

Phosphorylation of cytosolic phospholipase A2 by IL-3 is associated with increased free arachidonic acid generation and leukotriene C4 release in human basophils

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Background: Human basophils secrete leukotriene C4 (LTC4) in response to various stimuli, and a short treatment with IL-3 enhances LTC4 release, although IL-3 alone does not induce LTC4 release. However, the mechanism of this priming effect of IL-3 for LTC4 generation remains unknown in human basophils.

Objective: This study was designed to explore the mechanisms by which short treatments with IL-3 enhance stimulated secretion of LTC4, with a focus on the activation of cytosolic phospholipase A2 (cPLA2).

Methods: The phosphorylation state of cPLA2 in human basophils was examined by its shift in electrophoretic mobility as detected by Western blotting. Free arachidonic acid (AA) and LTC4 were measured by gas chromatography–negative ion chemical ionization mass spectrometry and LTC4-specific RIA, respectively.

Result: Human basophils expressed cPLA2. IL-3, as well as the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate, caused a shift in the electrophoretic mobility of cPLA2, which indicated phosphorylation of cPLA2 and therefore its activation. Ionomycin at a concentration of 0.1 $\mu\text{g/mL}$ was used to induce a modest elevation of cytosolic calcium response ($[\text{Ca}^{2+}]_i$), no apparent cPLA2 phosphorylation, and little free AA and LTC4 generation. Pretreatment with IL-3 (1 to 10 ng/mL) markedly enhanced ionomycin (0.1 $\mu\text{g/mL}$)-mediated AA and LTC4 generation. The concentration dependence of cPLA2 phosphorylation by IL-3 and its effects on free AA and LTC4 generation were similar. The selective PKC inhibitors, bis-indolylmaleimide II and Ro-31-8220 inhibited the phorbol 12-myristate 13-acetate-mediated cPLA2 electrophoretic mobility shift, but not the IL-3-mediated shift, suggesting that the IL-3 effect is PKC independent. Both the anaphylatoxin split product of the C component C5 (C5a) and f-Met-Leu-Phe induced PKC-independent cPLA2 phosphorylation with a similar time course most notable for the absence of observable changes in cPLA2 phosphorylation before 30 seconds. These results suggested an explanation for the absence of free AA generation by C5a. When $[\text{Ca}^{2+}]_i$ was ele-

vated in response to C5a, there was no phosphorylation of cPLA2, and by the time cPLA2 became phosphorylated, $[\text{Ca}^{2+}]_i$ had returned to resting levels. Treatment with IL-3 preconditioned the cPLA2 by causing its phosphorylation so that the transient $[\text{Ca}^{2+}]_i$ response, which followed stimulation by C5a, could induce the generation of free AA and LTC4. **Conclusion:** Taken together, these results suggest that the effect of IL-3 for free AA generation and LTC4 release might be due to induction of cPLA2 phosphorylation. The studies demonstrated a need for synchronous cPLA2 phosphorylation and elevations in $[\text{Ca}^{2+}]_i$. (*J Allergy Clin Immunol* 1998;102:512-20.)

Key words: Human basophils, IL-3, arachidonic acid, leukotriene C4, cytosolic phospholipase A2

Secretion from human basophils is upregulated by a variety of cytokines of which IL-3 is generally the most efficacious. IL-3 is known to enhance the secretion of the 3 major classes of mediators: preformed mediators (eg, histamine), newly synthesized lipid mediators (eg, leukotriene C4 [LTC4]), and newly synthesized cytokines (eg, IL-4). In addition, IL-3 affects secretion induced by all known stimuli.¹⁻⁴ There appear to be 3 distinct phases to the changes induced by IL-3. Short treatments with IL-3 (5 to 15 minutes) result in modest enhancement of histamine, LTC4, and IL-4 secretion with no changes in the cytosolic calcium response ($[\text{Ca}^{2+}]_i$) that follows stimulation with a variety of secretagogues.¹ Treatment for 24 hours induces marked enhancement of LTC4 and IL-4 secretion that is accompanied by a marked increase in the $[\text{Ca}^{2+}]_i$ response.⁴⁻⁶ Finally, treatment for 3 to 5 days can convert nonreleasing basophils into releasing basophils.⁷ It seems likely that these distinct phases reflect progressive changes in basophil gene expression, although little is understood of the mechanisms for human basophils. A variety of studies from this laboratory have focused on the changes induced by IL-3 within the first few minutes of treatment. A particularly useful model of the effect of IL-3 within this time frame is its effect on the anaphylatoxin split product of the C component C5 (C5a)-induced LTC4 release. C5a induces marked histamine release from human basophils with little or no LTC4 release.^{1,8} After a 5-minute treatment with IL-3, LTC4 release resembles that of other complete secretagogues, such as f-Met-Leu-

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Abbreviations used

AA:	Arachidonic acid
C5a:	Anaphylatoxin split product of the C component, C5
[Ca ²⁺] _i :	Cytosolic calcium response
cPLA2:	Cytosolic phospholipase A2
fMLP:	f-Met-Leu-Phe
LTC4:	Leukotriene C4
MAPK:	Mitogen-activated protein kinase
PKC:	Protein kinase C
PMA:	Phorbol 12-myristate 13-acetate
PMSF:	Phenylmethylsulfonyl fluoride
sPLA2:	Secretory phospholipase A2
TBST:	Tris-buffered saline/Tween 2.0

Phe (fMLP) or anti-IgE antibody. The use of this particular model leads to questions about the mechanisms of free arachidonic acid (AA) generation and its subsequent metabolism to LTC4. In an effort to understand how IL-3 upregulates LTC4 secretion, it is necessary to determine the behavior of the various components of the LTC4 generating process.

