Chemoattractants Stimulate Phosphatidylinositol-4-phosphate Kinase in Human Polymorphonuclear Leukocytes

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Chemoattractant receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by phospholipase C is instrumental for leukocyte activation. Previous studies have demonstrated that chemoattractant treatment of intact polymorphonuclear leukocytes (PMN) causes a transient decrease in PIP$_2$ due to phospholipase C activation, followed by an increase in cellular PIP$_2$ levels. The present study determined whether chemoattractants altered the activities of the two enzymes responsible for the synthesis of PIP$_2$, phospha tidylinositol kinase, and phosphatidylinositol-4-phosphate (PIP) kinase. Incubation of intact PMN with the N-formylated peptide chemoattractant formyl-methionyl-leucyl-phenylalanine at 37°C caused a rapid (3 min), 2-fold stimulation of PIP kinase activity isolated from a particulate membrane fraction. The increase in PIP kinase was dose-dependent for a variety of N-formylated chemoattractants as well as leukotriene B$_4$. Lineweaver-Burk analysis showed that the $V_{\text{max}}$ of PIP kinase was increased 2-fold by formylmethionyl-leucyl-phenylalanine, without a significant change in the apparent $K_m$ of the enzyme for ATP. Phosphatidylinositol kinase was, however, not altered by any chemoattractants tested. Nonchemotactic activators of the oxidative burst in leukocytes such as phorbol myristate acetate and ionophore A23187 did not significantly alter PIP kinase, suggesting a specificity for chemotactic agents. These findings demonstrate direct, chemoattractant-induced stimulation of PMN PIP kinase which may serve to replenish the important phospholipid, PIP$_2$, in the membrane following its hydrolysis by phospholipase C.

The influx of calcium into human polymorphonuclear leukocytes (PMN) is a prerequisite for chemoattractant-induced activation of inflammatory functions such as chemotaxis, lysosomal enzyme release, and superoxide anion generation (1-3). Chemoattractant receptor-mediated increases in leukocyte intracellular calcium concentrations are thought to result as a consequence of the hydrolysis of membrane phosphoinositides such as phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-trisphosphate and diacylglycerol (4-6). The former molecule releases calcium directly from intracellular stores (7-10). Protein kinase C activation results from the stimulating actions of both diacylglycerol and calcium (11, 12), a process thought to be required for superoxide anion generation in human PMN (13, 14).

The molecular reservoir of these second messenger molecules, PIP$_2$, is formed by the phosphorylation of phosphatidylinositol (PI) and phosphatidylinositol 4-monophosphate (PIP) by two distinct enzymes, PI and PIP kinases, which we have recently characterized in human PMN (15, 16). Although the hydrolysis of PIP$_2$ by phospholipase C has been well studied, little is known of the synthesis and thus regeneration of this important phospholipid in leukocytes.

It has been demonstrated that incubation of intact PMN with the chemoattractant, formyl-methionyl-leucyl-phenylalanine (FMLP) results in a rapid decrease in PIP$_2$, followed by a slower increase in cellular levels of this phospholipid (4, 17). Similar observations have been noted in other receptor systems as in the action of epidermal growth factor on A431 tumor cells, except that increases in PIP$_2$ are noted following the initial receptor-mediated hydrolysis of PIP$_2$ (18). Epidermal growth factor was subsequently shown to directly activate the first enzyme in the sequence, PIP$_2$ kinase (18). More recently, Auger et al. (19) have shown that antiphosphotyrosine immunoprecipitates from platelet-derived growth factor-stimulated smooth muscle cells contain lipid kinases which phosphorylate phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to produce the novel phosphoinositides, phosphatidylinositol 3,4-diphosphate and phosphatidylinositol trisphosphate. These kinase activities are absent in immunoprecipitates of resting cells. Based on these observations, we sought to determine whether chemoattractants altered the activity of PI and/or PIP kinases in human PMN. We report here that chemoattractants cause an activation of PIP kinase, but not PI kinase. The activation of PIP kinase appears to be due to an increase in the $V_{\text{max}}$ of the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals—PI, PIP$_2$, Trizma base, ATP, Triton X-100, FMLP, leukotriene B$_4$ (LTB$_4$), A23187, phorbol myristate acetate (PMA), N-formyl-norleucyl-leucyl-phenylalanine-norleucyl-tyrosyllysine (fNle-Leu-Phc-Nle-Tyr-Lys), N-formyl-methionyl-methionyl-methionyl (Met-Met-Met), N-formyl-methionyl-phenylalanine (Met-Phe), phenylmethylsulfonylfluoride, leupeptin, EGTA, benzamidine, cycloheximide, cytochalasin B, and superoxide dismutase were from Sigma. Hank's balanced salt solution was from Gibco. [1-3H]$\text{ATP}$ (3000 Ci/mmol), [2,8,5'-3H]ATP, and inositol [2-3H]PIP$_2$ were obtained from Du Pont-New England Nuclear.

