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Role of Mitogen-Activated Protein Kinase-Mediated Cytosolic Phospholipase A2 Activation in Arachidonic Acid Metabolism in Human Eosinophils

Xiangdong Zhu,* Hiroyuki Sano,* Kwang Pyo Kim,† Akiko Sano,* Evan Boetticher,* Nilda M. Muñoz,* Wonhwa Cho,‡ and Alan R. Leff**

The objective of this investigation was to determine the role of secretory and cytosolic isoforms of phospholipase A2 (PLA2) in the induction of arachidonic acid (AA) and leukotriene synthesis in human eosinophils and the mechanism of PLA2 activation by mitogen-activated protein kinase (MAPK) isoforms in this process. Pharmacological activation of eosinophils with fMLP caused increased AA release in a concentration (EC50 = 85 nM) and time-dependent (t1/2 = 3.5 min) manner. Both fMLP-induced AA release and leukotriene C4 (LTC4) secretion were inhibited concentration dependently by arachidonic trifluoromethyl ketone, a cytosolic PLA2 (cPLA2) inhibitor; however, inhibition of neither the 14-kDa secretory phospholipase A2 by 3-(3-acetamido-1-benzyl-2-ethylindolyl-5-oxy)propanephosphonic acid nor cytosolic Ca2+-independent phospholipase A2 inhibition by bromoeno lactone blocked hydrolysis of AA or subsequent leukotriene synthesis. Pretreatment of eosinophils with a mitogen-activated protein/extracellular signal-regulated protein kinase (ERK) kinase inhibitor, U0126, or a p38 MAPK inhibitor, SB203580, suppressed both AA production and LTC4 release. fMLP induced phosphorylation of MAPK isoforms, ERK1/2 and p38, which were evident after 30 s, maximal at 1–5 min, and declined thereafter. fMLP stimulation also increased cPLA2 activity in eosinophils, which was inhibited completely by 30 μM arachidonic trifluoromethyl ketone. Preincubation of eosinophils with U0126 or SB203580 blocked fMLP-enhanced cPLA2 activity. Furthermore, inhibition of Ras, an upstream GTP-binding protein of ERK, also suppressed fMLP-stimulated AA release. These findings demonstrate that cPLA2 activation causes AA hydrolysis and LTC4 secretion. We also find that cPLA2 activation caused by fMLP occurs subsequent to and is dependent upon ERK1/2 and p38 MAPK activation. Other PLA2 isozymes native to human eosinophils possess no significant activity in the stimulated production of AA or LTC4.


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was attributed to cPLA2 activation (21, 22). In thrombin-stimulated astrocytes, JNK has been implied in cPLA2 activation (23). The role of the specific MAPK isoform in cPLA2 activation in human eosinophils has not been determined.

FMLP, a tripeptide purified from bacteria, has a variety of biological effects on human eosinophils. These include degranulation, adhesion, chemoattraction, superoxide synthesis, and leukotriene synthesis and release (24–27). FMLP acts through a specific G protein-coupled cell surface receptor. This promotes a rapid and transient increase in intracellular Ca2+ in human eosinophils (28). Furthermore, many of the functional effects elicited by FMLP are mediated by a pertussis toxin-sensitive mechanism, indicating the involvement of one of more members of the Gi or Go family of heterotrimeric GTP-binding proteins (29, 30). However, the signaling pathway leading to AA metabolism after FMLP receptor occupation has not been defined in human eosinophils.

The objective of this study was to characterize the isoform of cPLA2 involved in arachidonic hydrolysis during activation of human eosinophils. Studies also were performed to determine the contribution of MAPK activation in AA hydrolysis in FMLP-stimulated human eosinophils. We found that both ERK1/2 and p38 MAPK are involved in cPLA2 activation, AA release, and subsequent leukotriene C4 (LTC4) synthesis. We found further that other cPLA2 isoforms native to human eosinophils possess little or no activity in stimulated production of AA or its metabolites.