AA is the precursor for a variety of proinflammatory lipid mediators, including the leukotrienes and prostaglandins, and the production of these potent agents is controlled by the availability of free AA.⁹ The release of AA from membrane phospholipids is a regulated process that is catalyzed by phospholipase A2 (PLA2) and that occurs in many cell types in response to a variety of physiologic and pharmacologic agonists. There is considerable controversy over the type of PLA2 involved; both secretory and cytosolic phospholipase 2 (sPLA2 and cPLA2, respectively) have been implicated. We have suggestive evidence of a role of sPLA2 in LTC4 generation¹⁰ but have not yet explored a role for cPLA2.

cPLA2 has been purified from several sources, and 1 form has been cloned.¹¹⁻¹³ On the basis of the partial amino acid sequence of the enzyme, the molecular mass based on the predicted sequence is 85 kd, slightly lower than the apparent size determined by gel electrophoresis (~95 to 110 kd). cPLA2, which shows specificity for hydrolyzing phospholipids with AA at the sn-2 position,^{14,15} is posttranslationally regulated both by phosphorylation and by the level of intracellular calcium. These properties suggest that cPLA2 may be specialized in cellular signaling.^{16,17} Elevations in the [Ca²⁺]_i⁹ do enhance the catalytic activity of cPLA2, but these elevations are required for the binding of cPLA2 to phospholipid in membrane.^{18,19} Calcium-dependent membrane binding of cPLA2 occurs at nanomolar calcium levels and is mediated by a calcium-dependent lipid binding domain present at the amino terminus of cPLA2.^{12,19,20} In various cells the stimulated phosphorylation of cPLA2 occurs exclusively on serine residues.²¹⁻²³ cPLA2 is a substrate for mitogen-activated protein kinase (MAPK), and phosphorylation by MAPK induces a consistent increase in cPLA2 activity and decreases its electrophoretic mobility (gel shift).^{11,24} cPLA2 has 1 consensus site for MAPK at Ser-505.¹¹ Activation of cPLA2 caused by phosphorylation is often observed secondary

to protein kinase C (PKC) activation directly or indirectly by means of MAPK activation.^{12,21,25} An increase in [Ca²⁺]_i, which promotes association of the cPLA2 with the membrane, and phosphorylation of cPLA2 is required for full activation.^{11,26}

Although there are other means by which free AA may be generated, a role for cPLA2 in human basophil free AA generation seems likely. These studies were undertaken to explore whether IL-3 alters the activation state of cPLA2 and to explore the association between cPLA2 phosphorylation and the secretion of free AA and LTC4.

METHODS

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, phorbol 12-myristate 13-Acetate (PMA), NaF, Na₄P₂O₇, Na₃VO₄, 2-mercaptoethanol, NP-40, fMLP, and human recombinant C5a (Sigma, St Louis, Mo); crystallized human serum albumin (Miles Laboratories, Elkhart, Ind); FCS and RPMI 1640 containing 25 mmol/L HEPES (GIBCO-BRL, Grand Island, NY); Percoll (Pharmacia, Piscataway, NJ); Tris (hydroxymethyl)-aminomethane and Tween-20 (Bio-Rad, Hercules, Calif); leupeptin, dithiothreitol, and phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim, Indianapolis, IN); 4 α -phorbol 12-myristate 13-acetate (LC Laboratories, Woburn, Mass); peroxidase-labeled donkey anti-rabbit Ig antibody (Amersham Life Science, Arlington Heights, Ill); ionomycin, bis-indolylmaleimide II, and Ro-31-8220 (Calbiochem, La Jolla, Calif); Fura-2AM (Molecular Probes, Eugene, Ore); and recombinant human IL-3 (Biosource, Camarillo, Calif). Rabbit anti-cytosolic PLA2 antibody and recombinant human cytosolic PLA2 were provided by Dr Lisa Marshall (Smithkline Beecham, King of Prussia, Pa).

Buffers and media

PIPES-albumin-glucose (PAG) buffer contained 25 mmol/L PIPES, 110 mmol/L NaCl, 5 mmol/L KCl, 0.1% glucose, and 0.003% human serum albumin. PAGCM was PAG supplemented with 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. PAG-EDTA was PAG supplemented with 4 mmol/L EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% human serum albumin.

Basophil purification

Basophils were purified from residual cells of normal donors undergoing leukapheresis by using Percoll density gradient and countercurrent elutriation as previously described.⁵ In the experiments presented, basophil purities were greater than 80%, as determined by alcian blue staining.²⁷

Anti-IgE positive selection

For the preliminary studies of expression of cPLA2 in human basophils, these cells were purified further by positive selection with anti-IgE antibody. To minimize concerns that the positive selection technique might induce changes in cPLA2 status, this entire procedure was carefully carried out below 4° C and the cells were suspended in PAG-EGTA (50 μ mol/L). The cells were incubated for 10 minutes with 1 μ g/mL mouse anti-human IgE (TES-19) (kindly provided by Dr Frances Davis at Tanox Corp) in the presence of 4 mg/mL normal human IgG to block Fc γ R. After a subsequent 20-minute incubation with rat anti-mouse IgG2a+b paramagnetic beads (8 μ L per 10⁷ cells), the cells were passed through a MACS mini-column (MACS system; Miltenyi Biotec Inc, Sunnyvale, Calif). Flow through cells were collected, and the eluted basophil purities were found to be between 98% and 99.4%.

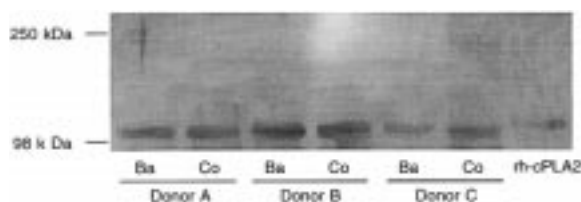


FIG 1. Expression of cPLA2 in human basophils and contaminating cells. Basophils (*Ba*) (basophil purity: 98% to 99.4%) and contaminating cells (*Co*) (basophil purity: <1%), which typically contaminate enriched basophil preparations (mainly lymphocytes and monocytes) were lysed at 2×10^7 cells/mL. Lysates containing 2×10^5 cells or human rh-cPLA2 (10 ng) were subjected to Western blot analysis as described in Methods section.

