THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 53, pp. 41491–41500, December 31, 2010

Eosinophil Cysteinyl Leukotriene Synthesis Mediated by Exogenous Secreted Phospholipase A_2 Group X*[S]

Received for publication, June 9, 2010, and in revised form, October 15, 2010 Published, JBC Papers in Press, October 25, 2010, DOI 10.1074/jbc.M110.153338

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Secreted phospholipase A_2 group X ($sPLA_2$-X) has recently been identified in the airways of patients with asthma and may participate in cysteinyl leukotriene (CysLT; C_4, D_4, and E_4) synthesis. We examined CysLT synthesis and arachidonic acid (AA) and lysophospholipid release by eosinophils mediated by recombinant human $sPLA_2$-X. We found that recombinant $sPLA_2$-X caused marked AA release and a rapid onset of CysLT synthesis in human eosinophils that was blocked by a selective $sPLA_2$-X inhibitor. Exogenous $sPLA_2$-X released lysophospholipid species that arise from phospholipids enriched in AA in eosinophils, including phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine as well as plasmenyl phosphatidylcholine and phosphatidylethanolamine. CysLT synthesis mediated by $sPLA_2$-X but not AA release could be suppressed by inhibition of $cPLA_2\alpha$. Exogenous $sPLA_2$-X initiated Ser^{505} phosphorylation of $cPLA_2\alpha$, an intracellular Ca^{2+} flux, and translocation of $cPLA_2\alpha$ and 5-lipoxygenase in eosinophils. Synthesis of CysLTs in response to $sPLA_2$-X or lysophosphatidylcholine was inhibited by p38 or JNK inhibitors but not by a MEK 1/2 inhibitor. A further increase in CysLT synthesis was induced by the addition of $sPLA_2$-X to eosinophils under conditions of *N*-formyl-methionyl-leucyl-phenylalanine-mediated $cPLA_2\alpha$ activation. These results indicate that $sPLA_2$-X participates in AA and lysophospholipid release, resulting in CysLT synthesis in eosinophils through a mechanism involving p38 and JNK MAPK, $cPLA_2\alpha$, and 5-lipoxygenase activation and resulting in the amplification of CysLT synthesis during $cPLA_2\alpha$ activation. Transactivation of eosinophils by $sPLA_2$-X may be an important mechanism leading to CysLT formation in the airways of patients with asthma.

Eosinophils are important effector cells of airway inflammation and hyperresponsiveness in asthma, in part through the production of cysteinyl leukotrienes (CysLTs[3]; C_4, D_4, and E_4) (1) that are increased in the airways of patients with asthma (2–4). Eosinophils efficiently synthesize CysLTs because they contain the critical enzyme LTC_4 synthase that is found in close association with 5-lipoxygenase (5-LO) and 5-LO-activating protein during activation (5–7). The first rate-limiting step in this pathway is phospholipase A_2 (PLA_2)-mediated release of unesterified arachidonic acid (AA) from the *sn*-2 position of membrane phospholipids. It is well known that group IVA cytosolic PLA_2 (*i.e.* $cPLA_2\alpha$) plays a major role in endogenous CysLT synthesis in myeloid cells (8, 9); however, 10 mammalian secreted PLA_2s ($sPLA_2$s) have been identified, and at least some of them may coordinate eicosanoid synthesis along with $cPLA_2\alpha$ (10–12). Among these $sPLA_2$s, groups V and X have unique functional capacity to initiate cellular eicosanoid synthesis (13, 14). Studies on $sPLA_2$ group V ($sPLA_2$-V) indicate that this enzyme initiates CysLT synthesis by human eosinophils in the absence of $cPLA_2\alpha$ activation (15, 16).

Recent studies have focused attention on $sPLA_2$s in asthma, particularly $sPLA_2$ group X ($sPLA_2$-X). Total $sPLA_2$ activity is increased in the bronchoalveolar lavage (BAL) fluid (17) and peripheral blood (18) of patients with asthma, and there is an increase in $sPLA_2$ activity in BAL and nasal lavage fluid following allergen challenge in patients with asthma and allergic rhinitis (19–21). We recently demonstrated that $sPLA_2$ group X ($sPLA_2$-X) is increased in the airways of asthmatics with exercise-induced bronchoconstriction (22) and further increased after exercise challenge, a stimulus known to induce CysLT production in the airways (23). Deletion of the $sPLA_2$-X gene in a murine model of asthma inhibits the development of airway inflammation, hyperresponsiveness, and structural remodeling (24). These results suggest that transactivation of eosinophils by $sPLA_2$-X may be an important mechanism leading to CysLT formation in the airways of patients with asthma.

We used recombinant human $sPLA_2$-X to activate CysLT synthesis and AA release in human eosinophils isolated

* This work was supported, in whole or in part, by National Institutes of Health Grants R01HL089215 (to T. S. H.), R01HL50040 (to M. H. G.), and R37HL036235 (to M. H. G.). Some of the results of this study have been previously reported in abstract form (25).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3 and Figs. 1–5.

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[3] The abbreviations used are: CysLT, cysteinyl leukotriene; 5-LO, 5-lipoxygenase; AA, arachidonic acid; BAL, bronchoalveolar lavage; $cPLA_2$, cytosolic phospholipase A_2; LTB_4 and LTC_4, leukotriene B_4 and C_4, respectively; LysoPC, lysophosphatidylcholine; $sPLA_2$, secreted phospholipase A_2; $sPLA_2$-IIA, -V, and -X, $sPLA_2$ group IIA, V, and X, respectively; fMLP, N-formyl-methionyl-leucyl-phenylalanine; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

from donors with a physician diagnosis of asthma and/or allergy. Enzyme inhibitors selective for sPLA$_2$-X and cPLA$_2\alpha$ were used to determine the contribution of the different PLA$_2$ enzymes to CysLT synthesis. Intracellular signaling and cPLA$_2\alpha$ activation mediated by sPLA$_2$-X were assessed by an intracellular calcium assay and cPLA$_2\alpha$ phosphorylation. Because lysophospholipids are known to activate cPLA$_2\alpha$, we used liquid chromatography-tandem mass spectrometry to determine the lysophospholipids species released from human eosinophils by sPLA$_2$-X. Our goals were to determine 1) whether exogenous sPLA$_2$-X participates in CysLT synthesis in humans eosinophils, 2) whether the mechanism of sPLA$_2$-X-mediated CysLT synthesis is dependent on the enzymatic activity of sPLA$_2$-X mediating the release of free AA, 3) the identities of lysophospholipid species generated by sPLA$_2$-X-mediated activation of eosinophils, 4) whether activation of cPLA$_2\alpha$ and 5-LO are involved in sPLA$_2$-X-mediated CysLT synthesis, 5) which MAPK signaling pathways lead to sPLA$_2$-X- and lysophospholipid-mediated CysLT synthesis, and 6) whether sPLA$_2$-X increases CysLT synthesis in eosinophils under conditions of cPLA$_2\alpha$ activation.