Materials and Methods

Materials

The cPLA2 inhibitor arachidonic trifluoromethyl ketone (AACOCF3) and iPLA2 inhibitor bromoelone lactone (BEL) were purchased from Biomol (Plymouth Meeting, PA). The sPLA2 inhibitor, 3-(3-aceatamide-1-benzyl-2-ethylindolyl-5-oxo)propane phosphonic acid (LY117727), was kindly donated by N. Roehm (Eli Lilly, Indianapolis, IN). The p38 inhibitor SB203580 was purchased from Upstate Biotechnology (Lake Placid, NY). Eosinophil isolation materials were obtained from Miltenyi Biotec (Sunnyvale, CA). FMLP was purchased from Sigma (St. Louis, MO). SB202474 and Ras farnesyltransferase inhibitors farnesyl protein transferase (FPT) inhibitor III and manumycin A were purchased from Calbiochem (San Diego, CA). The purified cPLA2, and polyclonal anti-cPLA2, antisera were obtained as previously described (4). [5,6,8,9,11,12,14,15-3H]AA (sp. act., 100 Ci/mmol) was palmityl-2-[14C]arachidonyl phosphatidycholine (PAPC) were purchased from New England Nuclear (Boston, MA). Anti-phospho-ERK1/2 Ab and MEK inhibitor, U0126, were purchased from Promega (Madison, WI). Anti-ERK1/2, anti-phospho-p38 MAPK, anti-phospho JNK, anti-p38 MAPK, p38 kinase assay kit, and anti-IN12 were purchased from New England Biolabs (Beverly, MA). Goat anti-rabbit Ig conjugated with HRP was purchased from Amersham (Arlington Heights, IL).

Isolation of human eosinophils

Eosinophils were isolated by a method modified from Hansel et al. (31). The method is based on Percoll centrifugation (density 1.089 g/ml) to isolate granulocytes, hypotonic lysis of RBCs, and, finally, immunomagnetic depletion of eosinophils by the magnetic cell separation system (MACS particles). Eosinophil purity of ≥98% was routinely obtained, as assessed by Wright-Giemsa staining. Cells were kept on ice until use.

Immunoblot analysis of MAPK

Eosinophils (2–3 × 106/group) were preincubated with cytochalasin B for 2 min, and then stimulated with FMLP for various times, and the reaction was stopped by centrifugation at 12,000 × g for 10 s. The pellets then were lysed in 80 μl lysis buffer (20 mM Tris-HCl, 30 mM Na3PO4, 50 mM NaF, 40 mM NaCl, 5 mM EDTA, pH 7.4) containing 1% Nonidet P-40, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMFSF, 2 mM Na3VO4, and 0.5% deoxycholic acid. After 10 min on ice, the samples were centrifuged at 12,000 × g for 20 min to remove nuclear and cellular debris. The supernatant was mixed with 14 μl of 6× sample buffer and boiled for 5 min. The samples were collected and saved at −70°C.

Samples were subjected to SDS-PAGE, using 10% acrylamide gels under reducing condition (15 mM/100 μl). Electrotransfer of proteins from the gels to polyvinylidene fluoride membrane was achieved using a semidry system (400 mA, 60 min). The membrane was blocked with 1% BSA for 60 min, then incubated with 1/5000 anti-phosphorylation-specific ERK1/2 Ab, 1/1000 anti-ERK1/2 Ab, 1/1000 anti-phosphorylation-specific p38 MAPK, 1/1000 anti-p38 Ab, 1/1000 anti-phosphorylation-specific JNK, or 1/1000 anti-JNK Ab diluted in TBST overnight. The membranes then were washed three times for 20 min with TBST. Goat anti-rabbit IgG conjugated with HRP was diluted 1/3000 in TBST and incubated with polyvinylidene fluoride membrane for 60 min. The membrane was again washed three times with TBST and assayed by an ECL chemiluminescence system (Amersham).

