in PMN cytosol (data not shown). Hence, we observed that MAFP significantly blocked cPLA₂ activity in the cytosol of human PMN in vitro, a finding consistent with the capacity of MAFP to inhibit fMLP-induced AA release from intact cells.

No role for iPLA₂ in fMLP-induced PMN AA release
We used the suicide substrate HELSS (44) to study the role of iPLA₂ in PMN AA release. HELSS has previously been shown to selectively constrain the activity of iPLA₂ and to be a poor inhibitor of cPLA₂ activity (26). At a concentration that significantly decreased PMN iPLA₂ activity (42) and obliterated PMN superoxide production (45), HELSS failed to inhibit iMLP-induced AA mass release (Fig. 4) or to have any effect on neutrophil sPLA₂ activity (Fig. 5). Hence, we observed that MAFP significantly blocked cPLA₂ activity in the cytosol of human PMN in vitro, a finding consistent with the capacity of MAFP to inhibit fMLP-induced AA release from intact cells.

A potential role for an intracellular or cell-associated sPLA₂-like activity in PMN AA release
We have provided evidence that extracellular sPLA₂ and iPLA₂ make little or no contribution to AA release (Figs. 1 and 4), and that the function of cPLA₂ appears to be required for fMLP-induced AA release (Figs. 3 and 4). As a control for the AA mass release experiments with the soluble cPLA₂ inhibitor MAFP, we examined the effects of the putative sPLA₂-specific inhibitors SB203347 and Scalaradial (23, 24), which, like MAFP, readily penetrate intact cells. We noted that pretreatment with SB203347 or Scalaradial inhibited AA mass release (Fig. 4), findings consistent with a role for an intracellular or cell-associated sPLA₂ in this process. In this regard, activity measurements showed that approximately 70% of cellular sPLA₂ remained within the cell following stimulation with fMLP (Fig. 1A), and thus was inaccessible to LY311-727 or DTT, sPLA₂ inhibitors that failed to affect fMLP-induced AA mass release (Fig. 2B). Hence, we considered whether an intracellular or cell-associated sPLA₂-like molecule could play a role in PMN AA metabolism. To evaluate this hypothesis, we permeabilized PMN with SLO to deliver LY311-727 or 3F10 directly to the putative intracellular or cell-associated sPLA₂. As noted above, LY311-727 and 3F10 inhibit neutrophil sPLA₂ activity by approximately 98% and 80%, respectively (Fig. 5). [³H]AA release from permeabilized cells treated with DMSO or DTT were used as controls. After PMN were permeabilized with SLO, incubation with either LY311-727 or 3F10 resulted in a 50% inhibition of [³H]AA release (Fig. 7, +SLO), or vehicle (−SLO). Neutrophils were then treated with vehicle or fMLP in the presence of DMSO, LY311-727, or 3F10. Following centrifugation, the release of [³H]AA was measured, as described in Materials and Methods. Exposure of SLO-permeabilized cells (i.e., +SLO) to fMLP induced a mean release of 2598 cpm, which was arbitrarily taken as 100%. Results shown are the mean ± SE of four determinations. Different lower case letters indicate differences by the Tukey-Kramer HSD test, p < 0.05.
Discussion
We have systematically examined the roles of extracellular and intracellular or cell-associated sPLA₂-like enzymes, cPLA₂ and iPLA₂, in AA release using pharmacological and biochemical techniques. Our intention was to utilize an integrated approach in an attempt to establish the location and identity of the PLA₂ isomers that play a role in AA release from fMLP-stimulated human PMN.