EXPERIMENTAL PROCEDURES

Materials—$CHCl_3$ and CH_3OH (HPLC grade) and *n*-hexane and isopropyl alcohol were obtained from Fisher. Ficoll-Paque PLUS (d 1.077) was from GE Healthcare (Piscataway, NJ). Antibodies for immunomagnetic selection were from Miltenyi Biotec (Auburn, CA). [^{3}H]AA was purchased from American Radiolabeled Chemicals (St. Louis, MO). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was from Sigma-Aldrich. Antibodies directed against cPLA$_2$ and Ser^{505}-phosphorylated cPLA$_2$ were from Cell Signaling Technologies (Beverly, MA), and the antibody directed against β-actin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluo4-AM was purchased from Invitrogen. Inhibitors of MEK 1/2 (U0126), p38 (SB203580), and JNK (SP600125) were purchased from EMD Biosciences (San Diego, CA). Rabbit anti-5-LO antiserum was purchased from Cayman Chemical, and rabbit anti-cPLA$_2\alpha$ anti-serum was purchased from Santa Cruz Biotechnology, Inc. Cy3 goat anti-rabbit IgG was from Jackson ImmunoReasearch (West Grove, PA). Deuterated lysophospholipid internal standards were prepared as described (26).

Isolation of Human Peripheral Blood Eosinophils—The University of Washington Institutional Review Board approved the study, and written informed consent was obtained from all participants. Peripheral blood eosinophils were obtained from volunteers with a physician diagnosis of asthma and/or allergy and $\geq 1.2 \times 10^5$ eosinophils/ml of peripheral blood. Granulocytes were isolated from peripheral blood by density gradient centrifugation followed by hypotonic lysis of red blood cells. Eosinophils were removed from the granulocyte fraction by negative immunomagnetic selection. The purity of eosinophils was determined by differential counts of Romanowski-stained (Diff-Quick) cytospin preparations. Eosinophil viability was assessed by trypan blue exclusion.

Preparation of Recombinant sPLA$_2$-X—Recombinant human sPLA$_2$-X protein was produced in an *Escherichia coli* expression system followed by procedures to form disulfide bonds and refold the protein to its native form (27). The purity of the sPLA$_2$-X protein was confirmed by HPLC and SDS-PAGE analysis, and the molecular weight agreed with the calculated value within 0.8 atomic mass units (27). Analysis of purified sPLA$_2$-X using a cell-based assay of IL-8 production by HEK293T cells transfected with TLR4, CD14, and MD2 that express IL-8 in response to lipopolysaccharide but not other TLR ligands showed that the purified protein was devoid of lipopolysaccharide (supplemental Table 1).

Selective PLA$_2$ Inhibitors—Because human eosinophils contain sPLA$_2$ group IIA (sPLA$_2$-IIA) (28), we used a sPLA$_2$ inhibitor, known as ROC-0929, that is selective for sPLA$_2$-X and does not inhibit other mammalian sPLA$_2$s at nanomolar concentrations (29). The compound ROC-0929 is an analog of the well known sPLA$_2$ inhibitor LY315920 (29). Docking studies revealed that the isobutyl group of ROC-0929 sterically excludes this compound from the active site of sPLA$_2$-IIA, but not the active site of sPLA$_2$-X, resulting in >80-fold difference in inhibitory potency of ROC-0929 between the sPLA$_2$-X and sPLA$_2$-IIA enzymes (29). The structure of the inhibitors and inhibitory activity against the full set of human recombinant sPLA$_2$ enzymes are shown in supplemental Table 2. To test for off target effects, we used a control compound, known as ROC-0428, that differs from ROC-0929 by one methyl group and is essentially devoid of sPLA$_2$ inhibition (29) (supplemental Table 2). The inhibitory activities of the ROC-0929 inhibitor and the control compound (ROC-0428) at relevant molar concentrations are shown in supplemental Table 3. Inhibition of cPLA$_2\alpha$ was conducted with Pyr-2 (pyrrophenone) and Wyeth-2 (giripladib) (30, 31).

Measurement of AA and CysLT Release by Eosinophils—The release of AA and CysLT from eosinophils was determined in 24-well plates coated with 0.01% BSA. For CysLT release, eosinophils were resuspended in HBSS with Ca^{2+} and Mg^{2+} at a concentration of 1.5×10^5 cells/well in a 5% CO_2 incubator at 37 °C. The cells were preincubated with either inhibitor or DMSO control for 20 min at 37 °C and then stimulated with sPLA$_2$-X, heat-denatured sPLA$_2$-X, or, in some cases, fMLP as a positive control. Other studies were conducted with co-activation of eosinophils by combinations of fMLP and sPLA$_2$-X. The synthesis of eicosanoids was stopped by the addition of 4 volumes of iced methanol with 0.2% formic acid. An ELISA measured CysLT levels after removal of the methanol by evaporation (Cayman Chemical, Ann Arbor, MI). For AA release studies, eosinophils (1.8×10^5 cells/well) were resuspended in RPMI with 0.01% BSA, and the cells were incubated for 24 h with [^{3}H]AA (0.1 μCi/well). After the unincorporated AA was washed three times with HBSS with 0.01% BSA, the cells were preincubated with inhibitors for 20 min at 37 °C and then stimulated with sPLA$_2$-X and controls. Supernatants were submitted to scintillation counting after being centrifuged to remove detached cells. The remaining eosinophils were detached with 0.25% trypsin with EDTA for 30 min at 37 °C, pelleted, and submitted to scintillation counting. AA release was expressed as a percentage of counts/min in the supernatant to the total counts/min in the cells and supernatant. Each experiment was conducted with two repli-

cates of each condition and repeated at least three times using eosinophils from different donors.

Measurement of cPLA$_2$ Phosphorylation—Phosphorylation of cPLA$_2\alpha$ in eosinophils (2.0×10^6 cells) stimulated with DMSO control, 100 nM sPLA$_2$-X, or 100 nM fMLP for 20 min was determined by the total and Ser^{505}-phosphorylated cPLA$_2\alpha$ measured by Western blot. The cells were pelleted and treated with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 with protease and phosphatase inhibitors) on ice for 30 min. Equal amounts of cell lysate were heated for 10 min in LDS loading buffer with DTT and β-mercaptoethanol and separated on 4–12% BisTris gel under reducing conditions. The resolved proteins were transferred onto polyvinylidene fluoride membrane using a semi-dry apparatus, blocked with 5% nonfat milk, and incubated with rabbit polyclonal antibodies specific for cPLA$_2\alpha$ and Ser^{505} phospho-cPLA$_2\alpha$ overnight at 4 °C. The membrane was also reprobed for β-actin to quantify total cellular protein transferred to the membrane. ECL visualized the proteins of interest.

Confocal Microscopy—Eosinophils (1.0×10^6 cells/well) were allowed to adhere to coverglasses coated with 0.01% BSA and treated with DMSO vehicle control, sPLA$_2$-X (100 nM), or fMLP (100 nM) for 20 min at 37 °C in a 5% CO_2 incubator. The cells were fixed in 3.7% formaldehyde in PBS at room temperature for 30 min. Eosinophils were permeabilized and blocked with 0.1% Triton X-100 with 3% BSA. For the cPLA$_2$ immunostaining, the cells were treated with a rabbit anti-cPLA$_2$ polyclonal antibody diluted 1:50 in 1% BSA. For the 5-LO immunostaining, the cells were treated with a rabbit anti-5-LO polyclonal antibody diluted 1:100 in 1% BSA. The secondary antibody was a Cy3-labeled goat anti-rabbit IgG diluted 1:800 in 1% BSA. Immunostained cells were visualized with a Zeiss LSM510 confocal microscope.