Assay of p38 kinase activity

p38 kinase activity was assayed with a p38 MAPK assay kit following the manufacturer’s instructions (New England Biolabs). Briefly, eosinophils (2–3 × 106/group) were preincubated with various concentrations of SB203580 or SB202474 for 30 min, treated with cytochalasin B for 2 min, and then stimulated with FMLP for 1 min. The reaction was stopped by centrifugation, and the pellets were solubilized in 200 μl lysis buffer, as and after removing the nuclear and cellular debris, cells were precipitated with 1 μg anti-phospho (Thr180/Tyr182) p38 MAPK Ab bound to agarose hydrazide beads. The immune complexes were then washed twice with 500 μl lysis buffer and twice with 500 μl kinase buffer (25 mM Tris, pH 7.5, 2.5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2), and resuspended in 50 μl kinase buffer containing 100 μM ATP, 1 μM substrate GST/activating transcription factor (ATF)-2. The kinase reactions were conducted at 30°C for 30 min and terminated by the addition of 10 μl × 6X Laemmli sample buffer before SDS-PAGE. Phosphorylation of GST/ATF-2 substrate was detected by immunoblotting with anti-phospho (Thr180/Tyr182) ATF-2 Ab.

Determination of cPLA2 enzyme activity

cPLA2 enzyme activity was modified from Kim et al. (32). Briefly, 2 × 106 cytochalasin B-pretreated eosinophils were stimulated with or without 100 nM FMLP for various times. The reaction was stopped by centrifugation, and the pellets and the resuspended cells were subjected to 70 μl sonication buffer (20 mM Tris, pH 8.2, 5.2 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMFSF, 2 mM Na3VO4, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly (10 s) at 100°C, and then centrifuged for 2 min at 100,000 × g. The supernatant was added to 30 μl of 5X SD sample buffer, followed by 110 μl H2O, vortexed for 20 s, and then centrifuged at 12,000 × g. Upper layer (180 μl) was transferred to 800 μl hexane containing 25 mg silicone gel. A total of 750 μl of samples was then mixed with 2 ml scintillation fluids, and the radioactivity was counted in a liquid scintillation counter. cPLA2 activity was expressed as percentage of nonstimulated control (cpm of activated eosinophils/cpm of nonstimulated eosinophils) × 100.

Measurement of AA release

Eosinophils were incubated in RPMI media containing 5% FBS and 0.5 μg/ml 1H]AA. After a 2-h incubation period, labeled medium was aspirated, and eosinophils were washed twice with 500 μl HBSS containing 0.2% BSA. Uptake of [1H]AA by eosinophils occurred in a time-dependent manner, reaching its maximum (61 ± 2.3% of the total added [1H]AA) after 30-min incubation at 37°C. Maximal incorporation remained constant ≥2-h incubation. Aliquots of 106 eosinophils were preincubated with or without U0126, SB203580, AACOCF3, BEL, LY317172 for 30 min, or fMLP, cytochalasin III, manumycin A for 60 min. Cells were then preincubated with 5 μg/ml cytochalasin B for 2 min before stimulation by FMLP for additional 10 min at 37°C. The addition of cytochalasin B was used to promote AA metabolism, as described previously (24). The reactions were terminated by centrifugation at 12,000 × g for 1 min. Supernatants were collected, and pellets were lysed in 1% Triton X-100. [1H]AA release was measured by scintillation counting and expressed as percentage of total AA incorporation (100 × cp. of supernatant + cpm of pellet).

LTC4 assay

Aliquots of 250,000 eosinophils were preincubated with various concentrations of AACOCF3, LY311727, BEL, U0126, SB203580, or SB202474

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for 30 min, and then incubated with cytochalasin B for 2 min before being stimulated by fMLP for additional 10 min at 37°C in a final volume of 250 μl HBSS. The reactions were terminated by centrifugation at 12,000×g for 1 min. Aliquots of supernatants were assayed with a commercial enzyme immunoassay kit, as described previously (24).

