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Involvement of Cytosolic Phospholipase A2 and Secretory Phospholipase A2 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 A2 (sPLA2), calcium-independent PLA2, and cytosolic PLA2 (cPLA2) in arachidonic acid (AA) release from fMLP-stimulated human neutrophils. While fMLP induced the release of extracellular sPLA2 activity and AA, 70% of sPLA2 activity remained associated with the cell. Treatment with the cell-impermeable sPLA2 inhibitors DTT or LY311-727, or the anti-sPLA2 Ab 3F10 all inactivated extracellular sPLA2 activity, but had minimal effect on neutrophil AA mass release. In contrast, coinubation 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 cPLA2, a finding consistent with cPLA2 phosphorylation, and stimulated the translocation of cPLA2 from cytosolic to micosomal and nuclear compartments. The role of cPLA2 was further evaluated with the cPLA2 inhibitor methyl arachidonoyl fluorophosphonate, which attenuated cPLA2 activity in vitro and decreased fMLP-stimulated AA mass release by intact neutrophils, but had no effect on neutrophil sPLA2 activity. Inhibition of calcium-independent PLA2 with haloenol lactone suicide substrate had no effect on neutrophil cPLA2 activity or AA mass release. These results indicate a role for cPLA2 and an intracellular or cell-associated sPLA2 in the release of AA from fMLP-stimulated human neutrophils.

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Phospholipase A2 (PLA2) hydrolyzes arachidonic acid (AA) from the sn-2 position of glycerophospholipids (1). The hydrolysis of AA by PLA2 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 PLA2 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 PLA2 isoforms have been described in human neutrophils. An 85-kDa cytosolic PLA2 (cPLA2) that selectively hydrolyzes glycerophospholipids with AA in the sn-2 position and translocates to the nucleus in a Ca2+-dependent manner in response to agonists (3, 4) has been identified in PMN (5). Neutrophils also have Ca2+-independent PLA2 (iPLA2) activity (6). iPLA2 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 PLA2 (sPLA2) that are stored in granules (9–11). sPLA2 isoforms have six to eight disulfide bridges, require micromolar to millimolar levels of Ca2+ 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. sPLA2 isoforms, in contrast to cPLA2 and iPLA2, 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 cPLA2 directly mediates AA release includes the demonstration that cPLA2 is activated in response to bacterial agonists (18), that pharmacological inhibitors of cPLA2 attenuate PMN AA release (19), and that macrophages from mice in which the gene for cPLA2 was disrupted exhibited decreased AA release in response to PMA and calcium ionophores (20). Evidence that sPLA2 directly mediates AA release includes the observation that exogenous addition of group V sPLA2 to PMN (21) or recombinant synovial PLA2 to murine macrophages (21, 22) directly results in AA release. In addition, treatment with the cell-permeable sPLA2 inhibitors SB203347 and Scalardial prevented Ca2+-ionophore-stimulated AA mass release (23, 24). While these studies support a role for sPLA2 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 P388D1 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 that iPLA2 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, Na2HPO4, K[HPO4], MgCl2, and CaCl2, were obtained from Mallinckrodt (Paris, KY). Neutrophils were obtained from healthy volunteers after informed consent was obtained. Sodium orthovanadate, 2 mM DFP, 1 mM PMSF, 1 mM benzamidine, and Sep-Pak silica columns were obtained from Waters (Milford, MA). The DB-23 cyanopropyl gas chromatography column was purchased from J&W Scientific (Folsom, CA). The PLA2 inhibitors HELSS and MAFP 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 Scantibodies (Santa Cruz, CA). The PLA2 inhibitor 3-(3-acetaminido-1-benzyl-2-ethylindolyl-5-oxy)propanephosphonic acid (LY311-727) was the kind gift of Dr. E. Mihelich ( Lilly Research Laboratories, Indianapolis, IN), and the lysosomal-type Ca2+-independent PLA2 inhibitor 1-hexadecyl-3-trifluorylcglycerol-2-phosphomethanol (M33) (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). [3H]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), decreases nonspecific background activity 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 MAFP, 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 × 105 cells/ml in KRPD with 0.25% fatty acid-free human albumin and 1 mM CaCl2. Following resuspension of PMN, cells were resuspended in KRPD with CaCl2 and albumin, neutrophils were equilibrated for 5 min at 37°C. Cells were then subjected to either 0.1% DMSO or 5 μM cytochalasin B for 2 min and 100 nM IMLP for 3 min at 37°C. Experiments were terminated by centrifugation for 10 s at 14,000g.

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 IMLP, and the extracellular sPLA2 was collected in the supernatant by brief centrifugation at 14,000 × g. The supernatant was concentrated over a Centriicon 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 PLA2-deficient strain of E. coli (pLDA1) was radiolabeled during the log growth phase with [3H]AA, washed three times in KRPD with 0.25% fatty acid-free BSA, autoclaved and rewarshed 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 MAFP (10 μM), HELSS (16 μM), LY311-727 (10 μM), or 3F10 (1 μg/ml) in sPLA2, reactions were initiated by the addition of 25 μl of cell lysates, cell supernatants, or sPLA2 from acid-extracted neurothrophils. 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) or vehicle (KRPD) for 2.5 min in the presence of 5 μM cytochalasin B before stimulation with IMLP. 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 (Beckman 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 chlorormethanol (2:1, v/v) and 0.01% butyraldehyde hydroxytoluene. A total of 8 pmol of the internal standard deuterated AA ([2D]AA) was then added to each sample. After vortexing and addition of 1.5 ml NaCl solutions were centri

Cell fractionation

A total of 6 × 107 cells were lysed in 600 μl 20 mM HEPES, pH 7.4, 150 mM KCl, 5% glycerol, 1 mM EGTA, 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 HELOSS, 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 tetrahydronafuran 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% bromophenol 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) skim milk powder and 1% (v/v) goat normal serum. The blot was 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 (lumino), 0.65 μM 4-hydroxycinnamic acid (p-carboxylic 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 apparent 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 enriched 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 at 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 cPLA₂ translocated to nuclear and microsomal fractions was phosphorylated (41).