Selective Kinase Inhibitors—Eosnophils (1.5×10^5 cells/well) resuspended in HBSS with Ca^{2+} and Mg^{2+} were preincubated with either DMSO control or MEK 1/2 (U0126 10 μM), p38 (SB203580 30 μM), or JNK (SP600125 20 μM) for 20 min at 37 °C in a 5% CO_2 incubator and then stimulated with sPLA$_2$-X (100 nM), lysophosphatidylcholine (LysoPC) (10 μM), or fMLP (100 nM). The synthesis of eicosanoids was stopped by the addition of 4 volumes of iced methanol with 0.2% formic acid, and the levels of CysLTs were measured by ELISA. Each experiment was conducted with two replicates of each condition and repeated three times using eosinophils from different donors.

Measurements of Cytoplasmic Ca^{2+} in Eosinophils—Cell-wide changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in eosinophils were measured in cells loaded with the fluorescent Ca^{2+} indicator fluo4-AM. Eosinophils were loaded with this Ca^{2+} indicator as described (32). Briefly, cells at a concentration of 2×10^5 cells/200 μl of HBSS with Ca^{2+} and Mg^{2+} were incubated for 30 min in the presence of 5 μM fluo4-AM at room temperature. $[Ca^{2+}]_i$ was imaged using a Bio-Rad Radiance 2100 confocal system coupled to an inverted Nikon TE2000 microscope equipped with a ×60 (numerical aperture = 1.4) lens. Images were acquired every 5 s under control conditions and after cells were treated with sPLA$_2$-X (100 nM), followed by sPLA$_2$-X (200 nM), fMLP 100 nM, and then A23187 (10 μM). Background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (F_0).

Lysophospholipid Analysis—For lysophospholipid analysis, eosinophils (2.0×10^6 cells) were resuspended in HBSS with Ca^{2+} and Mg^{2+} in 0.01% BSA-coated microcentrifuge tubes and treated with DMSO control, fMLP (100 nM), or sPLA$_2$-X (100 nM) for 20 min at 37 °C. The cells were immediately frozen and stored at −80 °C prior to lipid extraction. Each sample was spiked with deuterated internal lysophospholipid standards and then extracted under neutral conditions with $CHCl_3/CH_3OH$ (2:1, v/v), followed by acidification of the remaining aqueous phase with 0.3 M citric acid and further extraction into PBS-saturated $CHCl_3/CH_3OH$ (2:1, v/v). Liquid chromatography electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) with a normal phase column quantified lysophospholipid species. Full details of this analysis have been reported (26).

Statistical Analysis—The release of AA and CysLTs over time after stimulation was assessed with the repeated measures analysis of variance. The change in AA and CysLTs from baseline was assessed with a paired t test. Comparisons of the dose response and between conditions with and without inhibitors were made by analysis of variance. *Post hoc* comparisons were made between control and other conditions with Dunnett's multiple comparison test, and among multiple conditions with Tukey's test. A Kruskal-Wallis test with Dunn's *post hoc* tests was used to compare intensity of the protein on Western blot and to test for the additive effects of sPLA$_2$-X and fMLP on CysLT synthesis.

RESULTS

Subject Characteristics—We isolated peripheral blood eosinophils 36 times from a total of 19 donors for these studies. The mean age of the donors was 29.2 years old, and 74% were female. The study population was 68% Caucasian and 32% Asian. The mean concentration of eosinophils in peripheral blood was 2.67×10^5 eosinophils/ml. The mean number of eosinophils isolated from peripheral blood was 1.17×10^7 eosinophils/donor, with 98.8% viability and 99.7% eosinophils on the cytospin preparation.

Exogenous sPLA$_2$-X Mediates Concentration-dependent CysLT Release by Eosinophils—We measured the effects of recombinant human sPLA$_2$-X added to peripheral blood eosinophils from asthmatics. Exogenous sPLA$_2$-X caused a time-dependent increase in [^{3}H]AA release from eosinophils treated with 100 nM sPLA$_2$-X that reached a plateau about 15 min after the addition of the recombinant enzyme (Fig. 1*A*). Eosinophils treated with 100 nM sPLA$_2$-X had a marked increase in CysLT synthesis that reached a maximum at 20 min after the addition of the enzyme (Fig. 1*B*). Synthesis of CysLTs following the addition of the sPLA$_2$-X was plotted as the change in CysLT synthesis over baseline because there was variability in the unstimulated eosinophil CysLT synthesis among subjects enrolled in the study (data not shown). Exogenous sPLA$_2$-X approximately doubled the CysLT synthesis

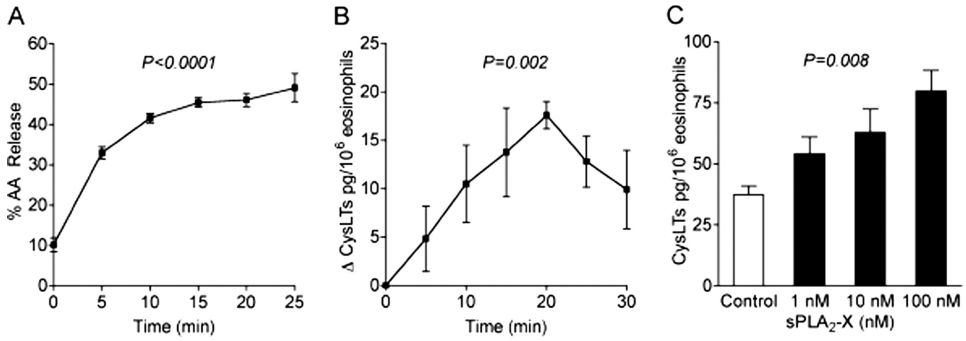

FIGURE 1. **Time course and concentration dependence of sPLA$_2$-X mediated eicosanoid synthesis by eosinophils.** *A*, following treatment with 100 nM exogenous sPLA$_2$-X, there was an increase in [^{3}H]AA release by eosinophils over time that reached a plateau 15 min after addition of the enzyme ($p <$ 0.0001). The [^{3}H]AA release is expressed in terms of percentage of total [^{3}H]AA incorporated. *B*, eosinophil synthesis of CysLTs increased over time after treatment with 100 nM exogenous sPLA$_2$-X, reaching a maximum 20 min after the addition of the enzyme ($p = 0.002$). *C*, the levels of CysLTs following treatment of eosinophils for 20 min with buffer control or 1, 10, or 100 nM sPLA$_2$-X increased with increasing concentrations of the recombinant enzyme ($p =$ 0.008). Error bars, S.E.

