Regulation of eosinophil function by phosphatidylinositol-specific PLC and cytosolic PLA2

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Sano, Akiko, Xiangdong Zhu, Hiroyuki Sano, Nilda M. Muñoz, Evan Boetticher, and Alan R. Leff. Regulation of eosinophil function by phosphatidylinositol-specific PLC and cytosolic PLA2. Am J Physiol Lung Cell Mol Physiol 281: L844–L851, 2001.—We examined the regulatory role of cytosolic phospholipase A2 (cPLA2) and phosphatidylinositol (PI)-specific phospholipase C (PLC) in the degranulation of human eosinophils and leukotriene (LT) C4 synthesis. Activation with formyl-Met-Leu-Phe + cytochalasin B (fMLP/B) caused a time-dependent release of eosinophil peroxidase (EPO) and LTC4, which was inhibited by pertussis toxin. By immunoblotting, eosinophil PLC-β2 and -γ2 isoforms were identified, and PLC activation was measured as a function of inositol 1,4,5-trisphosphate concentration. Stimulated release of EPO and intracellular Ca2+ concentration was inhibited by ET-18-OCH3, a PI-PLC inhibitor, whereas trifluoromethylketone (TFMK), a cPLA2 blocker, had no inhibitory effect. Both TFMK and ET-18-OCH3 attenuated stimulated arachidonate release and LTC4 secretion, suggesting that activation of both PLC and cPLA2 is essential for LTC4 synthesis caused by fMLP/B. The structurally unrelated protein kinase C inhibitors bisindolylmaleimide, Ro-31–8220, and Go-6976 all blocked fMLP/B-induced EPO release but not LTC4 secretion. 1,2-Bis(2-Aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester, an intracellular Ca2+ chelator, suppressed both EPO release and LTC4 secretion. We found that fMLP/B-induced LTC4 secretion from human eosinophils is regulated by PI-PLC through calcium-mediated activation of cPLA2. However, cPLA2 does not regulate eosinophil degranulation.

arachidonate; protein kinase C; calcium concentration; phospholipase C isoforms

EOSINOPHIL ACTIVATION AND SECRETION of lipid mediators such as platelet-activating factor (PAF), leukotriene (LT) C4, and granular proteins are thought to have a major role in the pathophysiology of eosinophil-associated disease including human asthma (2, 8, 17, 23). However, the signaling pathways that regulate eosinophil secretion of preformed granular proteins and the synthesis and release of lipid mediators are incompletely characterized.

Recent evidence suggests that G protein or protein tyrosine kinase couples cell surface receptors to phospholipase C (PLC) (11). G protein-dependent PLC activation appears to involve the PLC-β2 isoform, which is known to be activated by N-formyl-Met-Leu-Phe (fMLP) in neutrophils (6, 7). In certain cases, receptor-mediated activation of PLC is sensitive to pertussis toxin (PTX), which catalyzes ADP-ribosylation of certain G protein α-units in intact cells, resulting in the uncoupling of these G proteins from cell surface receptors (22). By contrast, receptor-activated protein tyrosine kinases induce phosphoinositide hydrolysis by phosphorylating either PLC-γ1 or PLC-γ2. The activated PLC catalyzes the hydrolysis of phosphatidylinositol (8, 11) biphosphate into two intracellular second messengers, inositol 1,4,5-trisphosphate ([Ins(1,4,5)]P3) and 1,2-diacylglycerol (DAG), which, in turn, increase intracellular Ca2+ concentration ([Ca2+]i) and activate protein kinase C (PKC), respectively (1). Although the specific transmembrane signaling events for triggering eosinophil degranulation have not been elucidated, it has been suggested that PLC is involved in eosinophil degranulation induced by PAF (15) or immobilized immunoglobulin (13).