Cell Preparation—Peripheral blood human PMN were isolated by sedimentation in 3% dextran (Pharmacia LKB Biotechnology Inc.), 0.9% NaCl, followed by centrifugation on Lymphoprep (Oregon Tek-
Cell Treatment and Membrane Preparation—Isolated PMNs (9.5 x 10^7/mL) were suspended in Hank's balanced salt solution containing 10 mM HEPES buffer and 4.2 mM NaHCO₃, pH 7.4 (HBSS). The cells were warmed to 37 °C for 10 min prior to the addition of HBSS alone or containing the indicated stimulant. Following additional incubation times at 37 °C, the reactions were stopped by the addition of a 10-fold excess of ice-cold HBSS and the cells were immediately centrifuged at 550 x g for 10 min, 4 °C. Cell pellets were suspended in 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4, containing 10 mM benzamidine, 1 pg/ml leupeptin, 5 pg/ml a2-macroglobulin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EGTA as protease inhibitors. The cells were disrupted using a tissue homogenizer-Sonifier (Brinkmann Polytron) for 15 s twice on ice and centrifuged at 200 x g for 5 min to remove nuclei and undisrupted cells. The supernatant was centrifuged at 100,000 x g for 1 h, and the resulting pellet was suspended in 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4, plus protease inhibitors. Protein was determined using the bicinchoninic acid reagent (Pierce Chemical Co.).

Assay of PI and PIP Kinases—PI and PIP kinases were assayed as described previously by monitoring the transfer of 32P from [γ-32P]ATP (442 cpm/pmol) to exogenously added PI (5 pg) or PIP (7.5 pg) (15). Assays contained 75 μg of membrane protein, 0.01% Triton X-100, and 0.1 mM [γ-32P]ATP in 50 mM Tris-HCl, 10 mM MgCl₂, pH 7.4, in a total volume of 0.1 ml unless otherwise specified. Reactions were carried out at 25 °C for 5 min and were linear over this time period. Reactions were terminated by the addition of 2.5 ml of chloroform/methanol/2.4 N HCl (1:1:0.5, v/v) and extracted by the method of Schect (21). The organic phase was washed with 2 ml of methanol/1 N HCl (1:1, v/v), applied to neomycin affinity columns and the phosphoinositides eluted as described previously. Results are expressed as picomoles of phosphoinositide formed per min/mg of protein, calculated assuming 1 mol of 32P was incorporated per mol of 32PIP and 1 mol of 32PIP₂ was incorporated per mol of PIP₂ formed. Statistics were performed using the Student’s t test for unpaired samples.

Assay of ATP Hydrolyzing Activity—The hydrolysis of [2,8,5'-3H]ATP was measured by incubating duplicate samples of 5 μCi of labeled ATP and unlabeled ATP (0.1 mM) in the presence of 7.5 μg of phosphomonoesterase activity was measured by incubating inositol phosphate, ATP, and adenosine, AMP, ADP, and ATP standards. The plate was developed in a solvent system consisting of n-butyric acid/H₂O/methanol followed by rapid centrifugation and the supernatant fluid was counted by liquid scintillation spectroscopy.

Assay of PI and PIP Hydrolyzing Activity—The hydrolysis of [2,8,5'-3H]PIP, by phosphomonoesterase activity was measured by incubating inositol phosphate, ATP, and adenosine, AMP, ADP, and ATP standards. The plate was developed in a solvent system consisting of n-butyric acid/H₂O/ammonium hydroxide (66:33:1). Standards were visualized with UV light and the spots were scraped into vials and counted by liquid scintillation spectroscopy.

Assay of Protein Synthesis—Protein synthesis was quantified as the trichloroacetic acid-precipitable radioactivity from human PMN which had been incubated in the presence of [3H]leucine (Du Pont-New England Nuclear) for 30 min at 37 °C (22).