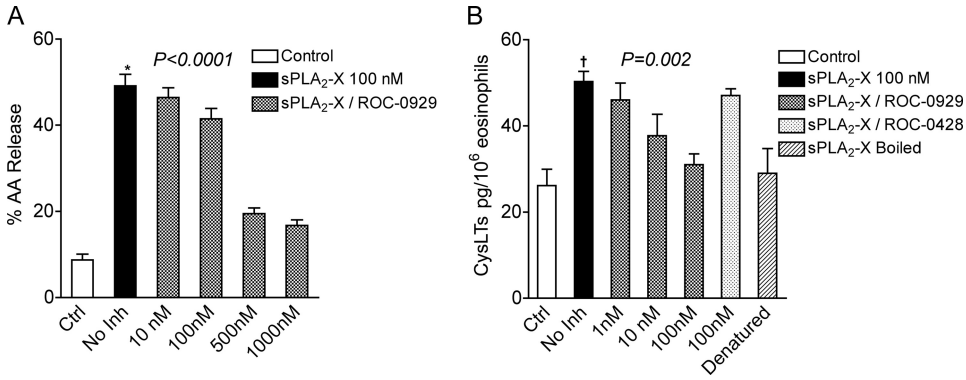

FIGURE 2. **Effects of sPLA$_2$ inhibitors on AA release and CysLT synthesis from sPLA$_2$-X-treated eosinophils.** *A*, eosinophils treated with 100 nM exogenous sPLA$_2$-X (*black bar*) had significant AA release relative to buffer control (*white bar*; *, $p = 0.01$). The sPLA$_2$-X-specific inhibitor (ROC-0929) caused a dose-dependent decrease in [^{3}H]AA release by eosinophils following treatment with 100 nM exogenous sPLA$_2$-X at inhibitor concentrations ranging from 10 to 1000 nM ($p < 0.0001$). *B*, eosinophils also had a significant increase in CysLT synthesis after treatment with 100 nM exogenous sPLA$_2$-X (*black bar*) relative to buffer control (*white bar*; †, $p = 0.002$). The synthesis of CysLTs by eosinophils after treatment with 100 nM sPLA$_2$-X was inhibited in a dose-dependent manner by increasing concentrations of the ROC-0929 inhibitor ranging from 1 to 100 nM ($p = 0.002$). Treatment with a structurally related inhibitor devoid of sPLA$_2$-X inhibitory activity (ROC-0428) had no effect on sPLA$_2$-X-mediated CysLT synthesis. Eosinophils did not synthesize CysLTs in response to 100 nM sPLA$_2$-X that had been heated for 10 min (*Denatured*). *Error bars*, S.E.

over baseline, whereas the release of AA from eosinophils was more than tripled after the addition of sPLA$_2$-X. Based on the time course of AA release and CysLT synthesis, we then assessed the dependence of CysLT synthesis by eosinophils on the molar concentration of sPLA$_2$-X 20 min after the addition of the enzyme. At concentrations of sPLA$_2$-X ranging from 1 to 100 nM, the synthesis of CysLT synthesis by eosinophils increased with increasing concentrations of sPLA$_2$-X (Fig. 1*C*).

CysLT Synthesis Mediated by Exogenous sPLA$_2$-X Is Blocked by a Selective sPLA$_2$-X Inhibitor—We used a selective inhibitor of sPLA$_2$-X that does not inhibit the endogenous sPLA$_2$-IIA (28, 29). Pretreatment of eosinophils with the ROC-0929 inhibitor resulted in dose-dependent inhibition of [^{3}H]AA release 20 min after treatment with 100 nM exogenous sPLA$_2$-X (Fig. 2*A*). Similarly, eosinophil CysLT synthesis in response to 100 nM of sPLA$_2$-X was inhibited in a dose-dependent manner by the ROC-0929 inhibitor (Fig. 2*B*). No inhibition of sPLA$_2$-X-mediated CysLT synthesis was observed with the control ROC-0428 inhibitor, which is structurally similar but unable to bind to the active site of sPLA$_2$-X (supplemental Tables 2 and 3). We also demonstrate that CysLT synthesis was not increased when heat-denatured sPLA$_2$-X was added to human eosinophils. It is notable that 100 nM ROC-0929 only partially inhibited AA release but inhibited CysLT synthesis nearly completely, suggesting that CysLT synthesis mediated by sPLA$_2$-X is not strictly dependent on the concentration of free AA. Taken together, these results indicate that sPLA$_2$-X rather than a trace impurity in the recombinant preparation of sPLA$_2$-X is responsible for AA release and CysLT synthesis by a mechanism involving the active site of the sPLA$_2$-X enzyme.

Generation of Lysophospholipids by Exogenous sPLA$_2$-X—Because of the marked AA release and evidence of cPLA$_2\alpha$ activation, we assessed the release of lysophospholipid species mediated by sPLA$_2$-X in eosinophils as a potential mechanism of cPLA$_2\alpha$ activation. As compared with unstimulated eosinophils as well as fMLP (100 nM)-treated eosinophils, exogenous sPLA$_2$-X caused prominent release of lysophosphatidylinositol (LysoPI), lysophosphatidylethanolamine (LysoPE), lysophosphatidylserine (LysoPS), and LysoPC species (Fig. 3). The complete analysis of lysophospholipid species is presented in supplemental Fig. 3), which also shows the release of smaller quantities of 16:0 and 18:0 lysophosphatidylglycerol but no significant release of lysophosphatidic acid. Eosinophils treated with sPLA$_2$-X also generated 16:1, 18:1 and 18:2 plasmenyl LysoPC species, 16:1 and

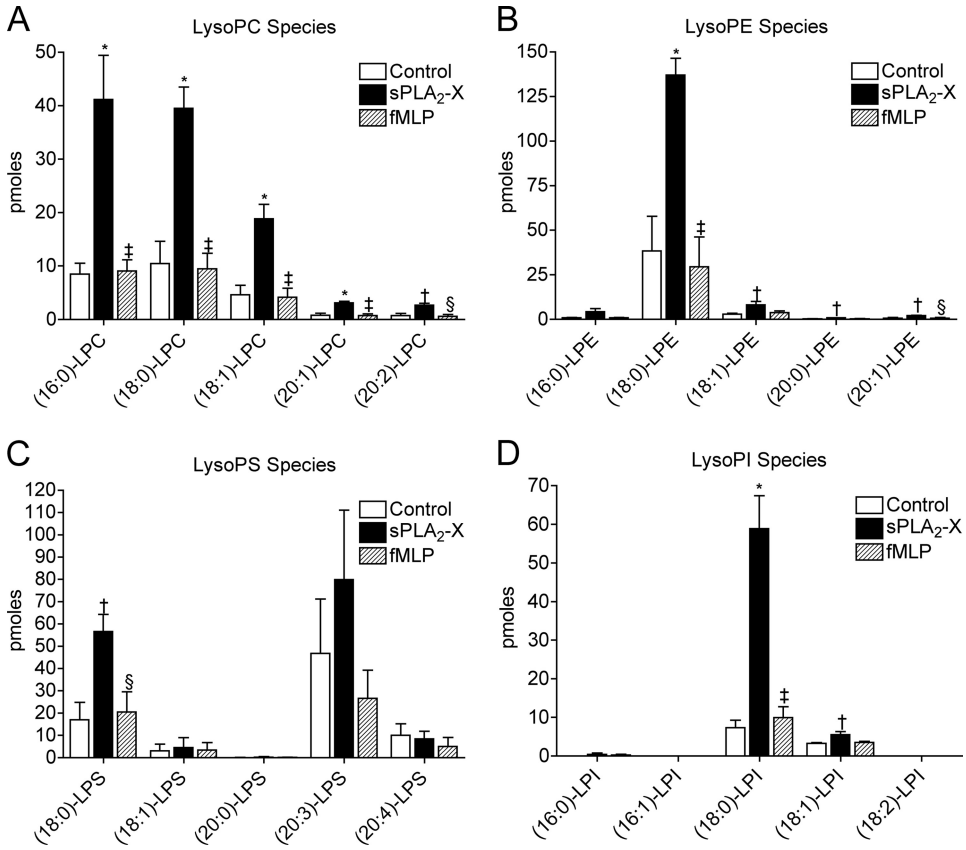

FIGURE 3. **Exogenous sPLA$_2$-X-mediated generation of lysophospholipids by eosinophils.** *A–D*, treatment of eosinophils with sPLA$_2$-X (100 nM) initiated the generation of LysoPC species (*A*), lysophosphatidylethanolamine (*LysoPE*) species (*B*), lysophosphatidylserine (*LysoPS*) species (*C*), and lysophosphatidylinositol (*LysoPI*) species (*D*) relative to control conditions (*white bar*) and relative to eosinophils treated with fMLP (100 nM). *, $p \leq 0.01$ and †, $p \leq 0.05$ overall. ‡, $p \leq 0.01$ and §, $p \leq 0.05$ *versus* fMLP. The complete analysis of lysophospholipid species is presented in supplemental Figs. 3 and 4. *Error bars*, S.E.