**Statistical analysis**

All data are expressed as mean ± SEM. Differences between groups were assessed by paired t test. Where more than two groups were compared, differences among groups were assessed by one-way ANOVA. Where differences were found, comparisons among groups were made by Fisher’s least-protected difference test. Statistical significance was claimed where p < 0.05.

**Results**

**Effects of fMLP on AA release in eosinophils**

fMLP caused [3H]AA release from purified eosinophils in a concentration-dependent manner with an EC50 value of 8.5 ± 1 nM (Fig. 1A). Nonstimulated eosinophils release minimal amount of [3H]AA during 10-min incubation period. A significant increase in [3H]AA release was observed at 10 nM fMLP (4.3 ± 0.4% vs 0.7 ± 0.03% of total incorporation for nonstimulated control, p < 0.001), and increasing the fMLP concentrations provoked a corresponding increase in AA release, reaching maximum of 7.3 ± 0.4% at 1 μM (Fig. 1A).

fMLP caused [3H]AA release in a time-dependent manner with a t1/2 of 3.5 ± 0.5 min (Fig. 1B). With 1 μM fMLP, 63% of maximal AA release was elicited in 5 min, and at 10 min AA release reached its plateau value of 7.1 ± 0.8% (p < 0.001 vs 0.6 ± 0.03% for control). Similar dose-response curve and time course were observed in fMLP-induced LTC4 production (data not shown).

**Effects of inhibitors of sPLA2, cPLA2, or iPLA2 on fMLP-induced [3H]AA release and LTC4 production**

To determine which of the three PLA2 subtypes was responsible for the production of AA and LTC4 in fMLP-stimulated eosinophils, the effect of selective inhibitory agents on AA metabolism was assessed. Eosinophils were preincubated with AACOCF3, a cPLA2 and iPLA2 inhibitor (33, 34); LY311727, a sPLA2 inhibitor (35, 36); or BEL, an iPLA2 inhibitor (34), before stimulation by 1 μM fMLP. Nonstimulated eosinophils released minimal amounts of AA and undetectable amount of LTC4. AACOCF3 inhibited both fMLP-induced [3H]AA release (Fig. 2A) and LTC4 production (Fig. 2B) in a concentration-dependent manner. The fMLP-stimulated net AA release (background subtracted) was decreased significantly from 6.6 ± 0.8% to 2.7 ± 0.7% (p < 0.05) at 10 μM AACOCF3, and was further blocked to 1.5 ± 0.4% at 30 μM AACOCF3 (p < 0.01). Similarly, LTC4 release was decreased from 1120 ± 97 pg/ml to 429.4 ± 235.4 pg/ml (p < 0.05) with 10 μM AACOCF3, and was further blocked to 50 ± 10.4 pg/ml (p < 0.001) with 30 μM AACOCF3. In additional studies, the blocking effects of the cPLA2 inhibitor, surfactant, were examined (32). At 10 μM, surfactant significantly inhibited fMLP-stimulated AA and LTC4 release (data not shown). By contrast, neither the sPLA2 inhibitor, LY311727, nor the iPLA2 inhibitor, BEL, had any inhibitory effect on the stimulated AA or LTC4 production (Fig. 2, A and B), demonstrating that cPLA2, but neither sPLA2 nor iPLA2,
is involved in the fMLP-induced AA metabolism in human eosinophils.

**FMLP-induced activation of cPLA₂ in eosinophils**

To demonstrate further the role of cPLA₂ in fMLP-induced AA release, we next examined whether fMLP induced cPLA₂ activation. fMLP increased cPLA₂ activity by 23 ± 6.9% after 1 min, and was maximal after 5 min (60.7 ± 8.8%, p < 0.01), decreasing slightly thereafter (Fig. 3A). cPLA₂ activity in fMLP-stimulated eosinophil lysates was inhibited by AACOCF₃ in a concentration-dependent manner (Fig. 3B). cPLA₂ activity decreased to 30 ± 4.6% of buffer-treated control with 10 μM AACOCF₃ and 10.9 ± 1.9% with 30 μM AACOCF₃ (p < 0.01 vs control for both comparisons).

