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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 2*

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 = 8.5 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 acid trifluoromethyl ketone, a cytosolic PLA₂ (cPLA₂) inhibitor; however, inhibition of neither the 14-kDa secretory PLA₂ by 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propanephosphonic acid nor cytosolic Ca²⁺-independent PLA₂ inhibition by bromoelactone 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 acid 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 isoforms native to human eosinophils possess no significant activity in the stimulated production of AA or LTC₄.

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 isofrom in cPLA2 activation in human eosinophils has not been determined.

1MLP, a tripeptide purified from bacteria, has a variety of biological effects on human eosinophils. These include degranulation, adhesion, chemotraction, superoxide synthesis, and leukotriene synthesis and release (24–27). 1MLP 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 1MLP are mediated by a pertussis toxin-sensitive mechanism, indicating the involvement of one of more members of the G or G, family of heterotrimetric GTP-binding proteins (29, 30). However, the signaling pathway leading to AA metabolism after 1MLP receptor occupation has not been defined in human eosinophils.

The objective of this study was to characterize the isoform of PLA2 involved in arachidonic hydrolysis during activation of human eosinophils. Studies also were performed to determine the contribution of MAPK activation in AA hydrolysis in 1MLP-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 PLA2 isofroms 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 bromomelale lactone (BEL) were purchased from Biomol (Plymouth Meeting, PA). The sPLA2 inhibitor, 3-(3-acetamido-1-benzyl-2-ethylindolyl-5-oxo)propane phosphonic acid (LY311727), 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). 1MLP 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 antiserum in human eosinophils has not been determined.

Methods

Materials and Methods

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 monocytes by the magnetic-activated cell sorting procedure using anti-CD16-coated 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 1MLP 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 Na3P04, 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 PMSF, 2 mM Na3VO4, and 0.5% deoxycholic acid. After 10 min on ice, the sample was centrifuged at 12,000 × g for 20 min to remove nuclear and cellular debris. The supernatant then 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 μA/gel). Electrottransfer of proteins from the was gels to polyvinylidene fluoride membrane was achieved using a semidry transferring condition (15 mA/gel). Immunoblotting with anti-phospho (Thr71) Laf was detected by chemiluminescence (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 1MLP for 1 min. The reaction was stopped by centrifugation, and the pellets were solubilized in 200 μl lysis buffer, as and after removing nuclear and cellular debris, cells were washed three times with TBST and assayed by an ECL chemiluminescence system (Amersham).

Determination of cPLA2 enzyme activity

cPLA2 activity assay was modified from Kim et al. (32). Briefly, 2 × 106 cytochalasin B-pretreated eosinophils were stimulated with or without 1MLP for various times. The reaction was stopped by centrifugation, and the pellets were resuspended in 70 μl sonication buffer (20 mM Tris, pH 8, 2.5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 2 mM Na3VO4, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly (4 × 10 s, at a power setting of 3). Lysates were prewashed with 5 mM DTT on ice for 5 min to inactivate cPLA2, and 10 μl of 50 mM CaCl2 was then added to each sample. A total of 10 μl substrate ([14C]PAPC) was dried to a white film in a test tube containing vigorous vortex mixing. The reaction was initiated by adding 10 μl portion of the substrate (final concentration 9 μM) to cell lysate. The reaction was conducted for 30 min at 37°C and was stopped by adding 560 μl Dole’s reagent (heptane-isopropyl alcohol-1 N H2SO4, 400:390:10 by volume), followed by 110 μl H2O2, vortexed for 20 s, and then centrifuged at 12,000 × g. Upper layer (180 μl) was transferred to 800 μl hexane containing 25 mg silica 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 [3H]AA. After a 2-h incubation period, labeled medium was aspirated, and cells were incubated with 5 μg/ml cytochalasin B for 2 min before stimulation by 1MLP 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. [3H]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 for 30 min, treated with cytochalasin B for 2 min, and then stimulated with 1MLP for 1 min. The reaction was stopped by centrifugation, and the pellets were resuspended in 50 mM pH 8, 2.5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 2 mM Na3VO4, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly (4 × 10 s, at a power setting of 3). Lysates were prewashed with 5 mM DTT on ice for 5 min to inactivate cPLA2, and 10 μl of 50 mM CaCl2 was then added to each sample. A total of 10 μl substrate ([14C]PAPC) was dried to a white film in a test tube containing vigorous vortex mixing. The reaction was initiated by adding 10 μl portion of the substrate (final concentration 9 μM) to cell lysate. The reaction was conducted for 30 min at 37°C and was stopped by adding 560 μl Dole’s reagent (heptane-isopropyl alcohol-1 N H2SO4, 400:390:10 by volume), followed by 110 μl H2O2, vortexed for 20 s, and then centrifuged at 12,000 × g. Upper layer (180 μl) was transferred to 800 μl hexane containing 25 mg silica 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].
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 ACOOCF3, 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. ACOOCF3 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 ACOOCF3, and was further blocked to 1.5 ± 0.4% at 30 μM ACOOCF3 (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 ACOOCF3, and was further blocked to 50 ± 10.4 pg/ml (p < 0.001) with 30 μM ACOOCF3. In addition 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).