Western blot analysis for expression of cPLA2

High-speed cell pellets ($\approx 14,000 g$ for 5 to 10 seconds) were resuspended at 2×10^7 cells/mL in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA, 10 mmol/L EGTA, 5 mmol/L dithiothreitol, 1% NP-40, 1 mmol/L PMSF, 20 μ g/mL leupeptin, 100 μ g/mL aprotinin, and 10 mmol/L benzamidine). After 20 seconds of vortexing, the cell lysates were kept on ice for 20 minutes and microfuged for 15 minutes at $4^\circ C$. Supernatant was collected as a protein extract containing lysed cell components without nuclei. Extracts containing equal cell numbers (2×10^5 cell equivalents/lane) were diluted with an equal volume of 2 $\times$ loading buffer (0.125 mol/L Tris-HCl [pH 6.8], 4% SDS, 0.005% bromophenol blue, and 20% glycerol) (NOVEX, San Diego, Calif) containing 0.05% 2-mercaptoethanol and subjected to electrophoresis on 4% to 20% Tris glycine density gradient gels (NOVEX) at 135 V for 2 hours. Gels were then transferred to pure nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by Trans Blot (NOVEX) at 35 V for 4 hours. After transfer, membranes were immersed in TBST containing 5% nonfat dry skim milk (Carnation, Los Angeles, Calif) overnight to block nonspecific binding. Membranes were then washed 3 $\times$ for 5 minutes each with TBST. Immunoreactive proteins were detected by using anti-cPLA2 antibody, which was diluted in TBST (1/2000 dilution) containing 1% skim-milk. After a 4-hour incubation, membranes were washed with TBST and were incubated with peroxidase-labeled donkey anti-rabbit Ig antibody for 1 hour. After 5 $\times$ 10-minute washes, membrane-bound anti-rabbit Ig antibody was visualized by means of ECL Western blotting detection reagents (Amersham Life Science, Arlington Heights, Ill) and Hyper-ECL luminescence detection film (Amersham Life Science).

Electrophoretic separation of phosphorylated and nonphosphorylated cPLA2

After stimulation, the reactions were stopped by ice-cold PAG and microfuged for 5 to 10 seconds. Cell pellets were lysed in ice-cold hypotonic lysis buffer (20 mmol/L Tris [pH 7.5], 2 mmol/L EDTA, 2 mmol/L EGTA, 5 mmol/L dithiothreitol, 1 mmol/L PMSF, 10 mmol/L benzamidine, 100 μ g/mL aprotinin, 200 μ g/mL leupeptin, 50 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4 , and 1% NP-40). Extracts containing equal numbers of basophils (3×10^5 cell equivalents/lane) were diluted with an equal volume of 2 $\times$ loading buffer. To separate phosphorylated cPLA2 from nonphosphorylated cPLA2, samples were subjected to electrophoresis on 8% Tris glycine gels (NOVEX) at 150 V for 4 hours. After electrophoresis, proteins were transferred to nitrocellulose (35 V for 4 hours), and immunoblotting was performed as described above.

Fura-2AM labeling and $[Ca^{2+}]_i$ measurements

Basophils were labeled with 1 μ mol/L fura-2AM for 20 minutes at $37^\circ C$ in RPMI 1640 containing 0.32 mmol/L EDTA and 2% FCS (300,000 to 500,000 cells in 200 μ L). After washing once with 200 μ L PAG, the cells were resuspended in PAG for loading in the microscope chamber.¹ $[Ca^{2+}]_i$ was measured by digital video microscopy by using techniques previously described in detail.^{1,28} Briefly, 15 μ L of cells (20,000 to 30,000) were loaded onto the siliconized cover slip of the microscope chamber, and after settling, they were overlaid with 1 mL of PAGCM buffer. After warming to $37^\circ C$, monitoring of the cells was begun, and after 4 frames (each frame is a single ratio measurement of a field of 30 to 100 cells) of prechallenge $[Ca^{2+}]_i$ levels were acquired, the cells were challenged with 1 mL of stimulus in buffer. Data were then acquired for 50 to 150 frames at intervals of 1 to 10 seconds to determine the subsequent $[Ca^{2+}]_i$ response.

AA measurements

The mass of AA was determined by gas chromatography–negative ion chemical ionization mass spectrometry with modifications of described procedures.^{1,29} One hundred thousand cells were challenged in a final volume of 100 μ L of PAGCM at $37^\circ C$. The reactions were terminated with the addition of 1 mL acetone followed immediately by the addition of 15 to 20 ng of octodeuterated AA (as an internal standard). Reaction mixtures were transferred to silanized glass vials and were dried under dry N_2 . The carboxyl moieties of AA were converted to pentafluorobenzyl esters as described.¹ Once derivatized, the residue was dissolved in 60 μ L of dodecane for injection into the gas chromatograph. The GC/(NICI)MS conditions for measurement of derivatized AA have been described in previous reports.^{1,29}

LTC4 measurements

Fifty thousand cells were challenged in a final volume of 100 μ L of PAGCM at $37^\circ C$. The reactions were terminated with 400 μ L of ice-cold PAG-EDTA, and the suspensions were centrifuged in a microfuge at 14,000 rpm for 10 seconds. Aliquots (100 μ L) of supernatants were assayed with an LTC4-specific RIA as described previously.^{30,31}

RESULTS

Expression of cPLA2 in human basophils

To determine whether cPLA2 was present in human basophils and to determine the amount relative to contaminating cells in these preparations, the proteins obtained from purified basophils and contaminating cells were examined by Western blotting. As described in the Methods section, beginning with basophils at 70% to 90% purity, the basophils were further purified by positive selection with anti-IgE antibody. This procedure resulted in a fraction containing the contaminating cells (the cells that wash through the magnetized mini MACS column contain <1% basophils) and a fraction consisting of 98% to 99.4% basophils. The cell lysates from the 2 populations were examined, and basophils and contaminating cells were found to express essentially equivalent cPLA2 levels (Fig 1). On the basis of comparisons with dilutions of recombinant human cPLA2 protein analyzed in the same gels, human basophils expressed approximately 50 ng/ 10^6 cells. In subsequent experiments the contribution of cPLA2 from contaminating cells was

minimized by using basophils at greater than 80% purity (but without positive selection).