The intracellular 85-kDa cytosolic PLA2 (cPLA2) is an important enzyme in mediating agonist-induced arachidonic acid (AA) release and eicosanoid production (5, 18, 26). cPLA2 is posttranslationally regulated by both phosphorylation (14, 18, 19, 27) and an increase in intracellular calcium (3, 4, 25). The role of PLA2 in regulating eicosanoid syntheses in eosinophils is widely recognized; however, the role of phospholipases in regulating eosinophil degranulation remains to be defined. We have reported previously that PLA2 regulates both degranulation and LTC4 synthesis by use of two broad PLA2 inhibitors, mepaycin and 4-bromophenyl bromide (BPB) (28).

The objective of this study was to examine the mechanisms of eosinophil degranulation and LTC4 secretion
after G protein-coupled receptor activation by fMLP + cytochalasin B (fMLP/B). Our data suggest that phosphatidylinositol (PI)-specific PLC is the critical enzyme for both eosinophil degranulation and synthesis of the cysteinyl LTC₄. Inhibition of PI-PLC blocks fMLP/B-induced degranulation, whereas inhibition of cPLA₂ selectively inhibits LTC₄ secretion caused by fMLP/B. Our data demonstrate that PI-PLC-mediated calcium influx and PKC activation, but not cPLA₂-activation, are essential for eosinophil degranulation, whereas PI-PLC-mediated calcium influx and cPLA₂ activation are involved in LTC₄ synthesis and secretion from human eosinophils.

MATERIALS AND METHODS

Reagents. fMLP, cytochalasin B (B), and rabbit IgG were purchased from Sigma Chemical (St. Louis, MO). Anti-PLC-β1 and -β2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ET-18-OCH₃, PTX, bisindolylmaleimide, Ro-31–8220, Go-6976, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), and fura 2-AM were purchased from Calbiochem (San Diego, CA). LTC₄ enzyme immunoassay kits and arachidonyl trifluoromethylketone (TFMK) were purchased from Cayman Chemical (Ann Arbor, MI). Anti-rabbit IgG (horseradish peroxidase-linked whole antibody from donkey) and [5,6,8,9,11,12,14,15-3H]arachidonic acid and 3H-myoo-Ins(1,4,5)P₃ radioimmunoassay kits were purchased from Amersham Life Science (Arlington Heights, IL). All supplies for eosinophil isolation were obtained from Wright-Giemsa-stained cytocentrifuge preparations. The viability of isolated eosinophils was confirmed by trypan blue exclusion. Cell preparations contained ≥99% eosinophils and were kept on ice until used.

Measurement of eosinophil peroxidase release and LTC₄ secretion. The release of both eosinophil peroxidase (EPO) and LTC₄ was determined in experimental cells from the same experiments. Eosinophils (2.5 × 10⁶/250 μl) were first treated with either buffer, ET-18-OCH₃ (0.1–10 μM), TFMK (1–30 μM), PTX (1–300 ng/ml), bisindolylmaleimide (0.1–10 μM), Ro-31–8220 (0.1–10 μM), Go-6976 (0.01–1 μM), or BAPTA-AM (0.1–1 μM) before activation with 1 μM fMLP + 5 μg/ml cytochalasin B (fMLP/B). Thirty minutes later, the reaction was terminated by centrifugation, and the supernatant was collected and stored at −80°C until analyzed. Briefly, 50 μl of sample or EPO standard were mixed with 100 μl of substrate (final concentrations 0.1% hydrogen peroxide, 6 mM o-phenylenediamine, and 0.06% Triton X-100 dissolved in 60 mM Tris, pH 8.0) in a polystyrene 96-well microplate and incubated for 30 min at room temperature. Reactions then were stopped by adding 50 μl of 4 M H₂SO₄, and absorbance was measured at 490 nm in a thermoregulating microplate absorbance spectrophotometer (Thermo-max; Molecular Devices, Menlo Park, CA). Final EPO concentrations then were calculated from standard curves fitted by four-parameter analysis.