No direct role for iPLA₂ in fMLP-stimulated PMN AA release
The role of iPLA₂ in AA metabolism appears to vary in different cell lines. In pancreatic β cells, smooth muscle cells, and cardiomyocytes, treatment with the iPLA₂ inhibitor HELSS decreased agonist-stimulated AA release (46–48). HELSS has been shown to be approximately 1000-fold more selective for inhibition of iPLA₂ compared with cPLA₂ or sPLA₂ under some conditions, and has no effect on other enzymes involved in AA metabolism, including arachidonoyl-CoA synthetase, lysophosphatidylcholine: arachidonyl acyl transferase, or CoA-independent transacylase (26, 47, 49–51). Therefore, the results obtained with HELSS were interpreted to indicate a role for iPLA₂ in AA release from pancreatic β cells, smooth muscle cells, and cardiomyocytes. In addition, human embryonic kidney 293 cells transfected with iPLA₂ cDNA demonstrated increased basal release of [³H]AA and increased [³H]AA release in response to serum compared with control cells (52). In contrast, studies with P388D₁ macrophages in which HELSS or antisense iPLA₂ oligonucleotides were used to inhibit or deplete iPLA₂ demonstrated that iPLA₂ participates in phospholipid remodeling, and did not play a role in PAF-stimulated AA release from these cells. In the present study, we found that HELSS, at a concentration that significantly inhibits neutrophil iPLA₂ activity (42), had no significant effect on fMLP-stimulated AA release. We conclude that iPLA₂ did not significantly contribute to fMLP-stimulated PMN AA release under our assay conditions.

Central role for cPLA₂ in PMN AA release
Multiple studies have implicated cPLA₂ activation as a critical step in PMN AA release (18, 53–55). In agreement with these studies, immunoblot analysis of fMLP-stimulated cells demonstrated translocation of cPLA₂ from cytosolic to microsomal and nuclear fractions. In addition, exposure to fMLP resulted in a decrease in the electrophoretic mobility of cPLA₂, consistent with a role for cPLA₂ in AA release from these cells (25). As the reducing environment of the cytosol would inactivate cPLA₂, the roles of cPLA₂ in AA release may be explained by the finding that brief exposure to exogenous human plasma was followed by resistance to further hydrolysis of membrane phospholipids by either enzyme, a phenomenon that may be mediated by sPLA₂ binding to a membrane receptor (60). Alternatively, it is possible that the affinity of the surface sPLA₂ receptors rapidly changed in response to agonist stimulation, and that stimulated cells did not effectively bind endogenous extracellular sPLA₂. In support of this, we have previously shown that sPLA₂ expression on the surface of PMN increases 7-fold after 15 s of stimulation with fMLP, but that surface sPLA₂ expression returned to baseline levels within 1 min of stimulation (61). We conclude from our data that the sPLA₂ that is released from human PMN in response to fMLP did not directly mediate a major portion of the AA released from these cells.

Role for sPLA₂ in PMN AA release
We showed that stimulation with fMLP for 30 s resulted in a significant release of both sPLA₂ activity and [³H]AA from PMN. These findings are consistent with the notion that sPLA₂ released by PMN into the extracellular space can bind to the cell membrane and hydrolyze membrane glycerophospholipids (26). In support of this model, addition of exogenous recombinant synovial PLA₂ to P388D₁ macrophages or group V PLA₂ to unstimulated human PMN both led to a significant release of AA (21, 22). In addition, the agonist-induced translocation of phosphatidylserine and phosphatidylethanolamine from the intracellular to the extracellular face of the phospholipid membrane favors membrane hydrolysis by an extracellular sPLA₂, as sPLA₂ binding to lipid bilayers is promoted by negative charges (57, 58), and group V sPLA₂ preferentially hydrolyzes phosphatidylethanolamine vesicles compared with phosphatidylcholine vesicles (59). Our results did not strongly support a role for an extracellular sPLA₂ in PMN AA release, as inhibition of extracellular sPLA₂ activity with LY311-727 or DTT, which constrain the catalytic activity of group IIa PLA₂, caused an initial release of fatty acids from human white blood cells that was followed by resistance to further hydrolysis of membrane phospholipids by either enzyme, a phenomenon that may be mediated by sPLA₂ binding to a membrane receptor (60). Alternatively, it is possible that the affinity of the surface sPLA₂ receptors rapidly changed in response to agonist stimulation, and that stimulated cells did not effectively bind endogenous extracellular sPLA₂. In support of this, we have previously shown that sPLA₂ expression on the surface of PMN increases 7-fold after 15 s of stimulation with fMLP, but that surface sPLA₂ expression returned to baseline levels within 1 min of stimulation (61). We conclude from our data that the sPLA₂ that is released from human PMN in response to fMLP did not directly mediate a major portion of the AA released from these cells.