We then examined the effect of the cPLA₂ inhibitor MAF 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).

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PLA_2 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 PLA_2 activity in PMN cytosol was attributable to cPLA_2 activity, we controlled this assay against the presence of iPLA_2 activity using the iPLA_2-specific inhibitor HELSS and against sPLA_2 activity using the sPLA_2 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 iPLA_2 inhibitors (PACOCF_3 and MJ33) also failed to inhibit the PLA_2 activity in PMN cytosol (data not shown). Hence, we observed that MAFP significantly blocked cPLA_2 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 iPLA_2 in fMLP-induced PMN AA release
We used the suicide substrate HELSS (44) to study the role of iPLA_2 in PMN AA release. HELSS has previously been shown to selectively constrain the activity of iPLA_2, and to be a poor inhibitor of cPLA_2 activity (26). At a concentration that significantly decreased PMN iPLA_2 activity (42) and obliterated PMN superoxide production (45), HELSS failed to inhibit fMLP-induced AA mass release (Fig. 4) or to have any effect on neutrophil sPLA_2 activity (Fig. 5). These findings do not support a role for iPLA_2 in fMLP-stimulated AA release from intact cells.

A potential role for an intracellular or cell-associated sPLA_2-like activity in PMN AA release
We have provided evidence that extracellular sPLA_2 and iPLA_2 make little or no contribution to AA release (Figs. 1 and 4), and that the function of cPLA_2 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 cPLA_2 inhibitor MAFP, we examined the effects of the putative sPLA_2-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 sPLA_2 in this process. In this regard, activity measurements showed that approximately 70% of cellular sPLA_2 remained within the cell following stimulation with fMLP (Fig. 1A), and thus was inaccessible to LY311-727 or DTT, sPLA_2 inhibitors that failed to affect fMLP-induced AA mass release (Fig. 2B). Hence, we considered whether an intracellular or cell-associated sPLA_2-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 sPLA_2. As noted above, LY311-727 and 3F10 inhibit neutrophil sPLA_2 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), while DTT decreased [3H]AA release from permeabilized cells by approximately 40% (data not shown). When [3H]AA-labeled cells were not permeabilized with SLO, the cell-impermeable sPLA_2 inhibitor LY311-727 and the neutralizing anti-sPLA_2 Ab 3F10 failed to prevent [3H]AA release (Fig. 7, −SLO). Hence, we were able to provide some evidence that an intracellular or cell-associated sPLA_2-like molecule participates in fMLP-stimulated AA release.

FIGURE 6. Effect of PLA_2 inhibitors on neutrophil cPLA_2 activity. Cells were disrupted by sonication, and the cytosolic fraction was isolated by centrifugation. The effect of MAFP, HELSS, or LY311-727 on cPLA_2 activity 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 7. Effect of LY311-727 and 3F10 on [3H]AA release from permeabilized neutrophils. Cells were metabolically labeled with [3H]AA, washed, and treated with SLO toxin (+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 isoforms 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 arachidonyl-CoA synthetase, lysophosphatidylcholine: arachidonoyl 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, a finding consistent with cPLA2 phosphorylation (41), which is known to increase the catalytic activity of cPLA2 in vitro (55). Pretreatment of neutrophils with MAFF, which covalently binds to and inactivates cPLA2 (56), inhibited fMLP-induced AA mass release. In parallel studies conducted in vitro, MAFF obliterated the PLA2 activity in neutrophil cytosol. While these results are consistent with a role for cPLA2 in neutrophil AA release, they do not rule out the possibility that MAFF inhibited AA release through an effect on sPLA2. To evaluate this possibility, sPLA2 was partially purified from PMN by acid extraction with H2SO4, pH 1.6 (43), and the effect of MAFF on sPLA2 activity was assessed. Coincubating acid-extracted sPLA2 with MAFF had no effect on sPLA2 activity (Fig. 5), indicating that MAFF did not decrease neutrophil AA release through an effect on sPLA2. MAFF also inhibits iPLA2 in vitro (56), but studies with HELSS indicated that iPLA2 does not participate in neutrophil AA release. Therefore, the observation that fMLP stimulated cPLA2 phosphorylation and translocation, and that MAFF inhibited fMLP-stimulated neutrophil AA mass release and cPLA2 activity supports a central role for cPLA2 in governing AA release from human PMN.

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 constrains the catalytic activity of group IIa and group V sPLA2 (16, 17), only had a small inhibitory effect on AA mass release from fMLP-stimulated cells. This discrepancy may be explained by the finding that brief exposure to exogenous group IIa or group V 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 MAFF (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 though 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 likely to be 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 nonselective 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. The possibility that a Ca2+ concentration of 500 nM was used. In the present study, cells were simultaneously exposed to fMLP and 1 mM Ca2+, a concentration of Ca2+ 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 sPLA2 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 sPLA2 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) and final 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 an rhoa-activated, fMLP-stimulated, SLO-permeabilized PMN (62). 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 Ca2+ concentration of 500 nM was used. In the present study, cells were simultaneously exposed to fMLP and 1 mM Ca2+, a concentration of Ca2+ that supports maximal sPLA2 activity.

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