Activation of Group IV Cytosolic Phospholipase A2 in Human Eosinophils by Phosphoinositide 3-Kinase Through a Mitogen-Activated Protein Kinase-Independent Pathway

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Activation of Group IV Cytosolic Phospholipase A₂ in Human Eosinophils by Phosphoinositide 3-Kinase Through a Mitogen-Activated Protein Kinase-Independent Pathway

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Activation of group IV cytosolic phospholipase A₂ (gIV-PLA₂) is the essential first step in the synthesis of inflammatory eicosanoids and in integrin-mediated adhesion of leukocytes. Prior investigations have demonstrated that phosphorylation of gIV-PLA₂ results from activation of at least two isoforms of mitogen-activated protein kinase (MAPK). We investigated the potential role of phosphoinositide 3-kinase (PI3K) in the activation of gIV-PLA₂ and the hydrolysis of membrane phosphatidylcholine in fMLP-stimulated human eosinophils. Transduction into eosinophils of Δp85, a dominant negative form of class IA PI3K adaptor subunit, fused to an HIV-TAT protein transduction domain (TAT-Δp85) concentration dependently inhibited fMLP-stimulated phosphorylation of protein kinase B, a downstream target of PI3K. FMLP caused increased arachidonic acid (AA) release and secretion of leukotriene C₄ (LTC₄). TAT-Δp85 and LY294002, a PI3K inhibitor, blocked the phosphorylation of gIV-PLA₂ at Ser⁵⁰⁵ caused by fMLP, thus inhibiting gIV-PLA₂ hydrolysis and production of AA and LTC₄ in eosinophils. FMLP also caused extracellular signal-related kinases 1 and 2 nor p38 was inhibited by TAT-Δp85 or LY294002. Inhibition of 1) p70 S6 kinase by rapamycin, 2) protein kinase B by Akt inhibitor, or 3) protein kinase C by Ro-31-8220, the potential downstream targets of PI3K for activation of gIV-PLA₂, had no effect on AA release or LTC₄ secretion caused by fMLP. We find that PI3K is required for gIV-PLA₂ activation and hydrolytic production of AA in activated eosinophils. Our data suggest that this essential PI3K independently activates gIV-PLA₂ through a pathway that does not involve MAPK. The Journal of Immunology, 2003, 171: 4399–4405.

Phospholipase A₂ (PLA₂)³ is the rate-limiting enzyme involved in the conversion of membrane phospholipids to arachidonic acid (AA) and lysophospholipids (1, 2); further catalysis of AA initiates the synthesis of potent bioactive mediators, e.g., PGs and leukotrienes (LTs). The 85-kDa group IV cytosolic phospholipase A₂ (gIV-PLA₂) is an intracellular, nonsecreted phospholipase that is uniquely regulated by cell-signaling mechanisms activated by cell surface membrane receptors (3). Activated phosphorylation and migration of this cytosolic enzyme to the perinuclear membrane causes hydrolysis of phosphatidylcholine at the sn-2 position and production of AA.

 Activation of gIV-PLA₂ is a complex process requiring both phosphorylation and mobilization of intracellular Ca²⁺ to effect nuclear translocation of gIV-PLA₂ from cytosol to membrane (4), in which gIV-PLA₂ binds by its N-terminal Ca²⁺-dependent lipid-binding domain. The presence of a consensus phosphorylation site for mitogen-activated protein kinase (MAPK) at Ser⁵⁰⁵ of gIV-PLA₂ has suggested a role for MAPKs, including extracellular signal-regulated kinase (ERK) and p38 MAPK, in the phosphorylation and activation of this enzyme (5, 6). Other investigations have shown that phosphorylation of gIV-PLA₂ at the Ser⁷²⁷ site is caused by MAPK-interacting kinase 1 and on Ser⁵¹⁵ by calcium/calmodulin-dependent protein kinase II (7). These findings suggested that kinases other than the MAPKs are involved in gIV-PLA₂ activation.