Phosphorylation of cPLA2 with IL-3 and PMA

The phosphorylation of cPLA2 on the Ser-505 residue by MAPK was shown to activate the enzyme and to lead to a decreased electrophoretic mobility¹¹ and retardation of cPLA2 on SDS- polyacrylamide gels, and it has been used as an indicator of cPLA2 phosphorylation. This technique was used to detect cPLA2 phosphorylation in stimulated basophils. It has been shown in other cell types that activation of PKC leads to phosphorylation of cPLA2.^{12,21,25} Therefore PMA was used as a positive control for subsequent experiments. Basophils were treated with PMA or IL-3 for 15 minutes, and the electrophoretic mobility shift was examined by using 8% gels as shown in Fig 2, A. A similar shift in electrophoretic mobility for cPLA2 followed treatment with both PMA and IL-3. 4 α -PMA, a negative control for PMA, did not affect the shift in cPLA2 mobility (data not shown). Several previous studies have shown that treatment of cell lysates with potato acid phosphatase converts cPLA2 from the slower migrating (phosphorylated) form into the faster migrating (dephosphorylated) form. This was also found to be true for the cPLA2 derived from basophils stimulated with PMA or IL-3 (Fig 2, C). Previous studies have shown that more than 1 to 2 minutes of IL-3 treatment are required to prime C5a-mediated LTC4 release from basophils.⁸ Fig 2, B demonstrates that there is little apparent mobility shift in cPLA2 obtained from basophils treated for less than 1 minute with 10 ng/mL IL-3, but as found for priming of C5a-mediated LTC4 release, 5 to 15 minutes of IL-3 treatment results in a maximal shift in cPLA2 mobility. The relatively rapid onset of priming suggests that IL-3 does not induce de novo protein synthesis of cPLA2 within this time frame. To confirm this assumption, the effect of IL-3 on expression of cPLA2 was examined; cPLA2 levels remained constant throughout a 45-minute incubation with IL-3 (Fig 3).

Inhibition of cPLA2 phosphorylation by PKC inhibitors

Both IL-3 and PMA caused a mobility shift in cPLA2. To test whether IL-3-induced phosphorylation of cPLA2 results from the action of PKC, the cPLA2 mobility shift was examined in basophils stimulated with IL-3 in the absence or presence of PKC inhibitors bis-indolylmaleimide or Ro-31-8220. Basophils were preincubated with PKC inhibitors bis-indolylmaleimide II or Ro-31-8220 for 15 minutes at the concentrations shown in Fig 4 and then incubated with IL-3 (1 ng/mL) or PMA (10 ng/mL) for 15 minutes before lysis. We have previously determined that 500 nmol/L of either bis-indolylmaleimide II and Ro-31-8220 would completely inhibit PMA-induced histamine release from human basophils, indicating that these concentrations should be sufficient to inhibit PKC activity (data not shown). Both bis-indolylmaleimide II and Ro-31-8220 inhibited the PMA-

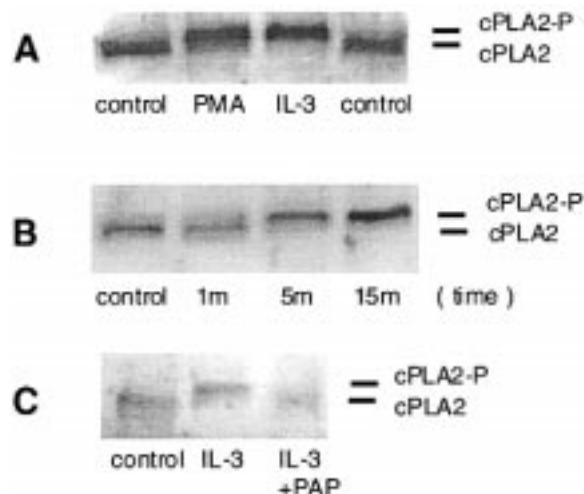


FIG 2. A, Electrophoretic mobility shift of cPLA2 incubated with IL-3 and PMA. Basophils were incubated with IL-3 (10 ng/mL) or PMA (100 ng/mL) for 15 minutes. Reactions were stopped by ice-cold PAG, and samples were microfuged. Cell pellets were resuspended with ice-cold hypotonic lysis buffer. The cPLA2 mobility shift was examined by Western Blot as described in Methods section. Blot shown is representative of 2 separate experiments. **B,** Time course of electrophoretic mobility shift of cPLA2 by IL-3. Basophils were incubated with IL-3 (10 ng/mL) for times indicated. cPLA2 mobility shift was examined by Western blot, as described above. Blot shown is representative of 2 separate experiments. **C,** Treatment of whole-cell lysate with potato acid phosphatase before analysis by electrophoresis and Western blotting. Three lanes show cPLA2 from unstimulated cells, cPLA2 from IL-3 stimulated cells, and cPLA2 from stimulated cells treated with acid phosphatase.

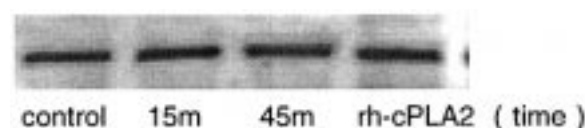


FIG 3. Effect of IL-3 on cPLA2 expression. Basophils were incubated with IL-3 (10 ng/mL) for times indicated. Reactions were stopped by ice-cold PAG, and samples were microfuged. Cell pellets were resuspended with ice-cold hypotonic lysis buffer. Expression of cPLA2 was examined by Western blot for expression, as described in Methods section.

mediated cPLA2 electrophoretic mobility shift at 500 nmol/L and 2 μ mol/L; however, neither compounds affected the IL-3-mediated electrophoretic mobility shift of cPLA2 (Fig 4). These results indicated that the IL-3 effect was PKC independent.