A separate aliquot of these same cells was used to measure the stimulated secretion of LTC₄, by use of an EIA kit (Cayman Chemical, Ann Arbor, MI).

Measurement of eosinophil Ins(1,4,5)P₃ concentration. Eosinophils (5 × 10⁶ cells/intervention) in Hanks’ balanced salt solution (HBSS) buffer were incubated for 20 min with ET-18-OCH₃ or TFMK before fMLP/B stimulation. The reaction was stopped at the indicated time for kinetic study or at 10 s for the other experiments by the addition of an equal volume of ice-cold 15% trichloroacetic acid and incubated for 20 min on ice. After centrifugation, the supernatant was removed and washed three times with 10 volumes of water-saturated diethyl ether. An aliquot of each neutralized supernatant (100 μl) was then used to determine the Ins(1,4,5)P₃ concentration. Ins(1,4,5)P₃ was measured by competitive radiobinding assay following manufacturer’s instructions (Amersham Life Science).

Measurement of eosinophil arachidonate release. Eosinophils (10⁷ cells/ml) were incubated with 0.5 μCi/ml [³H]arachidonate (AA) overnight and washed three times with HBSS containing 0.2% bovine serum albumin. The cells were divided into aliquots (10⁶ cells/100 μl final volume) and were treated with inhibitors at 37°C for 20 min before activation with fMLP/B activation. After centrifugation, a 90-μl aliquot of the supernatant was counted in 2 ml of scintillation solvent with the Beckman scintillation counter.

Measurement of eosinophil cPLA₂ activity. cPLA₂ activity was measured as described by Kim et al. (12). A total of 2 × 10⁶ eosinophils was preincubated with or without ET-18-OCH₃ or TFMK for 20 min before activation with either buffer alone or fMLP/B for 5 min. The pellet was resuspended in 70 μl of disruption buffer (20 mM Tris, pH 8.0, 2.5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na₃VO₄, 50 mM NaF, and 5 μg/ml pepstatin) and treated with 5 mM dithiothreitol on ice for 5 min to inactivate soluble PLA₂. The reaction was initiated by adding 10 μl of substrate ([14C]PAPC; final concentration 9 μM) to cell lysate for 30 min at 37°C. Extraction was performed by adding 560 μl of Dole’s reagent (heptane-isopropyl alcohol-1 N H₂SO₄; 400:390:10 vol/vol) to sample tube, followed by 110 μl of H₂O. The upper layer was transferred to 800 μl of hexane containing 25 mg of silica gel, and an aliquot (upper layer) was used to count the radioactivity with a liquid scintillation counter.

Immunoblotting analysis. An aliquot of treated eosinophils (2 × 10⁶ cells/intervention) was incubated for 20 min on ice with ice-cold lysis buffer (in mM: 20 Tris-HCl, 30 Na₂HPO₄, 50 NaF, 40 NaCl, and 5 EDTA, pH 7.4) containing 1% Nonidet P-40, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml chymostatin, 1 mM PMSF, 2 mM Na₃VO₄, and 0.5% deoxycholic acid. By centrifugation, nuclear and cellular debris were discarded, and the treated supernatant was boiled for 5 min. Proteins were subjected to 7% SDS-PAGE for detection of PLC isoforms. Membrane containing resolved proteins was exposed to 3% BSA in 20 mM Tris, 500 mM NaCl, and 0.05% Tween 20, pH 7.5 (TBS-T) before addition of primary antibodies (anti-rabbit IgG, anti-PLC-β2, and 1 μg/ml blocking peptide). Membranes were washed three times with TBS-T, and bound antibody was detected with horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody and developed using ECL Hyperfilm.
cells/sample) were dispensed in the cuvettes and allowed to equilibrate at 37°C for 2 min before preincubation with inhibitors. Twenty minutes later, fMLP/B was added, and fluorescence emission was continuously monitored for 5 min to determine the relative alternation in intensity. The concentration of free Ca²⁺ was maintained at 120 ± 20 μM by using 2 mM EGTA and 1.25 mM CaCl₂ in experimental buffer with eosinophils. The fluorescence intensity was monitored using excitation and emission wavelengths of 340 and 500 nm.