In this investigation, we considered the possible role of phosphoinositide 3-kinase (PI3K) as an alternative, and potentially essential, mechanism for gIV-PLA₂ phosphorylation and hydrolytic production of AA and its metabolites in fMLP-stimulated human eosinophils. The class IA PI3K is activated by tyrosine kinases and consists of a heterodimer composed of a 110-kDa (p110α, -β, -δ) catalytic subunit and an 85-kDa (p85α, p85β) adaptor protein (8). Class IB PI3K (PI3Kγ) consists of a 110-kDa protein (p110γ), which can be directly activated by the βγ subunits of G protein (9, 10). PI3K phosphorylates the D3 position of the inositol ring of phosphoinositide and its phosphorylated derivatives (11). PI3K is activated by a number of growth factors and other nonmitogenic stimuli and has been implicated in a wide variety of cellular functions, including mitogenesis, cell adhesion and motility, inflammatory mediator secretion, and protection from apoptosis (12, 13). PI3K has been reported to participate in the early events leading to activation or inactivation of MAPK, depending on cell type and

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3 Abbreviations used in this paper: PLA₂, phospholipase A₂; gIV-PLA₂, group IV cytosolic PLA₂; AA, arachidonic acid; LT, leukotriene; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; p70 S6 kinase; PKC, protein kinase C; Δp85, dominant negative form of p85α; WP85, wild-type p85α; PAPC, 1-palmitoyl-2-[¹⁴C]arachidonyl phosphatidylcholine; GFP, green fluorescent protein; PIP₃, phosphatidylinositol 3,4,5-trisphosphate.

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We also found that PI3K causes gIV-PLA2 phosphorylation and subsequent AA mobilization in fMLP-stimulated eosinophils.

To test our hypothesis, we constructed a TAT-dominant negative form of class IA PI3K regulatory subunit (TAT-p85) containing 6 His residues, 11 amino acids of HIV-TAT, and Δp85 (Fig. 1). Unlike the wild-type p85α (Wp85), Δp85 lacks the binding site for the catalytic p110 subunit (22). We transduced TAT-Δp85 fusion protein into fully differentiated human eosinophils and demonstrated that PI3K is required for gIV-PLA2 activation and subsequent AA mobilization in fMLP-stimulated eosinophils. We also found that PI3K causes gIV-PLA2 phosphorylation at Ser505 by a pathway that does not involve MAPK.

Materials and Methods

Reagents and Abs

Eosinophil isolation materials were obtained from Miltenyi Biotec (Sunnyvale, CA). Cytochalasin B, fMLP, and FITC were purchased from Sigma-Aldrich (St. Louis, MO). LY294002 was purchased from BioMol (Plymouth Meeting, PA). Ro-31-8220, Akt inhibitor, and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO). Ni-NTA columns were purchased from Qiagen (Valencia, CA). A 10,000 MWCO Slide-A-Lyzer dialysis cassette was purchased from Pierce (Rockford, IL). 1-Palmitoyl-2-[14C]arachidonyl phosphatidylcholine was purchased from New England Nuclear (Boston, MA), Thiazolyl blue (Promega, Madison, WI), and apoE (Hyclone, Logan, UT). Ni-NTA columns were purchased from Qiagen (Valencia, CA). A 10,000 MWCO Slide-A-Lyzer dialysis cassette was purchased from Pierce (Rockford, IL). 1-Palmitoyl-2-[14C]arachidonyl phosphatidylcholine was purchased from New England Nuclear (Boston, MA), Thiazolyl blue (Promega, Madison, WI), and apoE (Hyclone, Logan, UT). 

Isolation of human eosinophils

Eosinophils were isolated by a method modified from Hansel et al. (25). Percoll centrifugation (density 1.089 g/ml) was employed to isolate granulocytes and was followed by hypotonic lysis of RBC, and finally, immunomagnetic depletion of neutrophils by the magnetic cell separation system using anti-CD16-coated MACS particles. Eosinophil purity of >98% was routinely obtained, as assessed by Wright-Giemsa staining. Cells were kept on ice until use.