Enhancement of ionomycin-mediated AA generation and LTC4 release by IL-3

A high concentration of calcium ionophore has been previously shown to induce both AA and LTC4 release from human basophils,³² but we have recently found that lower concentrations of ionomycin, although causing significant elevations in $[Ca^{2+}]_i$, fail to induce LTC4 release and only low levels of free AA generation. For

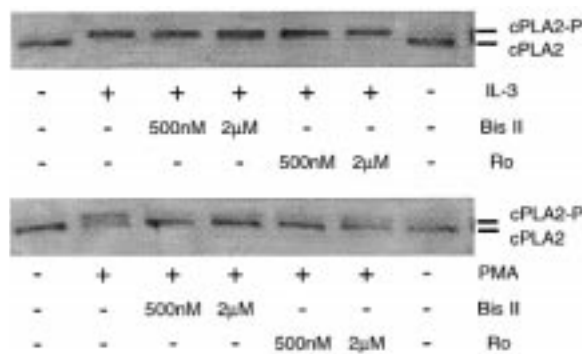


FIG 4. Inhibition of IL-3- or PMA-mediated cPLA2 electrophoretic mobility by PKC inhibitors. Basophils were incubated with bis-indolylmaleimide II (*Bis II*) or Ro-31-8220 (*Ro*) for 15 minutes and stimulated with IL-3 (1 ng/mL) or PMA (10 ng/mL) for 15 minutes. Reactions were stopped by ice-cold PAG and microfuged. Pellets were resuspended with ice-cold hypotonic lysis buffer. cPLA2 mobility shift was analyzed by Western blot as described in Methods section. Blot shown is representative of 2 separate experiments.

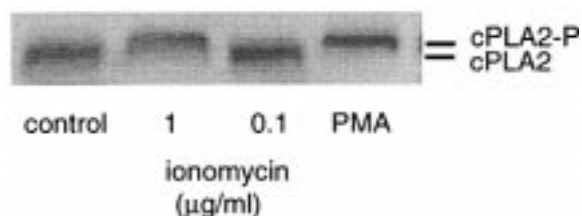


FIG 5. Electrophoretic mobility shift of cPLA2 incubated with 0.1 or 1 µg/mL ionomycin. Basophils were incubated with 0.1 or 1 µg/mL ionomycin or PMA (100 ng/mL) for 15 minutes. Reactions were stopped by ice-cold PAG, and samples were microfuged. Cell pellets were resuspended with ice-cold hypotonic lysis buffer. cPLA2 mobility shift was examined by Western blot as described in Methods section. Blot shown is representative of 2 separate experiments.

example, basophils (mean purity = $76\% \pm 3\%$, $n = 2$) were incubated with the different concentrations of ionomycin for 30 minutes. Ionomycin at 1 µg/mL generated 120 ± 10 pmoles/ 10^6 basophils net free AA and 24 ± 11 pmoles/ 10^6 basophils LTC₄, whereas 0.1 µg/mL ionomycin induced the generation of 20 ± 9 pmoles/ 10^6 basophils and undetectable LTC₄. Studies in other cell types indicate that the activity of cPLA₂ is dependent on both its phosphorylation and an elevation in the $[Ca^{2+}]_i$ to induce its translocation. Previous studies in human basophils indicate that 1 µg/mL ionomycin induced a $[Ca^{2+}]_i$ response that borders on exceeding the ability of fura-2 to measure free $[Ca^{2+}]_i$ accurately (>1 µmol/L), levels that exceed changes induced by physiologic stimuli. On the other hand, 0.1 µg/mL ionomycin induced a $[Ca^{2+}]_i$ response that was similar in magnitude to physiologic stimuli, approximately 100 nmol/L (net) (data not shown). We found that 1 µg/mL ionomycin also caused an electrophoretic mobility shift of cPLA₂, indicating that 1 µg/mL ionomycin induces cPLA₂ phosphoryla-

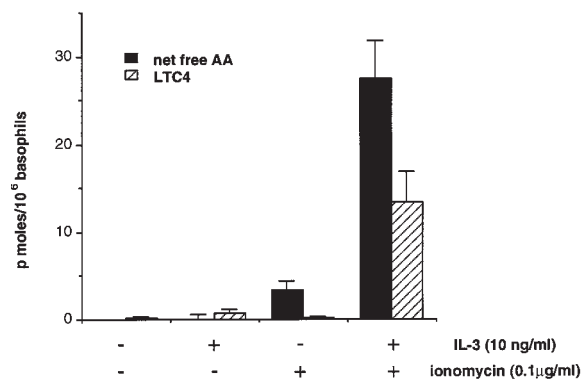


FIG 6. Effect of IL-3 on ionomycin-mediated free AA generation and LTC₄ release. Basophils were incubated with or without IL-3 (10 ng/mL) for 15 minutes and stimulated with or without ionomycin (0.1 µg/mL) for 30 minutes. Mass of free AA was determined by means of GC/(NICI)MS. LTC₄ was assayed with an LTC₄-specific RIA as described in Methods section. Values are means $\pm$ SEM of 7 experiments.

tion; however, 0.1 µg/mL ionomycin did not affect cPLA₂ mobility (Fig 5). Ionomycin (1 µg/mL)-mediated cPLA₂ phosphorylation was not inhibited by PKC inhibitors, such as bis-indolylmaleimide II and Ro-31-8220, indicating that ionomycin-mediated phosphorylation was not PKC dependent (data not shown). These results support the idea that both phosphorylation and a calcium elevation are required for cPLA₂ activity.

Further support was found by combining IL-3 with stimulation by a low dose of ionomycin. IL-3 has been previously noted to not result in elevations in $[Ca^{2+}]_i$, but as shown above, it does result in phosphorylation of cPLA₂. Therefore pretreatment of basophils with IL-3 before stimulation with 0.1 µg/mL ionomycin should induce significantly more AA and LTC₄ release. Human basophils were preincubated with 10 ng/mL IL-3 for 15 minutes and stimulated by 0.1 µg/mL ionomycin for 30 minutes. Ionomycin alone induced little free AA generation and no LTC₄ release, and IL-3 (10 ng/mL) alone did not induce free AA or LTC₄ release but in combination with ionomycin caused marked generation of both free net free AA (27 ± 4 pmoles/ 10^6 basophils) and LTC₄ (13 ± 4 pmoles/ 10^6 basophils) (Fig 6). Concentrations of PMA up to 100 ng/mL also did not induce a $[Ca^{2+}]_i$ elevation, but a combination of PMA and ionomycin caused significant free AA generation and LTC₄ release (data not shown). These results were consistent with the fact that both IL-3 and PMA cause phosphorylation of cPLA₂ and that the phosphorylation of cPLA₂ increases cPLA₂ catalytic activity.