RESULTS

Effects of Chemoattractants on PI and PIP Kinases in Human PMN—To determine whether the chemoattractant, FMLP, altered the activity of PI and PIP kinases in human PMN, intact cells were incubated at 37 °C for time intervals ranging from 30 s to 10 min with HBSS alone or containing 50 nM FMLP. Fig. 1 shows the effects of FMLP treatment on both PI and PIP kinases (Fig. 1A) and PI kinase (Fig. 1B) activities. PI kinase activity (Fig. 1A) was increased as early as 30 s following addition of FMLP to the cells, and maximal stimulation of 97% occurred at 3 min (p < 0.025). The increase in PIP kinase activity was noted during the entire 10 min incubation time. In contrast, PI kinase activity (Fig. 1B) was not altered by incubation of the cells with FMLP.

Fig. 2 shows the dose response effect of FMLP for stimulation of PMN PI and PIP kinases. Incubation of PMN at 37 °C for 3 min with concentrations of FMLP ranging from 1 nM to 0.1 μM caused a dose-dependent stimulation of PI kinase with a maximal increase in activity noted at a concentration of 50 nM, a dose which produces maximal chemotactic activity in a modified Boyden chamber chemotaxis assay (20). Of note, PI kinase activity was not altered at any concentration of FMLP tested.

To determine whether other formyl peptide chemoattractants were capable of stimulating PIP kinase activity in human PMN, cells were incubated with various concentrations of FMLP, Nle-Leu-Phe-Nle-Tyr-Lys, Met-Met-Met, and Met-Phe for 3 min at 37 °C prior to isolation of particulate membrane fractions which were then assayed for PIP kinase activity. Table 1 shows the concentration of peptide which produced maximal stimulation of PIP kinase activity. The order of potency for stimulation of PIP kinase was Nle-Leu-Phe-Nle-Tyr-Lys > FMLP > Met-Met-Met > Met-Phe. This order of peptide potency for stimulating PMN PIP kinase exactly parallels the order of potency of these peptides.
stimulation was dose-dependent, and increased PIP kinase activity in human PMN. The formyl peptide chemoattractants (24, 25) increased PIP kinase activity in human PMN. LTB₄ has been shown to bind to a class of cell surface receptors which are distinct from those that bind chemoattractants, FMLP and LTB₄, also altered PIP kinase activity in human PMN. LTB₄ has been shown to bind to a class of cell surface receptors which are distinct from those that bind formyl peptide chemoattractants (24, 25). Human PMN were incubated with HBSS alone or containing concentrations of FMLP ranging from 0.1 nM to 1 PM for 3 min at 37 °C, following which the particulate membrane fraction was isolated as described in "Experimental Procedures." Fig. 3 shows that LTB₄, ranging from 0.1 nM to 1.0 μM for 3 min at 37 °C following which PI (○) and PIP (○) kinase activities were measured in the presence of 0.1 mM [γ-³²P]ATP and 50 μg/ml PI or 75 μg/ml PIP, respectively. ○, ³²PIP₂ formed, ○, ³²PIP formed. The experiment shown is representative of three performed.

**TABLE I**

<table>
<thead>
<tr>
<th>Cells incubated with*</th>
<th>Concentration*</th>
<th>PIP kinase specific activity†</th>
<th>HBSS control†</th>
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<tr>
<td>HBSS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>/Nle-Leu-Phe-Nle-Tyr-Lys</td>
<td>10</td>
<td>46.6 ± 0.7</td>
<td>173</td>
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<tr>
<td>/Met-Leu-Phe</td>
<td>50</td>
<td>37.9 ± 0.2</td>
<td>140</td>
</tr>
<tr>
<td>/Met-Met-Met</td>
<td>100</td>
<td>46.1 ± 1.4</td>
<td>171</td>
</tr>
<tr>
<td>/Met-Phe</td>
<td>1000</td>
<td>47.6 ± 4.9</td>
<td>177</td>
</tr>
</tbody>
</table>

*PMNs were incubated with HBSS alone or the indicated peptide for 3 min at 37 °C, and the membrane fraction isolated as described in the text.  †The concentration indicated produced maximal stimulation of the PIP kinase activity. Assays were performed in the presence of 0.1 mM ATP (specific activity = 442 cpm/pmol) and 75 μg/ml PIP. Results shown are ± S.D. of duplicate samples in a representative experiment performed three times with similar results.

**FIG. 2.** Effect of FMLP on PMN PI and PIP kinases: dose response. Intact cells were incubated for 3 min at 37 °C with HBSS alone or containing concentrations of FMLP ranging from 0.1 nM to 1 μM. PI (○) and PIP (○) kinase activities were measured in the presence of 0.1 mM [γ-³²P]ATP and 50 μg/ml PI or 75 μg/ml PIP, respectively. ○, ³²PIP₂ formed, ○, ³²PIP formed. The experiment was performed three times with similar results.