Transduction of TAT-Δp85 into eosinophils

Transduction of eosinophils with TAT-Δp85 was assessed by the method described by Hall et al. (26). Briefly, either TAT-Δp85 or Δp85, which lacks the TAT protein transduction domain, was labeled with FITC according to manufacturer’s instructions. Eosinophils resuspended in calcium- and magnesium-free HBSS, pH 6.8, containing 1% BSA were incubated with 100 nM FITC-TAT-Δp85 or FITC-Δp85 for indicated times at 37°C. The pH of the sheath fluid of the flow cytometer was adjusted to 6.8 to quench FITC fluorescence on the outside of the cell, and at least 10,000 cells were analyzed on a FACScan (BD Biosciences, Mansfield, MA).

Measurement of AA release

Eosinophils were incubated in RPMI medium containing 5% FBS and 0.5 μCi [3H]AA. After a 2 h incubation period, labeled medium was aspirated, and unincorporated [3H]AA was washed away by HBSS containing 0.2% BSA. Uptake of [3H]AA by eosinophils occurred in a time-dependent manner, reaching its maximum (61 ± 2.3% of the total added [3H]AA) after 30-min incubation at 37°C. Maximal incorporation remained constant ≥2 h incubation. Aliquots of 10⁶ eosinophils were preincubated with or without TAT-Δp85, TAT-Wp85, TAT-GFP, LY294002, Ro-31-8220, Akt inhibitor, and rapamycin for 30 min. Cells were subsequently incubated with 5 μg/ml cytochalasin B for 2 min before stimulation with 1 μM fMLP for an additional 15 min at 37°C. The addition of cytochalasin B was used to promote AA secretion, as previously described (27). The reactions were terminated by centrifugation at 12,000 × g for 1 min. Supernatants were collected, and pellets were lysed in 1% Triton X-100. [3H]AA release was measured by scintillation counting and expressed as percentage of total AA incorporation ([100 × cpm of supernatant/cpm of supernatant + cpm of pellet]).

LTCA assay

Aliquots of 250,000 eosinophils were preincubated with various concentrations of TAT-Δp85, TAT-Wp85, TAT-GFP, LY294002, Ro-31-8220, Akt inhibitor, and rapamycin for 30 min, and then incubated with cytochalasin B for 2 min before being stimulated by fMLP for additional 15 min at 37°C in a final volume of 250 μl HBSS. The reactions were terminated by centrifugation at 12,000 × g for 1 min. Aliquots of supernatants were assayed with a commercial enzyme immunoassay kit, as previously described (27).

Immunoblot analysis of PI3K, PKB, ERK1/2, p38 MAPK, and gIV-PLA2

The reaction (10⁶ eosinophils/group) was stopped by centrifugation at 12,000 × g for 30 s. The pellets then were lysed in 40 μl lysis buffer (20 mM Tris-HCl, 30 mM Na3PO4, 50 mM NaF, 40 mM NAc, 5 mM EDTA, pH 7.4) containing 1% Nonidet P-40, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 2 mM Na3VO4, 0.5% deoxycholic acid. After 20 min on ice, samples were centrifuged at 12,000 × g for 2 min to remove nuclear and cellular debris. The supernatants then were mixed with 7 μl of 6× sample buffer and boiled for 5 min. The samples were collected and saved at −80°C.