**FMLP-induced activation of MAPK isoforms in eosinophils**

To assess the involvement of MAPKs in fMLP-induced AA release, we next investigated the effect of fMLP on ERK1/2, p38, and JNK activation in eosinophils. Phosphorylation of ERK1/2, p38, and JNKs is commonly used as an indicator of activation. As shown in Fig. 4A, fMLP caused time-dependent ERK1/2 phosphorylation, which was observed within 0.5 min, peaked at 1–5 min, and declined thereafter (top panel). The phosphorylation was not due to differences in ERK1/2 content in each treatment, as equal amounts of ERK1/2 were present for all treatment groups (bottom panel).

p38 MAPK phosphorylation followed the same kinetics, except for the presence of minimal constitutive phosphorylation in unstimulated eosinophils (Fig. 4B, top panel). Differences in phosphorylation were not due to differences in p38 content among treatment groups, as demonstrated by the equal loading of p38 for all treatment groups (Fig. 4B, bottom panel). No JNK phosphorylation was observed in fMLP-stimulated eosinophils (data not shown).

fMLP-induced ERK1/2 phosphorylation was inhibited concentration dependently by U0126, an inhibitor of ERK1/2 activation secondary to its inhibition of upstream MAPK kinase (MEK) (37, 38). ERK1/2 phosphorylation (top panel) and total ERK1/2 phosphorylation (bottom panel) were measured as in A (n = 3). D, Effects of SB203580 and SB202474 on fMLP-stimulated p38 MAPK activity. Eosinophils were preincubated with different concentrations of SB203580 and SB202474 for 30 min, incubated with cytochalasin B for 2 min, and stimulated with fMLP for 1 min. p38 kinase activity was measured by phosphorylation of substrate GST/ATF-2, as described in Materials and Methods. The result shown is representative of three different experiments. Con, Control.

Stimulation with fMLP also caused an increase in p38 activity (Fig. 4C), which was inhibited concentration dependently by SB203580 (39), a specific p38 MAPK inhibitor. Inhibition was observed at 10 μM and was complete at 30 μM SB203580. SB202474, an inactive analogue of SB203580 (39), did not inhibit
p38 activity at 3–10 μM. However, at 30 μM, it also suppressed fMLP-stimulated p38 activity in eosinophils.

**Effects of MAPK inhibitors on fMLP-induced [3H]AA and LTC₄ productions**

The involvement of MAPK in agonist-induced AA release has been reported in platelet as well as in other cells (21, 40, 41). In these studies, pharmacological inhibitors of MAPK were employed. To address the question of whether MAPK isoforms were involved in the events leading to fMLP-stimulated AA release, we measured the effect of the MEK inhibitor, U0126, and the specific p38 MAPK inhibitor, SB203580, on fMLP-stimulated AA and LTC₄ release. As shown in Fig. 5, both U0126 and SB203580 inhibited fMLP-induced AA release (Fig. 5A) and LTC₄ production (Fig. 5B) in a concentration-dependent manner. fMLP-stimulated AA release was decreased from 6.6 ± 0.8% to 1 ± 0.07% with 10 μM U0126, and to 1.4 ± 0.3% with 30 μM SB203580 (p < 0.01 for both comparisons). Similarly, fMLP-stimulated LTC₄ release was decreased from 1280.7 ± 97.1 pg/ml to 21.7 ± 3.1 pg/ml with 10 μM U0126, and to 13.6 ± 0.7 pg/ml with 30 μM SB203580 (p < 0.001 for both comparisons). These data demonstrate that both ERK1/2 and p38 MAPK are involved in fMLP-stimulated AA release in human eosinophils. SB202474 did not inhibit fMLP-stimulated LTC₄ release at ≤10 μM. However, at 30 μM, the same concentration causing inhibition of p38 kinase activity (see Fig. 4D), SB202474 partially inhibited LTC₄ release (p < 0.05 vs fMLP) (Fig. 5B).