18:2 plasmenyl lysophosphatidylethanolamine species, and 16:0 lyso-platelet-activating factor (supplemental Fig. 4). These results demonstrate prominent release of lysophospholipids. Because several lysophopholipid species are known to induce Ca^{2+} flux in cells (33), these results may explain the activation of cPLA$_2\alpha$ by sPLA$_2$-X.

Effects of sPLA$_2$-X Are Partially Mediated through cPLA$_2\alpha$—To determine whether cPLA$_2\alpha$ is involved in the sPLA$_2$-X-induced AA release and CysLT synthesis, we treated eosinophils with two different cPLA$_2\alpha$ inhibitors, Pyr-2 (pyrrophenone) and Wyeth-2 (giripladib), prior to the addition of 100 nM sPLA$_2$-X. Treatment of eosinophils with either cPLA$_2\alpha$ inhibitor did not significantly decrease AA release from eosinophils mediated by sPLA$_2$-X (Fig. 4*A*). In contrast, sPLA$_2$-X-mediated CysLT synthesis was inhibited in a dose-dependent manner by Pyr-2 in concentrations ranging from 1 to 10 μM and was similarly inhibited by 5 μM Wyeth-2 (Fig. 4*B*). Because sPLA$_2$-V activates eosinophil CysLT synthesis in the absence of cPLA$_2\alpha$ activation (15), we treated eosinophils with recombinant human sPLA$_2$-V and found that in contrast to sPLA$_2$-X, neither Pyr-2 (1–10 μM) or Wyeth-2 (5 μM) inhibited sPLA$_2$-V-mediated CysLT synthesis in eosinophils (supplemental Fig. 1). In eosinophils, fMLP causes a modest increase in AA release but marked increase in CysLT synthesis that can be inhibited by either of the cPLA$_2\alpha$ inhibitors Pyr-2 and Wyeth-2 (data not shown). As further evidence of cPLA$_2\alpha$ activation by sPLA$_2$-X, Western blots of cell lysates from eosinophils treated with 100 nM fMLP as a positive control or 100 nM sPLA$_2$-X demonstrate evidence of phosphorylation of cPLA$_2\alpha$ at Ser^{505} mediated by sPLA$_2$-X (Fig. 4*C*). The results were similar for total Ser^{505} phospho-cPLA$_2\alpha$ and for the ratio of Ser^{505} phospho-cPLA$_2\alpha$ to total cPLA$_2\alpha$. Reprobing the blots for β-actin revealed equal protein loading on each of the three blots used to assess cPLA$_2\alpha$ activation. Because an increase in $[Ca^{2+}]_i$ is normally required for cPLA$_2\alpha$ activation, we assessed the $[Ca^{2+}]_i$ in eosinophils following treatment with sPLA$_2$-X (Fig. 4*D*). The addition of sPLA$_2$-X (100 nM) caused a transient increase in $[Ca^{2+}]_i$ that was further increased in frequency by a higher concentration of sPLA$_2$-X (200 nM) and fMLP (100 nM). In contrast to either sPLA$_2$-X and fMLP, treatment of the eosinophils with ionophore (A23187 10 μM) caused a sustained increase in $[Ca^{2+}]_i$. These results indicate that CysLT synthesis more so than AA release in response to exogenous sPLA$_2$-X is mediated through activation of cPLA$_2\alpha$.

Effects of sPLA$_2$-X on cPLA$_2$ and 5-LO Translocation—To better understand the mechanism of sPLA$_2$-X-mediated CysLT synthesis, we conducted confocal microscopy studies of human eosinophils labeled with antibodies directed against cPLA$_2\alpha$ and 5-LO. For these studies, adherent eosinophils were treated with vehicle control containing DMSO, sPLA$_2$-X (100 nM), or fMLP (100 nM) as a positive control. In unstimulated cells, cPLA$_2$ immunostaining was faint and diffuse throughout the cells (Fig. 5*A*). Following treatment of the cells