Samples were subjected to SDS-PAGE, using acrylamide gels under reducing condition (15 mA/gel). Electrophoretic proteins from the gels to...
polyvinylidene fluoride membrane was achieved using a semidy system (400 mA, 60 min). The membrane was blocked with 1% BSA for 60 min, then incubated with 1/5000 anti-phosphorylated ERK1/2 Ab, 1/1000 anti-ERK1/2 Ab, 1/1000 anti-phosphorylated p38 MAPK Ab, 1/1000 anti-phosphorylated PKB Ab, 1/1000 anti-PKB Ab, 1/1000 anti-phosphorylated gIV-PLA2 Ab, 1 μg/ml anti-p38α MAPK Ab, 1/1000 anti-phosphorylated PKB Ab, 1/1000 anti-PKB Ab, 1/1000 anti-phosphorylated gIV-PLA2 Ab, 1 μg/ml anti-p38α MAPK Ab, 1/1000 anti-phosphorylated PKB Ab, 1/1000 anti-PKB Ab, 1/1000 anti-phosphorylated gIV-PLA2 Ab, 1 μg/ml anti-P38 p110α, p110β, p110γ, and p110δ, or 1 μg/ml anti-gIV-PLA2 Ab diluted in TBST overnight. The membranes then were washed three times for 20 min with TBST. Donkey anti-rabbit IgG conjugated with HRP was diluted 1/3000 in TBST and incubated with polyvinylidene fluoride membrane for 60 min. The membrane was again washed three times with TBST and assayed by an ECL chemiluminescence system (Amersham).

**Determination of gIV-PLA2 enzyme activity**

The assay for gIV-PLA2 activity was modified from the method of Kim et al. (28). Briefly, 2 × 10⁹ eosinophils were stimulated with 5 μg/ml cytochalasin B for 2 min and 1 μM fMLP for additional 5 min. The reaction was stopped by centrifugation, and the pellets were resuspended in 70 μl sonication buffer (20 mM Tris, pH 8.0, 2.5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotonin, 1 mM PMSF, 2 mM Na₃VO₄, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly (4 × 10 s, at a power setting of 3). Lysates were pretreated with 5 mM DTT on ice for 5 min to inactivate secretory PLA2, and 10 μl of 50 mM CaCl₂ was then added to each sample. A total of 10 μl substrate ([14C]PAPC) was dried under a stream of N₂ and resuspended in 200 μl 10% ethanol in H₂O with vigorous vortex mixing. The reaction was initiated by adding 10 μl portion of the substrate (final concentration 9 μM) to cell lysate. The reaction was generated for 30 min at 37°C and was stopped by adding 560 μl Dole’s reagent (heptane-isopropanol alcohol-1 N H₂SO₄, 400:390:10 by volume), followed by 110 μl H₂O, vortexed for 20 s and then centrifuged at 12,000 × g for 1 min hexane containing 25 mg silica gel. A total of 750 μl of sample then was mixed with 2 ml scintillation fluids, and the radioactivity was counted in a liquid scintillation counter.

**Statistical analysis**

All measurements were expressed as mean ± SEM. Variation between three or more groups was analyzed using ANOVA followed by Fisher’s least protected difference test. Statistical significance was claimed when ever p < 0.05.

**Results**

**Transduction of TAT-Δp85 into eosinophils**

To assess the efficacy of TAT-protein transduction, eosinophils were incubated with FITC-conjugated TAT-Δp85 for ≤2 h at a pH of 6.8, and samples were analyzed by flow cytometry. At this pH, FITC-labeled proteins that enter the cell fluoresce, whereas those external to the cell have their fluorescence quenched (26, 29). TAT-protein was transduced into >99% of eosinophils receiving the TAT-Δp85 transduction fusion product after 5 min incubation (Fig. 2A). The Δp85, which has no protein transduction domain of TAT, did not enter eosinophils (Fig. 2B). Eosinophils were >95% viable during the course of these experiments, as indicated by trypan blue exclusion (data not shown).

We next examined whether the transduced TAT-Δp85 functionally inhibited signal transduction pathways downstream of PI3K in eosinophils. Detection of phosphorylated PKB, a downstream effector of PI3K, was used as measurement for PI3K activation (30). PKB phosphorylation by fMLP stimulation was observed within 0.5 min, peaked up to 5 min, and declined thereafter (Fig. 3A). Phosphorylation of PKB was reduced ≥5 min after 100 nM TAT-Δp85 transduction (Fig. 3B). These results indicate that TAT-Δp85 is functionally active after 5 min of incubation and remains effective in blocking PI3K activation for at least 2 h. Consequently, a 30-min incubation time was used in the subsequent experiments.