**Reversal of inhibition of LTC₄ secretion by AA**

To determine the specificity of MAPK and cPLA₂ inhibitors in blocking eosinophil LTC₄ secretion, experiments were generated measuring LTC₄ secretion in activated eosinophils after treatment with exogenous AA. Treatment of eosinophils with AA reversed substantially the inhibitory effects of AACOCF₃, U0126, or SB203580 on secretion of LTC₄ (Fig. 6). In three experiments, activation of eosinophils with fMLP caused 771 ± 136.4 pg/ml LTC₄ secretion after 10 min, vs 2.1 ± 0.8 pg/ml for nonactivated eosinophils (p < 0.01). Incubation with 10 μM U0126, 30 μM SB203580, or 30 μM AACOCF₃ before activation with 1 μM fMLP almost completely inhibited LTC₄ secretion (p < 0.01). In eosinophils treated with either inhibitor, addition of 10 μM AA restored LTC₄ secretion to 649.7 ± 118.1 pg/ml for SB203580-treated cells, and 704.2 ± 181.7 pg/ml for AACOCF₃-treated cells (p = NS vs fMLP alone). Addition of AA substantially increased LTC₄ secretion for U0126-treated cells to 331.6 ± 90.1 pg/ml (p < 0.01); however, this was still less than fMLP-treated cells that received no inhibitor (p < 0.05). The incomplete restoration of U0126-inhibited LTC₄ release may be explained by the fact that MEK is also required for 5-lipoxygenase activation (42). Treatment with AA did not alter LTC₄ secretion in nonactivated eosinophils (data not shown). These results demonstrated MAPK and cPLA₂ inhibitors specifically blocked AA mobilization from fMLP-stimulated eosinophils.

**Effects of MAPK inhibition on fMLP-induced cPLA₂ activation**

To evaluate the possible biological significance of MAPK in causing fMLP-induced cPLA₂ activation, we tested the effects of U0126 and SB203580 on fMLP-stimulated cPLA₂ activity in eosinophils. Eosinophils were pretreated for 30 min with 30 μM SB203580 or 10 μM U0126, followed by stimulation with fMLP for 5 min. Inhibitors were present throughout the activation period. cPLA₂ activity increased from 0.5 ± 0.07 pM/10⁶ cells/30 min for nonstimulated eosinophils to 1.1 ± 0.4 pM/10⁶ cells/30 min after fMLP stimulation (p < 0.01). This increased activity was almost completely blocked by the MEK inhibitor, U0126, or the p38 inhibitor, SB203580 (p < 0.01 for both comparisons vs fMLP only, Fig. 7). These observations suggest that both ERK1/2 and p38 MAPKs are substantially involved in fMLP-stimulated cPLA₂ activation.

**Effects of Ras farnesylation inhibition on fMLP-stimulated AA release**

In three additional experiments, the role of the small GTP-binding protein, Ras, which is a known upstream kinase for ERK1/2 (43),

**FIGURE 5.** Effect of ERK1/2 or p38 MAPK inhibition on fMLP-stimulated AA release and LTC₄ production. [3H]AA-labeled eosinophils (A) or nonlabeled eosinophils (B) were preincubated with U0126, SB203580, or SB202474 for 30 min, and then incubated with cytochalasin B for 2 min before stimulation by fMLP for another 10 min. [3H]AA release (A) was measured by scintillation counting, and was calculated by subtraction of background (n = 3). LTC₄ production (B) was measured by enzyme immunoassay (n = 3–7). Results are presented as the mean ± SEM.