These studies used relatively pure basophil preparations ($>80\%$), but it was possible that free AA generation and LTC₄ release were derived from the contaminating cells (mainly monocytes and lymphocytes) when ionomycin was used.³² To determine the relative contribution of AA and LTC₄ from contaminating cells, free AA generation and LTC₄ release from contaminating cells and

basophils were examined. In this study pure basophils (basophils purity: $90\% \pm 3\%$, purified without positive selection) and contaminating cells (basophils purity: $14\% \pm 2\%$) were used. Equal cell numbers from the same cell preparation were examined for free AA generation and LTC4 release. IL-3 (10 ng/mL) did not cause net free AA generation from either basophils (0.75 ± 0.43 pmoles/ 10^6 cells) or contaminating cells (1.59 ± 0.36 pmoles/ 10^6 cells). Ionomycin (0.1 μ g/mL) induced greater net free AA generation from contaminating cells (12 ± 4 pmoles/ 10^6 cells) than from basophils (2.1 ± 1.0 pmoles/ 10^6 cells), which indicated that there should be some concern for the purity of the basophil preparations. However, IL-3 markedly increased ionomycin-induced net free AA generation from basophils (18 ± 4 pmoles/ 10^6 cells), but not that from contaminating cells (15 ± 6 pmoles/ 10^6 cells). Enhancement of ionomycin-mediated free AA generation by IL-3 was 1.28-fold in contaminating cells versus 5- to 129-fold in basophils. Similar characteristics were observed for LTC4 release (data not shown). cPLA2 phosphorylation by IL-3 was also examined in contaminating cells and compared with that in basophils. There was no induced shift of cPLA2 by IL-3 in contaminating cells because the cPLA2 in resting cells was already maximally shifted (data not shown).

The concentration dependency of the electrophoretic mobility shift induced by IL-3 and its effect on ionomycin-mediated free AA generation and LTC4 release were examined. Concentrations of IL-3 greater than 1 ng/mL caused a maximal cPLA2 shift, whereas 0.1 ng/mL appeared to induce only a partial shift (Fig 7, A). The concentration-dependent enhancement of ionomycin-induced free AA and LTC4 by IL-3 was similar to that of the cPLA2 mobility shift, being half maximal value at 0.1 ng/mL (Fig 7, B).

cPLA2 phosphorylation induced by C5a or fMLP

There are differences in the free AA generation and LTC4 release induced by C5a and fMLP stimulation. Previous studies have shown that C5a induces little or no free AA generation and LTC4 release from human basophils, whereas fMLP alone is a strong stimulus for both free AA generation and LTC4 release.^{1,32} IL-3 was found to convert the response to C5a to one that resembled fMLP. Interestingly, C5a and fMLP both caused an electrophoretic mobility shift in cPLA2 with a similar time course (Fig 8). Most notably, a significant cPLA2 mobility shift was not observed before 30 seconds, and the shift only reached a maximum from 1 minute to 15 minutes. Previous studies demonstrated that the C5a-mediated $[Ca^{2+}]_i$ elevation is rapid and transient, lasting 30 to 45 seconds,¹ whereas the fMLP-induced $[Ca^{2+}]_i$ is sustained. The dissociation between the C5a-induced $[Ca^{2+}]_i$ elevation and the phosphorylation of cPLA2 may explain the inability of C5a to induce free AA generation (see Discussion section). As shown in Fig 9, the shift in cPLA2 mobility induced by C5a and fMLP was not dependent on PKC activity because neither bis-indolyl-

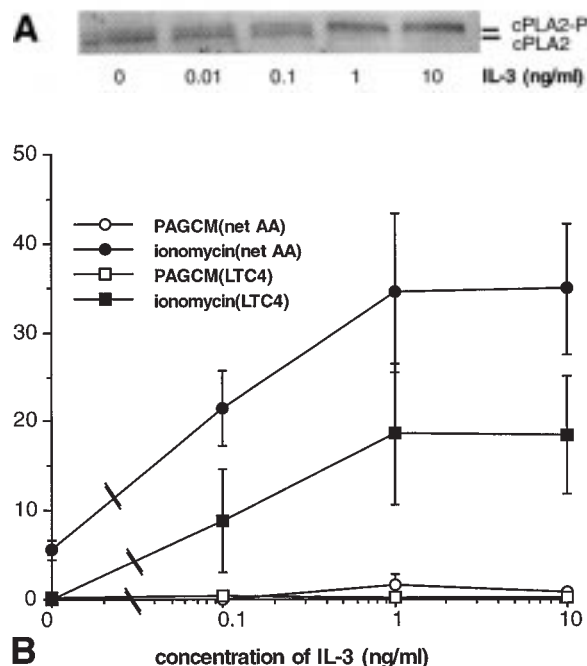


FIG 7. A, Dose-dependent electrophoretic mobility shift of cPLA2 incubated with IL-3. Basophils were incubated with indicated concentrations of IL-3 for 15 minutes, and cPLA2 mobility shift was examined by Western blot. Blot shown is representative of 2 separate experiments. B, Dose-dependent effect of IL-3 on free AA generation and LTC4 release. Basophils were incubated with indicated concentrations of IL-3 for 15 minutes and stimulated with or without ionomycin (0.1 μ g/mL) for 30 minutes. Average $\pm$ SEM of net release for 3 experiments is shown.