TAT-Δp85 caused concentration-dependent inhibition of fMLP-stimulated PKB phosphorylation (Fig. 3C). PKB phosphorylation was blocked completely by 100–300 nM TAT-Δp85. By contrast, TAT-Wp85 or TAT-GFP had no effect on fMLP-stimulated PKB phosphorylation. TAT-Δp85 alone had no effect on PKB phosphorylation. Accordingly, TAT-Δp85 functionally suppressed...
PI3K signal transduction pathways after transduction into eosinophils.

Effects of blockade of PI3K or PKC on fMLP-induced [3H]AA release and LTC4 production

We previously have shown that activation of eosinophils with fMLP caused AA and LTC4 release in a concentration- and time-dependent manner (31). To determine whether PI3K is required for the production of AA and LTC4 in fMLP-stimulated eosinophils, the effect of TAT-Δp85 or the selective PI3K inhibitor, LY294002, on AA metabolism was assessed. Eosinophils were preincubated with LY294002, TAT-Δp85, TAT-Wp85, and TAT-GFP before stimulation by 1 μM fMLP plus cytochalasin B. Nonstimulated eosinophils released minimal amounts of AA and undetectable amounts of LTC4. LY294002 substantially inhibited both fMLP-induced [3H]AA release (Fig. 4A) and LTC4 production (Fig. 4B). The fMLP-stimulated net AA release (background subtracted) decreased from 5.5 ± 0.3% to 4.4 ± 0.7% with 30 nM TAT-Δp85, and was further blocked to 1.7 ± 0.2% with 100 nM TAT-Δp85 (p < 0.01). Similarly, LTC4 release was blocked from 1052 ± 123 pg/ml to 476 ± 127 pg/ml (p < 0.01) with 30 nM TAT-Δp85 and further blocked to 85 ± 22 pg/ml (p < 0.01) with 100 nM TAT-Δp85.

We next investigated which downstream signal transduction molecules of PI3K might be involved in fMLP-induced AA metabolism. Known targets of PI3K are p70S6K (19), PKB (18), and molecules of PI3K might be involved in fMLP-induced AA and LTC4 production. [3H]AA-labeled eosinophils (A) or nonlabeled eosinophils (B) were preincubated with Akt inhibitor, rapamycin, or Ro-31-8220 for 30 min at 37 °C, and then incubated with cytochalasin B for 2 min before stimulation by 1 μM fMLP for another 15 min. [3H]AA was measured by scintillation counting (n = 4, A), and LTC4 production was measured by enzyme immunoassay (n = 4, B). Results are presented and error bars represent the mean ± SEM.

Effects of PI3K inhibition on fMLP-stimulated gIV-PLA2 activation

We further examined the direct effect of PI3K on gIV-PLA2 activation. Eosinophils were pretreated for 30 min with 30–100 nM TAT-Δp85 or 50 μM LY294002, followed by stimulation with fMLP for 5 min. gIV-PLA2 activity increased from 0.50 ± 0.02 pM AA/106 cells/min for nonstimulated eosinophils to 1.31 ± 0.10 pM AA/106 cells/min after 1 μM fMLP (p < 0.01). This increased activity was inhibited in a concentration-dependent manner by TAT-Δp85 and LY294002 (p < 0.01 for both comparisons.

FIGURE 4. Effect of PI3K inhibition on fMLP-stimulated AA release and LTC4 production. [3H]AA-labeled eosinophils (A) or nonlabeled eosinophils (B) were preincubated with TAT-Δp85, TAT-Wp85, TAT-GFP, or LY294002, a pharmacological PI3K inhibitor, for 30 min at 37 °C, and then incubated with cytochalasin B for 2 min before stimulation by 1 μM fMLP for another 15 min. [3H]AA was measured by scintillation counting (n = 4, A), and LTC4 production was measured by enzyme immunoassay (n = 4, B). Results are presented and error bars represent the mean ± SEM.

* p < 0.05 and ** p < 0.01 compared with fMLP control group.