**Assessment of eosinophil viability.** In all experimental studies, trypan blue exclusion analysis was performed to assess whether PTX, ET-18-OCH₃, TFMK, bisindolylmaleimide, or Ro-31–8220 affected eosinophil viability. Aliquots of 10⁶ eosinophils were incubated with various concentrations of these inhibitors at 37°C for 120 min (PTX) or for 20 min (ET-18-OCH₃, TFMK, bisindolylmaleimide, or Ro-31–8220). Cells remained >98% viable as determined using a hemocytometer.

**Statistical analysis.** Data are expressed as means ± SE. The difference between various treatment groups was assessed by ANOVA, and where differences were found between groups, statistical significance was determined using a Mann-Whitney unpaired t-test. Statistical significance was claimed when P < 0.05.

**RESULTS**

**EPO release and LTC₄ secretion: markers of eosinophil activation.** fMLP/B induced secretion of EPO and LTC₄ in concentration-dependent manner (Fig. 1). At 1 μM fMLP + 5 μg/ml cytochalasin B (fMLP/B), EPO release was 2,561 ± 979 vs. 121 ± 14 ng/ml for unstimulated cells (Fig. 1A, P < 0.05); LTC₄ secretion increased from 41 ± 33 pg/ml (buffer stimulated) to 1,368 ± 575 pg/ml (P < 0.05) (Fig. 1C). This concentration (1 μM) of fMLP, which caused substantial, but submaximal, release of both EPO and LTC₄, was used for all subsequent experiments.

A time-related increase in EPO release and LTC₄ secretion also was demonstrated after fMLP/B activation (Fig. 1, B and D). At 1 min, EPO release was maximal (3,043 ± 865 vs. 72 ± 16 ng/ml for unstimulated controls; P < 0.01) and was sustained thereafter. In contrast to the rapid release of preformed eosinophil granular protein, LTC₄ secretion was greatest at 10 min (1,476 ± 500 vs. 89 ± 82 pg/ml for unstimulated controls; P < 0.01). Neither buffer alone nor cytochalasin B (no fMLP) caused secretion of EPO or LTC₄.

**Inhibition of stimulated Ins(1,4,5)P₃ concentration, EPO release, and LTC₄ secretion by PTX.** PTX (1–100 ng/ml) caused concentration-dependent inhibition of Ins(1,4,5)P₃ production in eosinophils (Fig. 2A), EPO release (Fig. 2B), and LTC₄ secretion (Fig. 2C). Ins(1,4,5)P₃ concentration decreased from 0.84 ± 0.14 to 0.39 ± 0.10 pmol/10⁶ cells (P < 0.05), and EPO release of 1,782 ± 410 ng/ml was blocked completely to baseline (178 ± 114 ng/ml) after PTX (P < 0.05). Similarly, stimulated LTC₄ secretion of 1,223 ± 117 pg/ml also was blocked completely with 300 ng/ml PTX (P < 0.001 vs. stimulated cells, no PTX).

**PI-PLC isoforms in human eosinophils.** Bands of 120 and 140 kDa were detected on immunoblots after PLC-β2 antibody and -γ2 antibody staining; neither PLC-β1 (a weak band shown in lane 2 of Fig. 3A) was nonspecific because blocking peptide never reduced nonspecific because blocking peptide never reduced PLC-β1 bands were identified (Fig. 3, A and B). To confirm that each antibody was reacting with its intended target protein, PLC antibodies were preincubated with specific blocking peptide before immunoblotting. The 120- and 140-kDa bands were substantially reduced [Fig. 3, A (lane 4) and B (lane 4)].