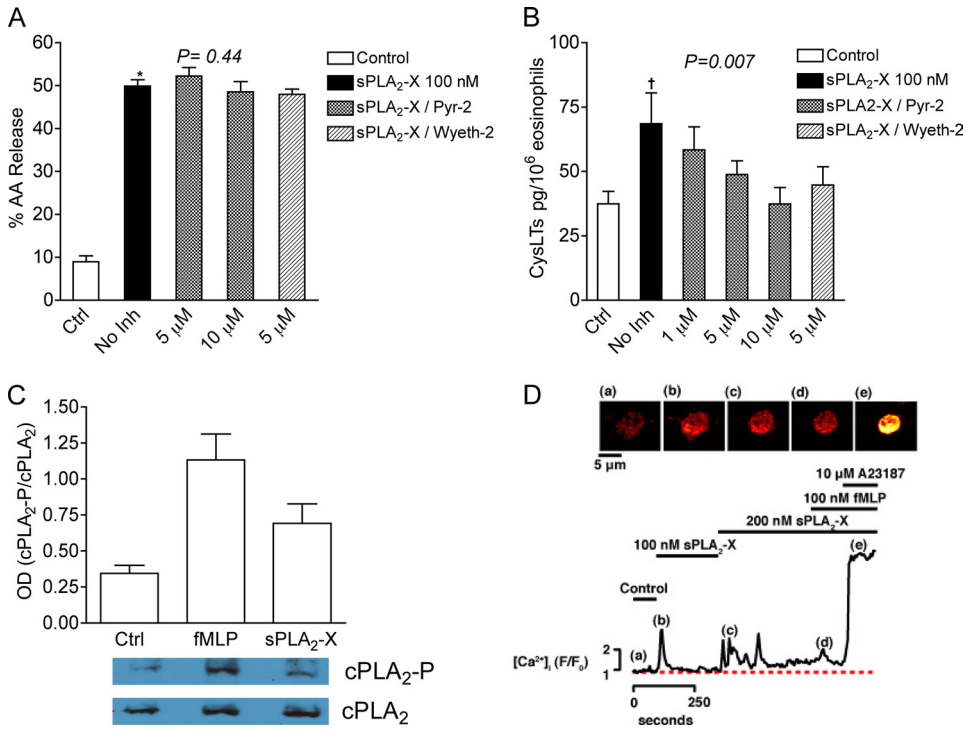

FIGURE 4. **Effects of sPLA$_2$-X mediated by cPLA$_2\alpha$ in eosinophils.** *A*, treatment of eosinophils with 100 nM exogenous sPLA$_2$-X for 20 min (*black bar*) increased [^{3}H]AA release over buffer control (*white bar*; *, $p = 0.001$). The cPLA$_2\alpha$ inhibitors Pyr-2 (5 and 10 μM) and Wyeth-2 (5 μM) failed to significantly decrease sPLA$_2$-X-mediated [^{3}H]AA release by eosinophils ($p = 0.44$). *B*, in contrast to AA release, the significant increase in CysLT synthesis by eosinophils following treatment with 100 nM sPLA$_2$-X (†, $p = 0.02$) was inhibited in a dose-dependent manner by Pyr-2 in concentrations ranging from 1 to 10 μM ($p = 0.007$) and by 5 μM Wyeth-2. *C*, Western blots of cell lysates from eosinophils treated with buffer control (*Ctrl*), 100 nM fMLP, or 100 nM sPLA$_2$-X demonstrate phosphorylation of cPLA$_2\alpha$ at Ser^{505} relative to total cPLA$_2\alpha$. An example blot from one of the three replicate blots from different subjects is shown at the *bottom*. *D*, cell-wide changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by fluo4-AM-loaded eosinophils, reported as the fluorescence intensity at each time point relative to the resting fluorescence (F/F_0). Relative to the buffer control (*a*), the addition of sPLA$_2$-X (*b*; 100 nM) caused an increase in $[Ca^{2+}]_i$ that was further increased by a higher concentration of sPLA$_2$-X (*c*; 200 nM) and fMLP (*d*; 100 nM). Ionophore (*e*; A23187, 10 μM) caused a sustained increase in $[Ca^{2+}]_i$. Images of the intracellular fluorescence at each point are shown at the *top* of the plot. *Error bars*, S.E.

with sPLA$_2$-X, immunostaining became prominent in the perinuclear space as well as focal cytoplasmic staining. Immunostaining in these regions was also found after treatment of eosinophils with fMLP as a positive control. Immunostaining for 5-LO was also observed to be faint and throughout the cytoplasm prior to stimulation of the cell but became more prominent and focal in perinuclear and cytoplasmic locations following treatment with sPLA$_2$-X (Fig. 5*B*). Similar immunostaining for 5-LO was observed for eosinophils treated with fMLP as a positive control. Appropriate controls for the immunostaining revealed little background immunofluorescence (supplemental Fig. 5).

Effects of Kinase Inhibitors on sPLA$_2$-X and LysoPC-mediated CysLT Synthesis—To further examine the signaling mechanism leading to CysLT synthesis in response to sPLA$_2$-X and to consider the effects that are mediated by lysophospholipids such as LysoPC, we determined if selective inhibitors of MEK 1/2 (U0126, 10 μM), p38 (SB203580, 30 μM), and JNK (SP600125, 20 μM) suppressed CysLT synthesis in response to sPLA$_2$-X (100 nM), LysoPC (10 μM), and fMLP (100 nM). Each of these kinase inhibitors is highly selective with little cross-inhibition of the other kinases at the concentrations utilized in this study (34–36). Relative to the maximum amount of CysLT generated by treatment of eosinophils with sPLA$_2$-X (mean 198.3 pg/ml), CysLT synthesis was not inhibited by a MEK 1/2 inhibitor but was significantly inhibited by p38 and JNK inhibitors individually (Fig. 6*A*). Relative to a base-line level of 20.4 pg/ml, eosinophils treated with LysoPC had an increase in CysLT level to 74.9 pg/ml ($p = 0.01$). Eosinophil CysLT synthesis mediated by LysoPC was inhibited by p38 and JNK inhibitors individually but not by a MEK 1/2 inhibitor (Fig. 6*B*). As a control for the experiment, we found that CysLT synthesis mediated by fMLP (mean 371.4 pg/ml) was inhibited by MEK 1/2, p38, and JNK inhibitors independently (Fig. 6*C*).

Additive Effects of sPLA$_2$-X-induced CysLT Synthesis in fMLP-stimulated Eosinophils—Because fMLP-stimulated eosinophils are known to synthesize CysLTs via cPLA$_2\alpha$, directing AA release via 5-LO/5-LO-activating protein toward CysLT synthesis, we determined if there was additional CysLT synthesis in fMLP-activated eosinophils by exogenous sPLA$_2$-X. Eosinophils treated with a low concentration of fMLP (10 nM) had a modest increase in CysLT synthesis that was further increased by the addition of sPLA$_2$-X at concentrations of 10 and 100 nM (Fig. 7*A*). A higher concentration of fMLP (100 nM) initiated robust CysLT synthesis in eosinophils that was further augmented by sPLA$_2$-X (10 and 100 nM) (Fig. 7*B*). Because the wide range of basal and stimulated CysLT production from the four eosinophil donors led to high variance in this analysis, the individual data are shown in supplemental Fig. 2. The eosinophils from all subjects had the same increase in CysLT synthesis in response to fMLP that

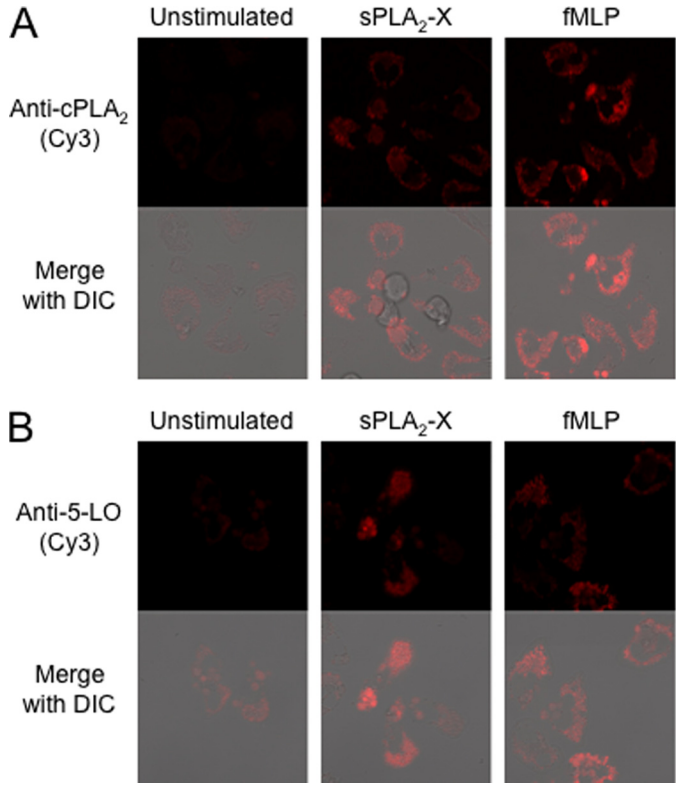

FIGURE 5. **Translocation of cPLA$_2$ and 5-LO in response to sPLA$_2$-X.** Eosinophils were allowed to adhere to BSA-coated coverslips and treated with vehicle alone (*Unstimulated*), sPLA$_2$-X (100 nM), or fMLP (100 nM). The fMLP-stimulated cells served as a positive control. Cells were fixed, permeabilized, and immunostained with antibodies directed against cPLA$_2$ (*A*) and 5-LO (*B*). Fluorescence from the secondary Cy3-labeled antibody was visualized with confocal microscopy. *A*, in unstimulated cells, the cPLA$_2$ immunostaining was faint and diffuse, but the immunostaining increased following treatment of the cells with sPLA$_2$-X and localized in the cells in the perinuclear space as well as punctate cytoplasmic staining. Similar immunostaining was observed for eosinophils treated with fMLP as a positive control. *B*, the 5-LO immunostaining in unstimulated cells was also faint and diffuse, but the immunostaining increased following treatment of the cells with sPLA$_2$-X in both perinuclear and focal intracytoplasmic locations. Similar immunostaining for 5-LO was observed for eosinophils treated with fMLP as a positive control.

was further increased in the presence of sPLA$_2$-X at both concentrations of fMLP. These results demonstrate that sPLA$_2$-X initiates additional CysLT synthesis when added exogenously to eosinophils that are already generating CysLTs via cPLA$_2\alpha$.