FIGURE 5. Effect of PI3K downstream pathway inhibition on fMLP-stimulated AA release and LTC4 production. [3H]AA-labeled eosinophils (A) or nonlabeled eosinophils (B) were preincubated with Akt inhibitor, rapamycin, or Ro-31-8220 for 30 min at 37 °C, and then incubated with cytochalasin B for 2 min before stimulation by fMLP for another 15 min. [3H]AA was measured by scintillation counting (n = 4, A), and LTC4 production was measured by enzyme immunoassay (n = 4, B). Results are presented and error bars represent the mean ± SEM.

* p < 0.05 and ** p < 0.01 compared with fMLP control group.
vs fMLP, only Fig. 6A). Western blot analysis showed the time-dependent phosphorylation of gIV-PLA2 at Ser505 caused by fMLP, which was observed at 0.5 min, peaked at 5–10 min, and declined thereafter (Fig. 6B). Pretreatment of eosinophils with TAT-Δp85 or LY294002 inhibited the phosphorylation of gIV-PLA2 caused by fMLP (Fig. 6C). A second band (Fig. 6C, lane 3), which was observed above the gIV-PLA2 in TAT-Δp85 pretreatment group, marked the TAT-Δp85 fusion protein. By contrast, neither TAT-Wp85 nor Akt inhibitor, p70S6K inhibitor rapamycin, or PKC inhibitor Ro-31-8220 had any effect on gIV-PLA2 phosphorylation. These results suggest that PI3K is required for fMLP-stimulated gIV-PLA2 activation.

**PI3K-caused gIV-PLA2 activation was MAPK independent**

As both ERK1/2 and p38 MAPK mediate fMLP stimulated gIV-PLA2 activation (31), we next examined whether ERK1/2 or p38 MAPK was utilized in PI3K-mediated activation of gIV-PLA2 caused by fMLP. Neither ERK1/2 nor p38 MAPK phosphorylation caused by fMLP was inhibited by TAT-Δp85 or LY294002 (Fig. 7), suggesting that PI3K-mediated activation of gIV-PLA2 does not use MAPK.

**PI3K isoform expression in human eosinophils**

In additional experiments, we determined the p110 isoforms of PI3K expressed in human eosinophils by Western blot analysis. Eosinophils expressed p110α, p110γ, p110σ, except for p110α (Fig. 8). The β isoform of PI3K was expressed as two distinct bands on Western blot analysis. As there has been no prior examination of this isoform in eosinophils, the precise reason for the double band was not established. PKB phosphorylation induced by fMLP/cytoschalin B was completely blocked by TAT-Δp85. This suggests that protein tyrosine kinase-regulated forms of PI3K, possibly P110β and p110σ, mediate fMLP-stimulated AA production.

**Discussion**

The objective of this investigation was to determine the potential role of PI3K in the phosphorylation and activation of gIV-PLA2 in inflammatory granulocytes. We further wished to determine whether PI3K-mediated phosphorylation of gIV-PLA2 could be

![FIGURE 6. Effect of PI3K inhibition on fMLP-induced gIV-PLA2 activation and phosphorylation at Ser505. A, Effect of PI3K inhibition on gIV-PLA2 activity. Eosinophils were preincubated with TAT-Δp85, TAT-Wp85, TAT-GFP, or LY294002 for 30 min, and treated with cytoschalin B for 2 min before stimulation by 1 μM fMLP for 2 min. gIV-PLA2 activity was measured, as described in Materials and Methods, using substrate [14C]JPC. Each point represents the mean ± SEM of three separate experiments. **, p < 0.01 compared with fMLP control group. B, Kinetics of gIV-PLA2 phosphorylation. Cytoschalin B-pretreated eosinophils were incubated with 1 μM fMLP for indicated times. Resultant cell lysates were separated on SDS-PAGE gels and probed with anti-Ser505 phosphorylated gIV-PLA2 (upper panel, gIV-PLA2-p) and with Ab for total gIV-PLA2 (lower panel, gIV-PLA2-t) to demonstrate equal loading in all lanes (n = 3). C, Effect on PI3K inhibition on gIV-PLA2 phosphorylation. Eosinophils were preincubated with TAT-Δp85 or LY294002 at 37°C for 30 min and then incubated with cytoschalin B for 2 min before stimulation with 1 μM fMLP for another 10 min. Ser505 gIV-PLA2 phosphorylation and expression was measured as previously described (n = 3). D, Effect of Akt inhibitor, rapamycin, Ro-31-8220, and TAT-Wp85 on gIV-PLA2 phosphorylation. Eosinophils were preincubated with indicated concentrations of these inhibitor for 30 min and then processed as in C (n = 3).**