**FIGURE 6.** Reversal of AACOCF₃-, U0126-, or SB203580-induced inhibition of eosinophil LTC₄ release by AA. Eosinophils were incubated with either inhibitor for 30 min, and further incubated with or without 10 μM AA for another 10 min, followed by stimulation with fMLP + cytochalasin B. LTC₄ production was measured by enzyme immunoassay, and results are presented as the mean ± SEM from three separate experiments. Con, control.
was investigated by using two structurally unrelated Ras farnesyl-
transferase inhibitors, FPT inhibitor III and manumycin (44, 45).
Activation of eosinophils with fMLP caused 6.9 ± 0.4% [%H]AA
release after 10 min, vs 0.7 ± 0.03% release for nonactivated
eosinophils (p < 0.01). Incubation with 100 μM FPT inhibitor III
or 10 μM manumycin before activation with 1 μM fMLP inhibited
[%H]AA release to <2% (Fig. 8, p < 0.01 for both groups).

**Discussion**
The objective of this investigation was to determine the relative
contribution of endogenous isoforms of PLA2 in the hydrolysis of
AA and subsequent downstream synthesis and secretion of LTC4
during metabolic activation of human eosinophils. Prior investiga-
tions have suggested that the 85-kDa cPLA2 plays a major role in
AA hydrolysis and downstream eicosanoid metabolism (46, 47),
and that this occurs as the result of transport of cPLA2 to nuclear
membrane in its phosphorylated state (48). Other investigations,
however, have identified a substantive role for the 14-kDa sPLA2
and/or the 80-kDa calcium-dependent iPLA2 (49, 50). In this
investigation, we examine the potential roles for all PLA2 isoforms
native to eosinophils as well as that of cPLA2. Multiple specific
blockers of each isoform were used, and cross-reactivity was ex-
cluded between sPLA2, iPLA2, and cPLA2. We found that cPLA2
plays a critical role in activated hydrolysis of AA and secretion of
LTC4 by human eosinophils.

We also examined the relationship between the upstream phos-
phorylation of ERK1/2 and p38 MAPKs on the subsequent cPLA2
activation, which is required for catalysis of phospholipid. Our
investigations demonstrated that cPLA2 inhibition prevents AA
hydrolysis and subsequent secretion of LTC4 for isolated human
eosinophils activated by fMLP. In these studies, fMLP was se-
lected because it is a widely studied activator of eosinophil activity
that causes both cell degranulation (26) and eosinophil secretion
of LTC4 (24). Because the physiological event(s) causing activation
of eosinophil secretion in human airways has not yet been identi-
fied, fMLP, which binds specifically to a G protein-coupled cell
surface receptor, has been used widely to replicate eosinophil ac-
tivation (27).

Eosinophils possess both the sPLA2 and cPLA2 (4, 5), and poss-
ibly iPLA2 (6). The role of specific PLA2 subtypes involved in
receptor-mediated AA mobilization has not been definitively elu-
cidated. Our data demonstrated that endogenous iPLA2 and sPLA2
play no significant role in fMLP-stimulated eosinophils. BEL is a
specific inhibitor of iPLA2, which does not inhibit sPLA2 or
cPLA2, and has been shown to block enzyme activity in A10
smooth muscle cells and P388D1 cells at concentrations ≤1–5 μM
(51, 52). Preincubiation of eosinophils with BEL at ≤10 μM had
no effect on AA or LTC4 release (Fig. 2); hence, iPLA2 has no
effect in fMLP-induced AA release from human eosinophils.
LY311727 is a structure-based sPLA2 inhibitor, and has been
shown to suppress the catalytic activity of both group IIa and
and group V sPLA2 with an IC50 of 23 and 36 μM, respectively (36,
53). Our data demonstrate that 10 μM LY311727 had no inhibitory
effect on fMLP-stimulated AA or LTC4 release (Fig. 2). This dem-
onstrates that endogenous 14-kDa sPLA2 activity is not required in
fMLP-induced AA hydrolysis or metabolism. However, this does
not exclude the antigenic properties of sPLA2 in AA release, as
suggested by others (54). Indeed, the minor role of other PLA2 in
AA release is suggested in Fig. 5, in which the IC50 for AA is
greater than the IC50 for LTC4 for both U0126 and SB203580.