Ins(1,4,5)P₃ concentration and inhibition by ET-18-OCH₃ and TFMK. PLC activation was measured as a function of Ins(1,4,5)P₃ production (Fig. 4A). In the unstimulated state, Ins(1,4,5)P₃ was 0.27 ± 0.08 pmol/10⁶ cells and increased to 0.71 ± 0.09 pmol/10⁶ cells 10 s after fMLP/B activation. Therefore, Ins(1,4,5)P₃

![Fig. 1. Formyl-Met-Leu-Phe + cytochalasin B (fMLP/B)-induced secretion of eosinophil peroxidase (EPO) and leukotriene (LT) C₄. A and C: EPO release and LTC₄ secretion are both concentration dependent. B and D: kinetics of fMLP/B-induced secretion of EPO and LTC₄. Data are means ± SE of 5 different experiments.](http://ajplung.physiology.org/)

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concentration decreased to baseline level within 2 min (0.32 ± 0.07 pmol/10^6 cells; P < 0.05). For eosinophils pretreated with selective PI-PLC inhibitor ET-18-OCH_3, Ins(1,4,5)_P_3 concentration decreased from 0.84 ± 0.08 to 0.43 ± 0.11 pmol/10^6 cells at 10 s (P < 0.01). In contrast, TFMK, a cPLA_2 inhibitor, had no inhibitory effect on Ins(1,4,5)_P_3 production after stimulation with fMLP/B.

**EPO release and inhibition by ET-18-OCH_3 and TFMK.** Pretreatment of cells with ET-18-OCH_3 attenuated the stimulated release of EPO from 1,926 ± 196 to 493 ± 151 ng/ml (P < 0.05) (Fig. 5). In contrast, EPO release caused by fMLP/B activation was unchanged even after treatment with the greatest concentration of TFMK. Cells remained viable throughout the experimental intervention (data not shown).

**Inhibition of stimulated AA release and LTC_4 secretion by ET-18-OCH_3 and TFMK.** Concentration-dependent inhibition of fMLP/B-induced AA release and LTC_4 secretion was demonstrated for eosinophils treated with ET-18-OCH_3 or TFMK (Fig. 6, A and B). ET-18-OCH_3 blocked the stimulated AA release from 379 ± 52 to 178 ± 80 cpm (P < 0.05); TFMK caused a decrease to 153 ± 34 cpm (P < 0.01 vs. fMLP/B activated, no TFMK). Stimulated LTC_4 secretion (no ET-18-OCH_3) was 2,450 ± 667 vs. 610 ± 398 pg/ml after 10 μM ET-18-OCH_3 (P < 0.05). Similarly, substantial inhibition of LTC_4 secretion was obtained for cells treated with TFMK (P < 0.01 vs. fMLP/B activated, no TFMK). The concentrations of ET-18-OCH_3 and TFMK used in these experiments had no effect on cell viability (data not shown).

**Inhibition of stimulated cPLA_2 activity by TFMK and ET-18-OCH_3.** To assess the effect of TFMK or ET-18-OCH_3 on fMLP/B-stimulated cPLA_2 activity, cPLA_2 activity was assessed in cells pretreated with various concentrations of TFMK and ET-18-OCH_3. TFMK inhibited fMLP-stimulated cPLA_2 activity in a concentration-dependent manner, with maximum inhibition of 73% of stimulated control at 30 μM. At the concentrations that blocked both fMLP/B-induced LTC_4 secretion and AA release, ET-18-OCH_3 had no significant inhibitory effect on stimulated cPLA_2 activity (Fig. 7), indicating that inhibition of LTC_4 secretion and [3H]AA release was not caused by inhibition of cPLA_2.

**Inhibition of stimulated EPO release and LTC_4 secretion by PKC inhibitors and intracellular Ca^{2+} chelator.** The regulatory role of PKC in stimulated EPO release and LTC_4 secretion was assessed using bisindolylmaleimide and Ro-31–8220, two structurally diff-
different broad spectrum of PKC inhibitors. Bisindolylmaleimide or Ro-31–8220 attenuated the stimulated EPO release (Fig. 8A) but not LTC₄ secretion (Fig. 8B). Go-6976, a Ca²⁺-dependent PKC-α and -β₁ isozyme inhibitor, also attenuated the stimulated EPO release.

