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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,* Wonthwa 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 trifluoromethyl ketone, a cytosolic PLA2 (cPLA2) inhibitor; however, inhibition of neither the 14-kDa secretory phospholipase A2 by 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxo)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 isoforms native to human eosinophils possess no significant activity in the stimulated production of AA or LTC4. The Journal of Immunology, 2001, 167: 461–468.

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egulation of leukotriene synthesis in human eosinophils depends upon initial hydrolysis of membrane phospho

lipid into arachidonic acid (AA)3 by phospholipase A2 (PLA2) (1). There are several forms of PLA2, including low mol-

ecule mass secretory PLA2 (sPLA2), an 85-kDa cytosolic PLA2 (cPLA2), and Ca2+-independent PLA2 (iPLA2) (2, 3). While sev-

eral PLA2 isoforms exist within eosinophils, the precise regulatory role of each has not been established (4–6). The 85-kDa cPLA2 has received specific attention because it is AA specific and ap-

pears to represent the enzyme that is distinctively regulated by cell-signaling mechanisms downstream of surface membrane rece-

ceptor (7). A model of dual regulation of cPLA2 has been proposed (8). Agents causing increase in cytosolic Ca2+ concentration have been associated with translocation of cPLA2 from cytosol to an intracellular membrane (9), in which cPLA2 can bind via its N-

terminal Ca2+-dependent lipid-binding domain. Translocation also facilitates access of cPLA2 to phospholipid substrate and, subse-

quently, access of free AA to metabolism by 5-lipoxygenase, which is thought to be a nuclear enzyme (10).

The enzymatic activity of cPLA2 is increased through phosphorylation by mitogen-activated protein kinase (MAPK) (11–13). There are at least three parallel MAPK pathways, the p44/p42 or extracellular signal-regulated protein kinase (ERK) 1/2 pathway, the c-Jun N-terminal kinase (JNK) pathway, and the p38 kinase pathway, which may be activated by diverse stimuli including stress, such as hyperosmolality (14–17). These subgroups are dis-

tinguished by both the sequence of the tripeptide dual phosphor-

ylation motif that is required for MAPK activation and the distinct subgroups of mitogen-activated protein/ERK kinases that activate the ERK group (MEK) and the JNK and p38 groups (MAPK ki-

nase of the stress group). The specific MAPK isoform involved in cPLA2 activation is controversial. Several studies have shown that cPLA2 activity is regulated by phosphorylation via ERK1/2 activation (12, 13, 18–20). However, in thrombin-stimulated platelets and TNF-α-stimulated human neutrophils, p38 MAPK activation

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3Abbreviations used in this paper: AA, arachidonic acid; AACOCF3, arachidonic trifluoromethyl ketone; ATF, activating transcription factor; BEL, bromoeno lactone; cPLA2, cytosolic group IV PLA2; ERK, extracellular signal-regulated protein kinase; FPT, farnesyl protein transferase; iPLA2, cytosolic Ca2+-independent PLA2; JNK, c-Jun N-terminal kinase; LTC4, leukotriene C4; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxo)propanephosphonic acid; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/ERK kinase; PAPC, 1-palmitoyl-2-14C]palmitoyl-l-14C]stearoylphosphatidylcholine; PLA2, phospholipase A2; sPLA2, secretory PLA2.
was attributed to cPLA₂ activation (21, 22). In thrombin-stimulated astrocytes, JNK has been implicated in cPLA₂ activation (23). The role of the specific MAPK isoform in cPLA₂ activation in human eosinophils has not been determined.

cPLA₂, 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). cPLA₂ 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 cPLA₂ are mediated by a pertussis toxin-sensitive mechanism, indicating the involvement of one of more members of the Gαi family of heterotrimeric GTP-binding proteins (29, 30). However, the signaling pathway leading to AA metabolism after cPLA₂ 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 cPLA₂-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 bromo-enol lactone (BEL) were purchased from Biomol (Plymouth Meeting, PA). The sPLA₂ inhibitor, 3-(3-acetamide-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). 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, antiseraum were obtained as previously described (4, 15, 16, 17, 26, 28, 31). 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.

**Isolation of human eosinophils**

Eosinophils were isolated by a method modified from Hansel et al. (31). 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 cPLA₂-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.

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**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 beads separation system 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.

**Immunohistological analysis of MAPK**

Eosinophils (2–3 × 10⁶/group) were preincubated with cytoscholain 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 PMSF, 2 mM Na3VO4, and 0.5% deoxycholic acid. After 10 min on ice, the sample was centrifuged at 12,000 × g for 2 min to remove nuclear and cellular debris. The supernatant was mixed with 14 μl of 6X 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 mA/gel). Electrophoretic transfer of proteins from the gel 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 again was washed three times with TBST and assayed by an ECL chemiluminescence system (Amersham).
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, surafactant, 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,

FIGURE 1. Concentration response and time course of fMLP-stimulated AA release. A, Concentration-dependent effect of fMLP on AA release. [3H]AA-labeled eosinophils were preincubated with cytochalasin B for 2 min at 37°C before stimulation by various concentrations of fMLP for 10 min. Results are presented as the mean ± SEM from five separate experiments. B, Time course of fMLP-stimulated AA release. Eosinophils were preincubated with cytochalasin B for 2 min, and then stimulated by 1 μM fMLP for the indicated times. Results are presented as the mean ± SEM from five separate experiments.

FIGURE 2. Effects of sPLA2, cPLA2, and iPLA2 inhibition on fMLP-stimulated AA release and LTC4 production. [3H]AA-labeled eosinophil s (A) or nonlabeled eosinophils (B) were preincubated with LY311727, AACOCF3, or BEL for 30 min at 37°C, and then incubated with cytochalasin B for 2 min before stimulation by 1 μM fMLP for another 10 min. [3H]AA release (A) was measured by scintillation counting and was calculated by subtraction of background release. LTC4 production (B) was measured by enzyme immunoassay. Results are presented as the mean ± SEM from four separate experiments.
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 3. cPLA2 activity in fMLP-stimulated eosinophil lysates. A. Time-dependent effect of fMLP on cPLA2 activity. Eosinophils were preincubated with cytochalasin B and then stimulated with 1 μM fMLP for indicated times. cPLA2 activity in the eosinophil lysates was measured, as described in Materials and Methods, using substrate [14C]PAPC, and expressed as percentage of nonstimulated eosinophils in three separate experiments. B. Effect of AACOCF3 on cPLA2 activity. fMLP/cytochalasin B-stimulated eosinophil lysates were incubated with the indicated concentrations of AACOCF3 for 10 min at 37°C and then assayed for cPLA2 activity. Data are normalized as percentage of buffer-treated controls from three independent experiments.

### 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 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 results shown are representative of three different experiments. Con, Control.
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. SB20474 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), SB20474 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), p38 activity at 3–10 μM. However, at 30 μM, it also suppressed fMLP-stimulated p38 activity in 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, fMLP 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, fMLP, 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 eosinophil 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 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 PLAs 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 inhibitor, surfactin (32, 55), also suppressed fMLP-induced eosinophil 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 occurs 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 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 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 cytochrome 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 phospholipases, in either the regulation phospholipid hydrolysis in the production of AA or in the generation of cytoeine leukotriene, as modeled by this system of pharmacological activation of human eosinophils.

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References

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