In these investigations, we found that the cPLA2 inhibitor, AA-
COCF3, substantially blocked fMLP-stimulated AA release and
subsequent LTC4 production (Fig. 2). AA-COCF3 also inhibits
iPLA2 in vitro (34), but studies with BEL indicated that iPLA2
does not participate in eosinophil AA release. Another cPLA2 in-
hibitor, surfactin (32, 55), also suppressed fMLP-induced eosino-
phil AA and LTC4 production (data not shown). We also found
that cPLA2 activation induced by fMLP follows the same kinetics
as that for AA release (Figs. 1B and 3). Our data therefore suggest
that fMLP-induced AA hydrolysis and LTC4 synthesis occur
mainly through activation of cPLA2.

ERK1/2 are associated with the activation of cPLA2 in many
cell systems (11, 12, 19, 20); however, different G protein-coupled
receptors can activate cPLA2 through ERK-independent pathways
(21–23). The data presented in this study show that fMLP stimu-
lation of eosinophils activates both ERK1/2 and p38 to activate
cPLA2. In our studies, ERK1/2 phosphorylation preceded cPLA2
activation (Figs. 3 and 4), and pretreatment of eosinophils with the
MEK inhibitor, U0126, inhibited cPLA2 activity (Fig. 7) and AA
release (Fig. 5) caused by fMLP. Pretreatment of eosinophils with
30 μM SB203580 also prevented fMLP-induced cPLA2 activity
(Fig. 7) and AA release (Fig. 5). Thus, our experiments provide
direct evidence that both ERK and p38 MAPKs are required for
cPLA2 activation and AA release in fMLP-stimulated eosinophils.
These results are consistent with recent findings in macrophages
and FcγRIIIa- or FcγRIIB-stimulated neutrophils (56, 57). How-
ever, our data were in contrast with those of Syrbu et al. (41), who
found in human neutrophils that fMLP-mediated ERK1/2/p38 are not
involved in cPLA2 phosphorylation. Taken together, the
findings from those studies and ours suggest that cell-specific pathways most likely are involved in the regulation of eicosanoid synthesis in different inflammatory cells.

Our study showed that relatively high concentrations of SB203580 (10–30 μM) are required to inhibit p38 activity and AA metabolism in fMLP-stimulated eosinophils. Similar concentrations also are required to inhibit p38 activity. This contrasts with the submicromolar concentration of SB203580 that is required to inhibit cytokine production in monocytes (39). However, our study is consistent with prior investigations, which have required high concentrations of SB203580 to suppress fMLP-induced respiratory burst activity and degranulation of neutrophils (58, 59). It should be noted that SB203580 (>5 μM) has recently been reported to affect 3-phosphoinositide-dependent protein kinase 1 activity, which is upstream kinase for protein kinase B (60). However, in fMLP-stimulated eosinophils, no protein kinase B phosphorylation was observed. This excluded the possibility of SB203580 acting on 3-phosphoinositide-dependent protein kinase 1. The concentrations of SB203580 required to block p38 activity in our study, as measured by blockade of ATF-2 phosphorylation, suggests that p38 is involved in cPLA2 activation during AA hydrolysis. Nevertheless, the possibility that SB203580 targets an enzyme other than p38 MAPK cannot be excluded. This is suggested by the inability of the inactive p38 MAPK inhibitor, SB20247, to block AA metabolism at concentrations greater than required for the active inhibitor.

We conclude that fMLP induces AA release from human eosinophils through the activation of cPLA2. ERK1/2 and p38 MAPK regulate cPLA2 activation, which is essential for AA hydrolysis and subsequent LTC4 secretion. Our data demonstrate no significant role for sPLA2, or iPLA2, the other endogenous phos- pholipases, in either the regulation of phospholipid hydrolysis in the production of AA or in the generation of cysteine leukotriene, as modeled by this system of pharmacological activation of human eosinophils.

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References