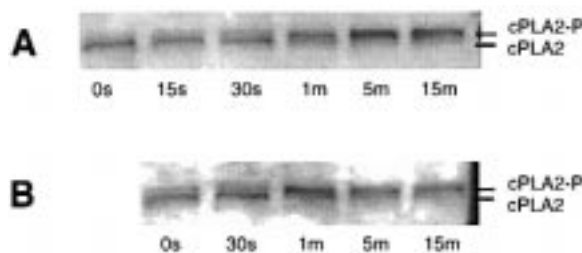


FIG 8. Electrophoretic mobility shift of cPLA2 incubated with C5a or fMLP. Basophils were incubated with C5a (50 ng/mL) (A) or fMLP (1 μ mol/L) (B) for times indicated, and cPLA2 mobility shift was examined by Western blot. Blot shown is representative of 2 experiments.

maleimide nor Ro-31-8220 inhibited the shift in mobility. Moreover, neither bis-indolylmaleimide II nor Ro-31-8220 inhibited fMLP-mediated free AA generation and LTC4 release (data not shown).

Because C5a could induce phosphorylation of cPLA2, although its $[Ca^{2+}]_i$ response was complete before this phosphorylation occurred, a combination of low-dose ionomycin, which does not induce cPLA2 phosphorylation but does generate a sustained $[Ca^{2+}]_i$ response, and

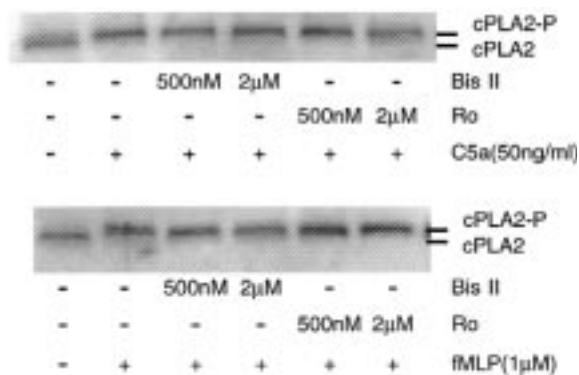


FIG 9. Inhibition of C5a- or fMLP-mediated cPLA2 electrophoretic mobility by PKC inhibitors. Basophils were incubated with bis-indolylmaleide II (*Bis II*) or Ro-31-8220 (*Ro*) for 15 minutes and stimulated with C5a (50 ng/mL) or fMLP (1 μ mol/L) for 5 minutes. cPLA2 mobility shift was analyzed by Western blot. Blot shown is representative of 2 experiments.

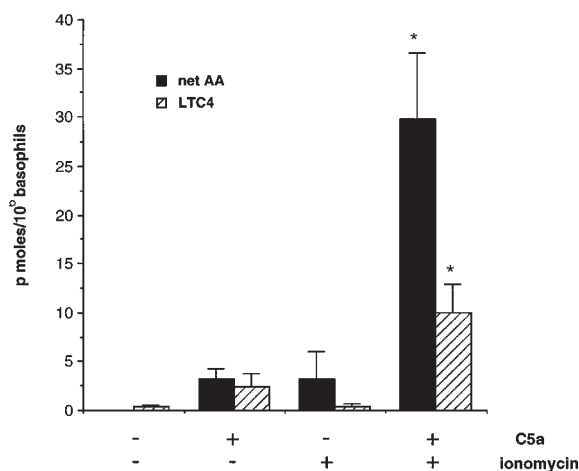


FIG 10. Effect of C5a on ionomycin-mediated free AA generation and LTC4 release. Basophils were incubated with or without C5a (50 ng/mL) and with or without ionomycin (0.1 μ g/mL) for 5 minutes simultaneously. Average $\pm$ SEM of net release for 4 experiments is shown. Asterisks indicate statistically significant difference from C5a-mediated free AA or LTC4 releases without treatment of ionomycin ($P < .05$ as determined by paired t test).

C5a should lead to significant free AA generation. Basophils were incubated for 5 minutes with or without C5a (50 ng/mL) and with or without ionomycin (0.1 μ g/mL) simultaneously. C5a or ionomycin alone caused only a small amount of net free AA (C5a: 3.1 ± 1.1 and ionomycin: 3.3 ± 2.7 pmoles/ 10^6 basophils) and LTC4 (C5a and ionomycin: 2.4 ± 1.3 and 0.39 ± 0.23 pmoles/ 10^6 basophils, respectively). However, a combination of C5a and ionomycin caused markedly more free AA (30 ± 7 pmoles/ 10^6 basophils) and LTC4 (10 ± 3 pmoles/ 10^6 basophils) generation (Fig 10). The enhancement of both free AA and LTC4 release caused by a combination of C5a and ionomycin was statistically signifi-

cant from those induced by C5a alone ($P < .05$ as determined by paired t test).

DISCUSSION

These studies demonstrate a correlation between the phosphorylation of cPLA2, as detected by a decrease in its electrophoretic mobility, and the generation of free AA and LTC4. If the phosphorylation of cPLA2 and its subsequent activity in generating free AA are requisites for LTC4 production, then these studies also suggest that the priming of LTC4 release by IL-3 may result from the action of IL-3 on a non-PKC-dependent pathway leading to cPLA2 phosphorylation.

In each of the presented experiments, the generation of both free AA and LTC4 correlated with the phosphorylation of cPLA2. Phosphorylation of cPLA2 increases its enzymatic activity,^{11,17,24} and Clark et al.¹⁷ suggested that, at least in the cell-free system, no accessory proteins are required for the increased activity of cPLA2 directly following phosphorylation. At this time, no other elements, except phosphorylation, have been shown to increase the enzymatic activity of cPLA2. The $[Ca^{2+}]_i$ induces the translocation of cPLA2 to phospholipid membranes, a requisite for this enzyme to act on the phospholipids.^{18,19} This combination of requirements forms the basis for an understanding of the observations presented. In Figs 6 and 7, we demonstrated that phosphorylation of cPLA2 caused by IL-3 treatment coupled with the modest calcium elevation induced by a low concentration of ionomycin operated synergistically to induce both free AA and LTC4 generation.