Possible role for an intracellular or cell-associated sPLA₂-like enzyme in PMN AA release
Previous studies have indicated that a sPLA₂ might play a role in AA release from within the cell (23, 24). Three independent lines of evidence were identified in this study that support this concept. First, we observed that approximately 70% of cellular sPLA₂ activity remained associated with the cell following stimulation with fMLP. Second, treatment with the cell-permeable sPLA₂ inhibitors SB203347 or Scalaradial both prevented AA mass release to an extent similar to MAFP (Fig. 4). However, studies with SB203347 or Scalaradial must be interpreted cautiously (24), as both of these inhibitors may constrain the activity of cPLA₂, and Scalaradial may affect AA release through inhibition of Ca²⁺ mobilization (25). As the reducing environment of the cytosol would inactivate sPLA₂ activity, sPLA₂ would likely have to be confined to intracellular granules (9) or some other intracellular location to retain catalytic activity. To examine the potential role of an intracellular or cell-associated sPLA₂ in AA release under our conditions, we used the highly specific sPLA₂ inhibitor LY311-727 (16) and the neutralizing anti-sPLA₂ mAb 3F10 (28). As neither LY311-727 nor 3F10 was able to enter cells effectively during the time frame of these studies (minutes), we permeabilized PMN with SLO so that the sPLA₂ inhibitors could gain direct access to the putative intracellular or cell-associated sPLA₂. The nonspecific reducing agent DTT, which inhibits sPLA₂, but not cPLA₂ or iPLA₂ activity, was used as a control. We found that both LY311-727 and
3F10 inhibited the release of [3H]AA from permeabilized PMN (Fig. 7) to an extent that was comparable with that of the nonspecific reducing agent DTT, thereby providing a third line of evidence that an intracellular or cell-associated sPLA2 participates in PMN AA release. In contrast to our results, Bauldry et al. showed that DTT had little effect on [3H]AA release from fMLP-stimulated, SLO-permeabilized PMN. This discrepancy may be explained by the fact that the cytosolic buffer used for the permeabilization studies conducted by Bauldry et al. (62) did not support sPLA2 catalytic activity, most likely because a Ca^{2+} concentration of 500 nM was used. In the present study, cells were simultaneously exposed to fMLP and 1 mM Ca^{2+}, a concentration of Ca^{2+} that supports maximal sPLA2 activity.

As SLO permitted a free diffusion of small molecules between cytoplasm and medium (35), it was not clear whether the [3H]AA release inhibited by LY311-727 or 3F10 in the permeabilized cell was in fact destined for extracellular release, or if the [3H]AA leaked out of the cell from intracellular compartments such as granules, through the SLO-induced pores. Since LY311-727 did not inhibit cPLA2 activity (Fig. 6), a finding consistent with a recent report (17), we conclude that a low m.w. sPLA2 activity remained within a noncytosolic compartment of the neutrophils, such as the granules (9), and participated in AA metabolism in response to the bacterial peptide fMLP.

In summary, our results demonstrate that cPLA2 plays a central role in fMLP-stimulated AA release from human PMN. We also present some evidence in support of a role for an intracellular or cell-associated sPLA2 in PMN AA release. The possibility that sPLA2 activity is regulated by the prior activation of cPLA2 (26) in human PMN is currently being evaluated.

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References