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** MAPK phosphorylation in fMLP-stimulated eosinophils. Time-dependent effects of fMLP on ERK1/2 (A) and p38 (B) phosphorylation. Cytochalasin B-pretreated eosinophils were incubated with 1 μM fMLP for indicated times. Eosinophils were lysed, and the lysates were mixed with sample buffer and loaded on 10% SDS-PAGE, followed by immunoblotting with anti-phosphorylation-specific ERK1/2 (A, top panel), anti-ERK1/2 (A, bottom panel), anti-phosphorylation-specific p38 Ab (B, top panel), or anti-p38 (B, bottom panel), as described in Materials and Methods. These results are representative of three experiments. C. Effects of U0126 on fMLP-stimulated ERK1/2 phosphorylation. Eosinophils were preincubated with different concentrations of U0126 for 20 min, incubated with cytochalasin B for 2 min, and stimulated with 1 μM fMLP for 2 min. ERK1/2 phosphorylation (top panel) and total ERK1/2 (phosphorylated + nonphosphorylated) (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 either compound 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.

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 was partially blocked at 3 μM U0126, and was completely blocked by 10 μM U0126 (Fig. 4C).

Stimulation with fMLP also caused an increase in p38 activity (Fig. 4D), 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-induced 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),...
was investigated by using two structurally unrelated Ras farnesyltransferase inhibitors, FPT inhibitor III and manumycin (44, 45). Activation of eosinophils with fMLP caused 6.9 ± 0.4% [³H]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 iMLP inhibited [³H]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 investigations 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 in AA release (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 excluded 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 phosphorylation 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, iMLP was selected 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 identified, iMLP, which binds specifically to a G protein-coupled cell surface receptor, has been used widely to replicate eosinophil activation (27).

Eosinophils possess both the sPLA₂ and cPLA₂ (4, 5), and possibly iPLA₂ (6). The role of specific PLA₂ subtypes involved in receptor-mediated AA mobilization has not been definitively elucidated. 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). Preincubation 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 Ia and group V sPLA₂ with an IC₅₀ of 23 and 36 nM, respectively (36, 53). Our data demonstrate that 10 μM LY311727 had no inhibitory effect on fMLP-stimulated AA or LTC₄ release (Fig. 2). This demonstrates 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 release 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 inhibitor, surfactin (32, 55), also suppressed fMLP-induced eosinophil AA and LTC₄ production (data not shown). We also found that cPLA₂ activation induced by iMLP 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 stimulation 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 iMLP. 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 FcγRIIA- or FcγRIIB-stimulated neutrophils (56, 57). However, our data were in contrast with those of Syrbu et al. (41), who found in human neutrophils that fMLP-mediated ERK1/2 and 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 ability of the inactive p38 MAPK inhibitor, SB202474, 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 phospholipases, in either the regulation of phospholipid hydrolysis in the production of AA or in the generation of cysteinyl leukotriene, as modeled by this system of pharmacological activation of human eosinophils.

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References

ROLE OF MAPK AND cPLA_2 IN EOSINOPHIL AA METABOLISM

468