A similar result could be found when basophils were stimulated with C5a and fMLP. Previous studies have outlined the circumstances under which C5a and fMLP induce either free AA or LTC4 generation. As noted in the Results section, the cytosolic calcium response after administration of C5a and fMLP differs. For C5a, a strong $[Ca^{2+}]_i$ response is transient, with $[Ca^{2+}]_i$ levels returning to resting within 30 to 45 seconds.¹ This transient response most likely results from the release of internal stores of calcium. For fMLP, there exists both a strong initial response, consistent with the release of internal stores, and a strong second phase, consistent with the influx of extracellular calcium.⁶ The generation of free AA after stimulation with fMLP is delayed. Histamine release is complete by 30 seconds, but both free AA and LTC4 release do not begin for approximately 30 to 60 seconds, after which release is rapid and occurs within 3 to 5 minutes.¹ Stimulation with fMLP in the presence of EGTA eliminates the second-phase calcium response and free AA and LTC4 generation but only marginally inhibits histamine release.⁶ When basophils are pretreated with 10 ng/mL IL-3, the primary effect on fMLP-induced release is an acceleration in the generation of free AA and LTC4, such that it begins within seconds of stimulation and is complete within 1 minute.¹ For C5a, treatment with IL-3 permits the generation of free AA and LTC4. The generation is also rapid, and LTC4 release is complete at precisely the time that the

$[Ca^{2+}]_i$ response returns to resting levels. IL-3 treatment does not alter the $[Ca^{2+}]_i$ responses that follow either C5a or fMLP.¹ These results can now be understood in the context of the results on cPLA2 phosphorylation. Both C5a and fMLP induce cPLA2 phosphorylation, but the observed shift in electrophoretic mobility is not apparent at times less than 30 seconds, and a full shift requires more than 1 minute. Furthermore, because the $[Ca^{2+}]_i$ response is required for cPLA2 translocation to the membrane,^{18,19} its late phosphorylation, relative to the $[Ca^{2+}]_i$ response, would preclude the generation of free AA after stimulation with C5a (provided that translocation were reversible). On the other hand, the second phase of the fMLP cytosolic calcium response would allow free AA generation.

Pretreatment of the basophils with IL-3 *preconditions* the cPLA2 by inducing its phosphorylation. Under these conditions, the $[Ca^{2+}]_i$ that followed stimulation with C5a was sufficient to induce free AA generation and cause its appearance within seconds of stimulation (ie, the $[Ca^{2+}]_i$ response and cPLA2 phosphorylation had been synchronized). For fMLP, the prephosphorylation of cPLA2 also accelerated free AA generation.

There appear to be at least 2 distinct pathways for cPLA2 phosphorylation. In these studies it is apparent that activation of PKC results in cPLA2 phosphorylation, whereas treatment with IL-3 results in cPLA2 phosphorylation that is not blocked by PKC inhibitors. In addition, stimulation with C5a and fMLP results in cPLA2 phosphorylation that is not blocked by PKC inhibitors. C5a and fMLP operate through pertussis toxin-sensitive G-proteins. In other cell types, G-protein-mediated activation causes activation of Ras, MEK (ERK kinase), ERK,³³ and activated ERK phosphorylates cPLA2. IL-3-stimulated phosphorylation and activation of MAPK has been shown in other cells.^{34,35} In human basophils IL-3 might also phosphorylate cPLA2 through MAPK activation, although the antecedent events for MAPK activation remain unknown. Indeed, in a manuscript under preparation, we have shown that the MEK inhibitor PD98059 effectively inhibits MAPK and cPLA2 phosphorylation and ablates AA and LTC4 release with no effect on histamine release.

In one respect the results presented concerning AA generation are in accord with our previous studies using sPLA2 inhibitors,¹⁰ in which the observable free AA was postulated to result from the action of cPLA2. However, these previous studies also suggested a role for sPLA2 in the generation of AA destined for LTC4 release. For example, 2 sPLA2 inhibitors, SB203347 or mAb (3F10) specific for sPLA2 (type II or V), would inhibit LTC4 release while having no effect on free AA generation. The working hypothesis that was derived from these observations was that the AA generation normally observed after stimulation resulted from the action of cPLA2 but that this free AA was not destined for LTC4 generation. Instead, the free AA generated by sPLA2 was tightly coupled to 5-lipoxygenase/LTC4 synthase such that the AA generated from the action of

sPLA2 was not normally observed. To further test this hypothesis, this normally unobservable free AA was made apparent by treating the cells with zileuton (blocking 5-lipoxygenase), thus preventing the metabolism of this free AA. Under these conditions, the mass of free AA that accumulated was similar to the lost mass of LTC4. Furthermore, treatment of the cells with mAb 3F10 or SB 203347 inhibited only the free AA that additionally accumulated because of the presence of zileuton. However, in our current studies the generation of LTC4 tracked well with the generation of free AA and the activation state of cPLA2, suggesting that cPLA2 generates the free AA destined for metabolism to LTC4. The current studies are strictly correlative; no good pharmacologic modulators of cPLA2 exist, and therefore a direct examination of its role is not yet possible. In our hands a series of unpublished efforts to downregulate cPLA2 in human basophils with anti-sense nucleotides have proven unsuccessful. This sort of conflicting data is not uncommon today because a role for either cPLA2 or sPLA2 has been found, sometimes in the same cell types.^{36,37} The data supporting a role for cPLA2 is appealing because there are several studies that demonstrate colocalization of cPLA2, 5-lipoxygenase-activating protein, 5-lipoxygenase, and LTC4 synthase on the cell's nuclear membrane, and the mechanism of cPLA2 activation is partially understood. To resolve this issue, additional studies in basophils will require specific pharmacologic inhibitors of cPLA2.

In summary, short treatments of basophils with IL-3 induce cPLA2 phosphorylation, and this correlates with both free AA generation and LTC4 release in response to C5a, fMLP, and ionomycin, suggesting that the priming effect of IL-3 might be due to induction of cPLA2 phosphorylation. The antecedent pathway leading to cPLA2 phosphorylation by IL-3 is not known, but it does not appear to be dependent on PKC activation. Furthermore, the studies shows that synchronous cPLA2 phosphorylation and elevations in $[Ca^{2+}]_i$ response are essential to generate free AA and LTC4, indicating that cPLA2 might be a major PLA2 to liberate AA for LTC4 in human basophils.

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