DISCUSSION

Disease models (24) and human studies (17, 22) indicate that sPLA$_2$-X may serve as an important mediator of asthma, but the function of sPLA$_2$-X in asthma is not fully understood. In the present study, we demonstrate that sPLA$_2$-X added exogenously to human eosinophils rapidly causes the release of a large portion of labeled AA and CysLT synthesis that is related to the amount of sPLA$_2$-X added exogenously to eosinophils. Both CysLT synthesis and AA release are inhibited by a specific, active site-directed inhibitor of sPLA$_2$-X, indicating that sPLA$_2$-X is responsible for AA release and CysLT synthesis. We found that sPLA$_2$-X causes marked lysophospholipid release from eosinophils, including marked release of LysoPC species known to induce a Ca^{2+} flux in eosinophils. A full analysis of the lysophospholipid species established that free fatty acids are predominantly released from species enriched in AA in human eosinophils, including phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine (37) as well as plasmenyl phosphatidylcholine and phosphatidylethanolamine species (38). We determined that sPLA$_2$-X-mediated CysLT synthesis but not AA release could be suppressed by inhibition of cPLA$_2\alpha$, suggesting that sPLA$_2$-X activates cPLA$_2\alpha$. Following treatment with sPLA$_2$-X, there is evidence of Ser^{505} phosphorylation of cPLA$_2\alpha$ and an intracellular Ca^{2+} flux in eosinophils and translocation of cPLA$_2$ and 5-LO to focal locations in the cytoplasm and in the perinuclear space. The MAPKs p38 and JNK are involved in the CysLT synthesis mediated by sPLA$_2$-X and LysoPC. The addition of sPLA$_2$-X to eosinophils during fMLP-mediated CysLT synthesis indicates that CysLT synthesis is further increased, suggesting that AA released by sPLA$_2$-X contributes to additional CysLT synthesis under these conditions. Taken together, these results indicate that sPLA$_2$-X initiates CysLT synthesis in eosinophils through AA

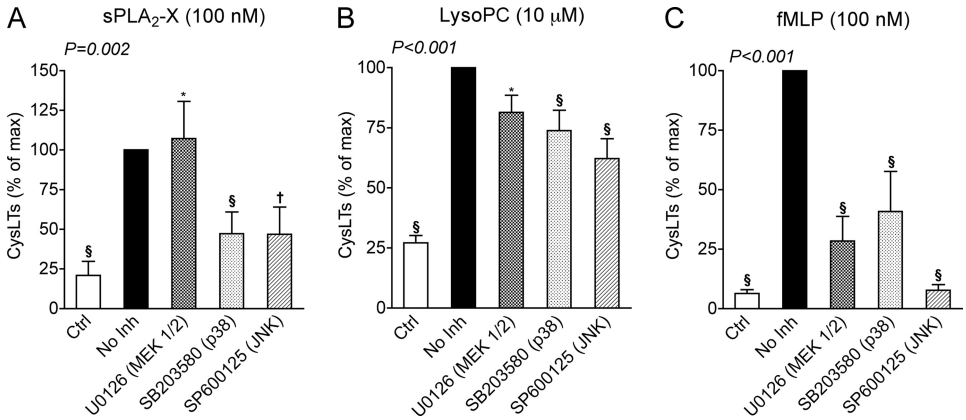

FIGURE 6. **Effects of kinase inhibitors on eosinophil CysLT synthesis.** *A*, relative to the maximum amount of CysLT generated by treatment of eosinophils with sPLA$_2$-X (100 nM) (*black bar*), CysLT synthesis was not inhibited by a MEK 1/2 inhibitor (U0126, 10 μM) but was significantly inhibited by a p38 inhibitor (SB203580, 30 μM) and by a JNK inhibitor (SP600125, 20 μM). *B*, relative to maximum CysLT synthesis by eosinophils treated with LysoPC (10 μM) (*black bar*), CysLT synthesis was not inhibited by a MEK 1/2 inhibitor (U0126, 10 μM) but was significantly inhibited by p38 (SB203580, 30 μM) and JNK inhibitors (SP600125, 20 μM). *C*, eosinophil CysLT synthesis after treatment with fMLP (100 nM) (*black bar*) was individually inhibited by MEK 1/2 (U0126, 10 μM), p38 (SB203580, 30 μM), and JNK (SP600125, 20 μM) inhibitors. *, p = not significant; †, $p < 0.05$; §, $p < 0.01$. *Error bars*, S.E.

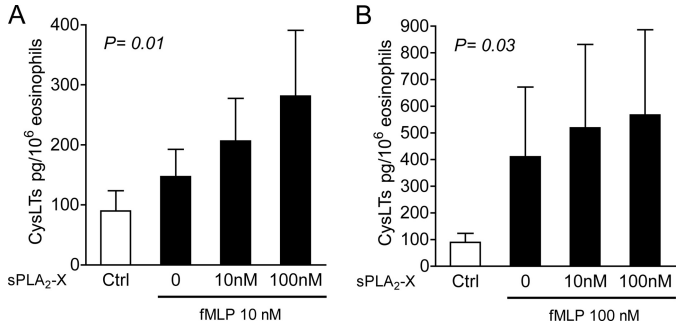

FIGURE 7. **Activation of CysLT synthesis in fMLP-stimulated eosinophils by sPLA$_2$-X.** *A*, in comparison with unstimulated eosinophils (*Ctrl*), eosinophils treated with fMLP (10 nM) had an increase in CysLT synthesis that was further increased by the addition of sPLA$_2$-X at concentrations of 10 and 100 nM ($p = 0.01$). *B*, at a higher concentration of fMLP (100 nM), eosinophil CysLT synthesis was also further augmented by sPLA$_2$-X at concentrations of 10 and 100 nM ($p = 0.03$). Additional plots of the individual data from each eosinophil donor are shown in supplemental Fig. 2. *Error bars*, S.E.

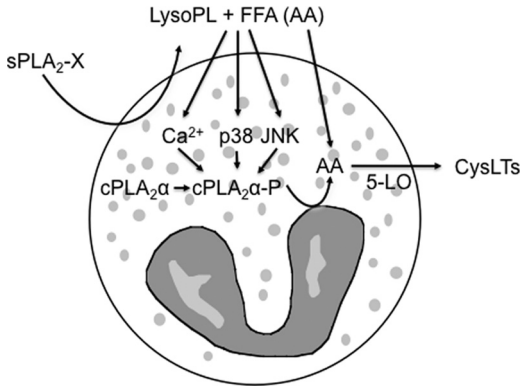

FIGURE 8. **Schematic representation of the events during sPLA$_2$-X-mediated CysLT synthesis by eosinophils.** sPLA$_2$-X causes the release of lysophospholipids (*LysoPL*) and free fatty acids (*FFA*), including AA from phospholipid species enriched in AA. sPLA$_2$-X causes CysLT synthesis that is dependent upon cPLA$_2\alpha$ and initiates a Ca^{2+} flux and cPLA$_2\alpha$ phosphorylation in eosinophils. Prior research has shown that LysoPC causes a Ca^{2+} flux in human eosinophils. We found that the sPLA$_2$-X causes a Ca^{2+} flux and that sPLA$_2$-X- and LysoPC-induced CysLT synthesis could be inhibited by p38 and JNK inhibitors but not by a MEK 1/2 inhibitor. Free AA released by sPLA$_2$-X may contribute to additional CysLT synthesis based on the observation that the addition of sPLA$_2$-X to eosinophils treated with fMLP leads to additional CysLT synthesis.

and lysophospholipid release through a mechanism involving cPLA$_2\alpha$ and resulting in the amplification of CysLT synthesis in cells that are actively synthesizing CysLTs induced by another stimulus (Fig. 8).