Fig. 4. A: time course of stimulated eosinophil Ins(1,4,5)P₃ generation. Ins(1,4,5)P₃ concentration in each extract was determined using the [³H]Ins(1,4,5)P₃ assay system (see MATERIALS AND METHODS). B: ET-18-OCH₃, but not trifluoromethylketone (TFMK), blocked the fMLP/B-induced Ins(1,4,5)P₃ generation to near baseline level. Data are means ± SE from 3 independent experiments.

Fig. 5. Blockade of stimulated EPO release by ET-18-OCH₃ and TFMK. A substantial inhibition of EPO release caused by fMLP/B activation after treatment with ET-18-OCH₃; TFMK had no inhibitory effect. Data are means ± SE (n = 5 donors).

Fig. 6. Inhibition of fMLP/B-induced LTC₄ secretion (B) and arachidonic acid (AA) release (A) by ET-18-OCH₃ and TFMK. A: comparable blockade of stimulated AA release was observed after pretreatment with ET-18-OCH₃ and TFMK (n = 7 donors). B: ET-18-OCH₃ and TFMK both blocked the stimulated LTC₄ secretion. Data are means ± SE from 7 independent experiments. AA value is shown as specific AA release by subtracting the control values from each experimental value (n = 4 donors).

Fig. 7. Stimulated cytosolic phospholipase A₂ (cPLA₂) activity. TFMK, but not ET-18-OCH₃, suppressed fMLP/B-induced cPLA₂ activity. Total cellular cPLA₂ activity assay was assessed as described in MATERIALS AND METHODS. Data are means ± SE of 4 separate experiments.

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but not LTC4 secretion (Fig. 8). These data suggest that PKCs, possibly the classical Ca\(^{2+}\)-dependent isozymes, were involved in fMLP/B-induced EPO release but not AA metabolism.

The role of intracellular Ca\(^{2+}\) in stimulated EPO release and LTC4 secretion was assessed using the cell membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM. BAPTA-AM suppressed both EPO and LTC4 release in fMLP/B-stimulated eosinophils concentration dependently (Fig. 8), indicating that [Ca\(^{2+}\)]\(_i\) was involved in both processes.

**Inhibition of stimulated calcium influx by ET-18-OCH\(_3\).** To determine whether Ins(1,4,5)P\(_3\) concentration was associated with the increase in [Ca\(^{2+}\)]\(_i\), after fMLP/B stimulation, [Ca\(^{2+}\)]\(_i\) influx was measured in eosinophils by means of the calcium-sensitive fluorescent dye fura 2-AM. As shown in Fig. 9, addition of fMLP/B caused a transient increase in intracellular calcium and declined to baseline level in <200 s; ET-18-OCH\(_3\) blocked the increase of [Ca\(^{2+}\)]\(_i\) completely.

**DISCUSSION**

The objective of this investigation was to assess the common and selective mechanisms by which PI-PLC causes both eosinophil degranulation and leukotriene synthesis in isolated human eosinophils. We have suggested previously that both functions were regulated by PLA2 (28). These data, however, were obtained before the development of more specific inhibitors of phospholipase isoforms. In these studies, we found that PI-PLC is critical to both fMLP/B-stimulated eosinophil degranulation and leukotriene synthesis and secretion. However, degranulation is likely regulated by PLC-\(\beta\) isoform, which is coupled to G protein (Fig. 3), whereas LTC4 synthesis is regulated by subsequent activation of cPLA2 by PI-PLC-induced [Ca\(^{2+}\)]\(_i\).