![FIGURE 7. Effect of PI3K inhibition on fMLP-stimulated ERK1/2 (A) and p38 MAPK (B) phosphorylation. Eosinophils were incubated with TAT-Δp85 or LY294002 for 30 min at 37°C, followed by addition of cytoschalin B for 2 min before stimulation by 1 μM fMLP for 1 min. Eosinophils were lysed, and the lysates were mixed with sample buffer and loaded on 10% SDS-PAGE, followed by immunoblotting with anti-phosphorylation-specific ERK1/2 Ab (A, top panel, ERK1/2-p), anti-total ERK1/2 Ab (A, bottom panel, ERK1/2-t), anti-phosphorylation-specific p38 MAPK Ab (B, top panel, p38-p), anti-p38 MAPK Ab (B, bottom panel, anti-p38-t). The results shown are representative of three different experiments.**

![FIGURE 8. PI3K isoform expression in human eosinophils. Eosinophils (10^6/lane) were lysed, the lysates were separated on 7.5% SDS-PAGE, and transferred to membrane. The membrane was cut into four separate lanes, and probed with anti-p110α, p110β, p110γ, and p110δ Ab. The results shown are representative of three different experiments.**

![PI3K isoform expression in human eosinophils](http://www.jimmunol.org/Downloaded_from/figure8.png)
activated independently of MAPK phosphorylation. We used HIV-TAT to transduce Δp85, a dominant negative form of class IA PI3K regulatory subunit (22), into fully mature human eosinophils to determine the role and mechanism by which PI3K could cause gIV-PLA₂ activation, hydrolysis of AA, and subsequent LTC₄ secretion. We also used LY294002, a pharmacological inhibitor of PI3K. We found this compound preferable to wortmannin, another commonly used PI3K inhibitor, because, unlike wortmannin, it has no inhibitory effect on gIV-PLA₂ (36), phospholipase D (37), or myosin L chain kinase (38).

We found that PI3K is likely to be required for gIV-PLA₂ activation and membrane hydrolysis to produce AA after activation of eosinophils with fMLP. Our data demonstrate that the time-course for PI3K activation (Fig. 3A) in eosinophils stimulated with fMLP corresponds precisely to that of gIV-PLA₂ phosphorylation (Fig. 6B) and the release of AA (31) from eosinophils. Inhibition of PI3K with a TAT-Δp85 and LY294002 also blocked the fMLP-induced phosphorylation of gIV-PLA₂ at Ser⁵⁰⁰ (Fig. 6C) and the subsequent activation of gIV-PLA₂ (Fig. 6A), resulting in attenuation of AA release (Fig. 4A) and LTC₄ synthesis (Fig. 4B).