We demonstrate here that as little as 1 nM exogenously added sPLA$_2$-X leads to detectable CysLT synthesis in human eosinophils. Both AA release and CysLT synthesis were monophasic in contrast to the biphasic release of AA and LTB_4 synthesis in neutrophils mediated by sPLA$_2$-V (39). Both AA release and CysLT synthesis could be inhibited in a dose-dependent manner by a selective active site-directed inhibitor of sPLA$_2$-X. It is known that eosinophils contain sPLA$_2$-IIA, but the sPLA$_2$-X inhibitor used in this study does not inhibit sPLA$_2$-IIA. In addition, exogenous sPLA$_2$-IIA does not contribute to eosinophil CysLT synthesis (40).

The marked AA release induced by sPLA$_2$-X was inhibited only by the sPLA$_2$-X inhibitor and not by either of the cPLA$_2\alpha$ inhibitors, whereas both the sPLA$_2$-X and cPLA$_2\alpha$ inhibitors attenuated CysLT synthesis. These results suggest that cPLA$_2\alpha$ is necessary for CysLT synthesis but that sPLA$_2$-X-mediated AA release contributes to additional CysLT synthesis in activated cells. The results also suggest that the contribution of total AA release from the lipolytic action of cPLA$_2\alpha$ is relatively small following sPLA$_2$-X addition to cells. The generation of lysophospholipid species from phosphatidylcholine and phosphatidylethanolamine shows that sPLA$_2$-X is acting on phospholipid species that are enriched in AA in human eosinophils (41). Endogenous ^{3}H-labeled AA is incorporated preferentially into 1-alkyl subclasses of phosphatidylcholine > phosphatidylethanolamine > phosphatidylinositol glycerophospholipd species and remodeled into phosphatidylethanolamine species prior to incorporation into endogenous eicosanoids (37). These results demonstrate that human sPLA$_2$-X efficiently mobilizes this pool of AA, resulting in CysLT synthesis due to the release of AA and other phospholipid products. An important observation in the present study is that even under conditions of strong cPLA$_2\alpha$ activation, CysLT synthesis is markedly increased by sPLA$_2$-X, suggesting that once 5-LO associates with 5-LO-activating protein and LTC_4 synthase in conjunction with a stimulus that activates cPLA$_2\alpha$, further CysLT synthesis occurs due to the increased substrate availability mediated by sPLA$_2$-X. Free AA is readily permeable across the cell membrane after release at the outer cell membrane, and the simple addition of free AA to eosinophils results in the synthesis of CysLTs and other eicosanoids (42).

The mechanism of CysLT synthesis mediated by sPLA$_2$-X also clearly involves activation of cPLA$_2\alpha$ as evidenced by the dose-dependent inhibition of CysLT synthesis by two specific cPLA$_2\alpha$ inhibitors as well as evidence of cPLA$_2\alpha$ translocation in response to sPLA$_2$-X. These results are in contrast to the mechanism the of sPLA$_2$-V-mediated CysLT synthesis by eosinophils that occurs in the absence of cPLA$_2\alpha$ phosphorylation and is not inhibited by $AACOCF_3$, an inhibitor of cPLA$_2\alpha$ and calcium-independent group VI PLA_2 (15). The absence of cPLA$_2\alpha$ involvement in sPLA$_2$-V-mediated CysLT synthesis in eosinophils was further confirmed in the present study with two specific cPLA$_2\alpha$ inhibitors. In this regard, the mechanism of sPLA$_2$-X-mediated eosinophil CysLT synthesis is similar to the effects of sPLA$_2$-V on neutrophil LTB_4 synthesis, where sPLA$_2$-V acts to release free fatty acids and lysophospholipids that activate cPLA$_2\alpha$ (39). A major difference between the group V and X enzymes is that sPLA$_2$-X does not bind heparin sulfate proteoglycan and is not internalized by this mechanism that has been described for sPLA$_2$-V and other heparin-binding sPLA$_2$s (43).

We found that exogenous sPLA$_2$-X causes marked generation of lysophospholipid species that was substantially greater than from fMLP-stimulated eosinophils. The predominant lysophospholipid species were LysoPC followed by lysophosphatidylethanolamine and lysophosphatidylinositol. These results are important because LysoPC causes eosinophil infiltration in the airways of guinea pigs (44), and lysophospholipids are increased in asthma and associated with airway hyperresponsiveness (45). Both LysoPC and lysophosphatidylinositol cause eosinophil adhesion at micromolar

concentrations, and LysoPC up-regulates the active conformation of the β_2-integrin CD11b (33). The marked liberation of AA and lysophospholipids may also serve as a signal for cPLA$_2\alpha$ activation via intracellular calcium flux. In human eosinophils, LysoPC causes a non-store-operated sustained Ca^{2+} influx (33). In human neutrophils, LysoPC causes cPLA$_2\alpha$ activation and a Ca^{2+} influx resulting in LTB$_4$ synthesis via sPLA$_2$-V acting on the outer plasma membrane (39). Lysophospholipid signaling in human neutrophils is mediated in part through signaling via the G2A G-protein-coupled receptor (46). The transient Ca^{2+} influx seen in the present study for both sPLA$_2$-X and for fMLP is more typical of a G-protein-coupled receptor mechanism that has been previously shown in the case of fMLP to be an inositol 1,4,5-trisphosphate-dependent signaling mechanism (47). Sphingosine 1-phosphate and lysophosphatidic acid both signal via G-protein-coupled receptors, but there is no direct pathway for sPLA$_2$-X action to lead to SIP generation, and lysophosphatidic acid was not released by sPLA$_2$-X treatment of eosinophils.

Ser^{505} is a consensus phosphorylation site for MAPKs, suggesting that sPLA$_2$-X or a product of sPLA$_2$-X activates cPLA$_2\alpha$ via MAPK-mediated phosphorylation. fMLP is well known to activate ERK 1/2 and p38 MAPK pathways and activate cPLA$_2\alpha$ via this mechanism in human eosinophils (48–50). We demonstrate here that JNK is also involved in CysLT generation mediated by fMLP in human eosinophils. In contrast, the ERK 1/2 pathway is not involved in either sPLA$_2$-X- or LysoPC-induced CysLT synthesis. Although not previously identified in human eosinophils, cPLA$_2\alpha$ activation in other cells has been attributed to JNK (51), and LysoPC has been implicated in the activation of JNK (52, 53). These results indicate that activation of p38 and JNK is involved in the sPLA$_2$-X-mediated synthesis of CysLTs in eosinophils and that CysLT synthesis may be largely a consequence of the generation of lysophospholipids such as LysoPC liberated in response to sPLA$_2$-X.

In summary, this study demonstrates that sPLA$_2$-X causes marked AA and lysophospholipid release, resulting in CysLT synthesis in eosinophils through a mechanism that involves activation of cPLA$_2\alpha$, and further amplifies CysLT synthesis in eosinophils that are actively synthesizing CysLTs. These results have important implications for the mechanism of CysLT formation in the airways of patients with asthma and allergic disease.

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