Our findings support the concept that eosinophil degranulation is regulated by PI-PLC activation. We found that EPO release corresponded temporally to PLC activation, as measured by Ins(1,4,5)P\(_3\) production (Figs. 1 and 4A). Both EPO release and Ins(1,4,5)P\(_3\) production occurred within 60 s; ET-18-OCH\(_3\), a selective PI-PLC inhibitor, at a concentration that inhibited Ins(1,4,5)P\(_3\) production, suppressed EPO release (24). Because ET-18-OCH\(_3\) induces rapid apoptosis in tumor cells, whereas normal cells remain unaffected (21), we demonstrated in this study that cell viability was always >95%, even at the greatest concentration used, thus excluding nonspecific cytotoxicity as an inhibitory mechanism. Calcium has been shown to play a major role in eosinophil degranulation (15, 16); ET-18-OCH\(_3\) at the concentration that suppressed Ca\(^{2+}\) influx, also inhibited EPO release.

In contrast to our previous observation (28), we found no evidence in this investigation for regulation of eosinophil degranulation by PLA2. In those previous studies, we used mepacrine and BPB to inhibit PLA2 activity. Although these inhibitors are structurally nonhomologous, they have global inhibitory activity on PLA2. We have also noted that mepacrine and BPB...
also likely have nonspecific inhibitory effects in cell secretion, the mechanism of which has not been defined. To address the role of cPLA2 in regulating eosinophil degranulation in this study, we used selective inhibitors of upstream activation of cPLA2 as well as selective inhibitors of cPLA2, which have been developed since the previous investigation. Although our data indicate a selective role for cPLA2 in cysteinyl LT synthesis in eosinophils, we find no evidence that cPLA2 regulates degranulation. Previous publications have suggested a potential role of sPLA2 in regulating eosinophil degranulation (9), and sPLA2 is blocked nonspecifically by mepacrine and BPB. However, preliminary observations have not indicated that selective blockade of sPLA2 causes substantial blockade of eosinophil degranulation during activation with fMLP/B (data not shown). Thus, although the use of selective blocking agents clearly demonstrates a role for intracellular calcium and PLC in regulating eosinophil degranulation, we do not find evidence for PL2-6 in this process as suggested in previous studies using less specific enzyme inhibitors.

We also examined the role of PKC in eosinophil degranulation and LTC4 secretion. Using three different PKC inhibitors (Fig. 8), we demonstrated suppression of fMLP/B-induced EPO release, suggesting that PKC, possibly the classical Ca2+-dependent isozymes, plays an important role in eosinophil degranulation. Our findings suggest that fMLP/B-induced eosinophil degranulation is mediated through a PTX-sensitive G protein that activates the PLC pathway (possibly the β isoform). The generation of Ins(1,4,5)P3 and DAG causes increase in [Ca2+]i (Fig. 9) and activation of PKC. Each step was shown to be required for stimulated eosinophil degranulation in these studies.

In contrast to degranulation of eosinophil protein, leukotriene release from stimulated eosinophils was regulated by both PI-PLC and cPLA2 activation. cPLA2 activation requires two signals, serine phosphorylation by mitogen-activated protein kinase and calcium influx (19). cPLA2 phosphorylation increases its enzymatic activity, whereas calcium influx causes translocation of cPLA2 to intracellular membranes (25, 27). TFMK, which selectively blocks cPLA2, also blocked LTC4 synthesis. Thus leukotriene synthesis caused by PI-PLC depended critically on calcium influx and also subsequently on cPLA2 activation. Similar results have been reported in human basophils (20).

We conclude that fMLP/B activates human eosinophils through PTX-sensitive G protein and activates PLC, possibly the β2 isoform. This causes generation of Ins(1,4,5)P3 and DAG, which, in turn, causes an increase in [Ca2+]i with subsequent PKC activation. Calcium and PKC activation is required for fMLP/B-induced eosinophil degranulation, whereas LTC4 secretion is regulated by PLC through calcium-mediated activation of cPLA2.

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