Class I PI3K isoforms have been implicated in phosphatidylinositol 3,4,5-trisphosphate (PIP₃) generation in response to external stimuli in a wide variety of cell types. Three isoforms of class I PI3K (p110α, p110γ, and p110δ; Fig. 8) are expressed in human eosinophils. Transduction of the TAT-Δp85 completely inhibited fMLP-induced activation of PKB in these cells, suggesting fMLP receptors in human eosinophils predominantly activate class IA PI3K via a G protein and nonreceptor tyrosine kinase cascade. Previous studies in neutrophils have yielded different results regarding isoforms of PI3K in chemoattractant-stimulated effector functions (39–41). Neutrophils from p110α null mice showed a profound defect in fMLP-induced PI3K generation, suggesting that p110α plays a major role in fMLP-mediated signaling (42, 43). However, in human neutrophils, genestein (100 μM), a broad protein tyrosine kinase inhibitor, attenuated fMLP-stimulated PIP₃ generation by ≥80%, indicating an essential role of class IA PI3K (40). Our data also demonstrate dependence upon class IA PI3K in mediation of LT synthesis (Fig. 4). Using microinjection with a nonhematopoietic cell line, Belis and Abo (44) have demonstrated that fMLP induces actin reorganization through class IA PI3K. These results suggest the contribution of PI3K in fMLP-stimulated cell signaling is species-, cell-, and function-dependent, and the differences in isoform utilization between human eosinophils and murine neutrophils are not completely resolved.

The exact mechanisms by which PI3K stimulates the activation of gIV-PLA₂ are not known. It has been established previously that the activation of gIV-PLA₂ is regulated by phosphorylation by ERK1/2 and p38 MAPK (5, 6, 31, 45, 46). Sakamaka et al. (47) have shown that wortmannin inhibits both MAPK activation and arachidonate release at almost identical IC₅₀ values in CHO cells. These results suggest that PI3K may be an upstream kinase for MAPK and gIV-PLA₂. However, in our study, neither Δp85 nor LY294002, at the concentrations that blocked fMLP-induced activation of gIV-PLA₂ (Fig. 6, A and C), blocked ERK1/2 and p38 phosho-tyr-ylation caused by fMLP (Fig. 7). Accordingly, our results suggest that PI3K-mediated gIV-PLA₂ activation was MAPK-independent.

We have reported previously that pharmacological inhibition of ERK1/2 or p38 MAPK blocks fMLP-induced activation of gIV-PLA₂ in human eosinophils (31). In addition to MAPK, the activation of PKC has been shown to play a role in gIV-PLA₂ activation (45, 48–50). Some studies suggest that PKC activates gIV-PLA₂ through ERK activation (48, 49), whereas other studies suggest PKC can activate gIV-PLA₂ directly (45, 50). Inhibition of the known potential targets of PI3K suggests that p70S6K, PKB, or PKC is not the downstream effector of PI3K for gIV-PLA₂-mediated release of AA. Accordingly, we suggest that at least two separate, independent pathways (PI3K and MAPK) are involved in fMLP-induced activation of gIV-PLA₂. An alternate explanation is that PI3K may directly stimulate the phosphorylation and activation of gIV-PLA₂ through the intrinsic serine kinase activity of the PI3K 110-kDa catalytic subunit (51, 52). This possibility has not yet been elucidated.

It is important to consider some specific limitations of our findings. Our data were performed in primary isolates of human eosinophils in vitro. In prior studies, we have shown that eosinophils have different surface receptors and respond differently to type-specific cytokines (53), and possess the LTC₄ synthase, which is absent in human neutrophils (54). Human eosinophils also differ in their chemotactic properties (e.g., to LTB₄) from murine eosinophils (55). Accordingly, it is not surprising that the PI3K isoform(s) mediating synthesis in human eosinophils might be different than those mediating secretory functions in murine neutrophils. Furthermore, our studies were conducted entirely in vitro, and the extrapolation of these findings to the in vivo state is not justified. Some significant questions also remain unanswered. Although we believe that it is likely that the PI3K and MAPK pathways converge, probably at gIV-PLA₂, to affect hydrolysis of membrane phospholipids into AA, the emphasis of this investigation was to establish the ability of PI3K to cause AA production and LT synthesis that was independent of MAPK. Accordingly, we did not investigate the potential additive, synergistic, or inhibitory functions resulting from the interaction of these two pathways, a decidedly complex interaction that will require substantial additional investigation.

We conclude that PI3K has a critical role in gIV-PLA₂ activation, AA release, and LTC₄ production in fMLP-stimulated eosinophils. Our data further demonstrate that PI3K regulates gIV-PLA₂ activation through a MAPK-independent pathway.

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