**FIG. 3.** Effect of LTB₄ on PMN PI and PIP kinases: dose response. Intact cells were incubated with HBSS alone or containing concentrations of LTB₄ ranging from 0.1 nM to 1.0 μM for 3 min at 37 °C following which PI (○) and PIP (○) kinase activities were measured in the presence of 0.1 mM [γ-³²P]ATP and 50 μg/ml PI or 75 μg/ml PIP, respectively. ○, ³²PIP₂ formed, ○, ³²PIP formed. The experiment shown is representative of three performed.

activity for 47.9 ± 1.1 pmol ³²PIP₂ formed per min/mg protein in the presence of buffer to 66.7 ± 0.2 pmol of ³²PIP₂ formed per min/mg protein in the presence of 50 nM LTB₄. This concentration of LTB₄ has been shown to maximally stimulate the chemotactic response in human PMN (24–26). As with the formyl peptide chemoattractants, no change in PI kinase activity was noted in membranes isolated from LTB₄-treated PMN.

**Kinetic Properties of the Chemoattractant-stimulated PMN PIP Kinase**—Particulate membrane fractions were prepared from human PMN incubated with or without 50 nM FMLP for 3 min at 37 °C and were assayed for PIP kinase activity in the presence of 75 μg/ml PIP and concentrations of ATP ranging from 0.01 to 2 mM ATP (22.1–4420 cpm/pmol). Fig. 4 shows a representative experiment in which PIP kinase activity is increased at all concentrations of ATP tested in membranes isolated from FMLP-treated cells. The inset (Fig. 4) shows Lineweaver-Burk analysis of this representative experiment. The V_max calculated as the mean ± S.E. of three identical experiments was 14.4 ± 2.6 pmol of ³²PIP₂ formed per min/mg protein in membranes isolated from HBSS-treated cells and 27.4 ± 4.4 pmol of ³²PIP₂ formed per min/mg protein in membranes isolated from FMLP-treated cells. There was no significant change noted in the apparent K_m for ATP of PIP kinase activity in membranes isolated from FMLP-treated cells (buffer, 0.07 ± 0.01 mM; FMLP, 0.09 ± 0.01 mM, n = 3).

**Effects of Nonchemoattractant Activators of the Human PMN Oxidative Burst on PIP and PI Kinase Activities**—Since chemoattractants cause stimulation of the oxidative burst in human neutrophils manifested by superoxide production and lysosomal enzyme release (27, 28), experiments were performed to determine whether nonchemoattractant activators of PMN were capable of stimulating PIP kinase activity. Fig. 5 shows a comparison of the amounts of superoxide produced by the chemoattractants, FMLP and LTB₄, as well as the phorbol ester and protein kinase C activator PMA and the calcium ionophore A23187. PMA was the most active stimulator of PMN superoxide production. Maximal stimulation of 3.65 nmol/10⁶ cells/10 min was noted in the presence of 0.1
Fig. 4. Kinetic properties of PMN PIP kinase activities in the presence and absence of chemoattractants. Particulate membrane fractions prepared from cells incubated with buffer alone (○) or 50 nM FMLP (○) for 3 min at 37 °C were assayed for PIP kinase in the presence of 75 μg/ml PIP and concentrations of [γ-32P]ATP ranging from 0.01 mM to 2.0 mM (specific activity 22.1-44.2 cpm/pmol). The inset shows the Lineweaver-Burk analysis of this representative experiment of three identical performed. The average V_{max} of PIP kinase in three experiments was 14.4 ± 2.6 pmol/32PIP formed per min/mg protein ± S.D. in the presence of buffer and 27.4 ± 4.4 pmol 32PIP formed per min/mg protein ± S.D. in the presence of FMLP (p < 0.025). The average apparent K_{m} values for ATP of the three experiments were 0.07 ± 0.01 mM (±S.E.M) for cells treated with buffer and 0.08 ± 0.01 mM for cells treated with FMLP (p > 0.2).

Fig. 5. Superoxide (O_{2}) production in PMN treated with cellular activators. Intact PMN were incubated with HBSS alone or the indicated stimulant for 10 min at 37 °C and superoxide production monitored by measuring the reduction of ferricytochrome c (1.245 mg/ml) in the presence and absence of superoxide dismutase (50 μg/ml). No reduction of cytochrome c above a buffer background was noted in cells treated with superoxide dismutase. Results shown indicate the amount of superoxide produced (nmol/10^6 cells/10 min) in the presence of the indicated activator minus the amount produced in the presence of the activator and superoxide dismutase.

μM PMA. Ionophore A23187 induced superoxide production maximally at a concentration of 10 μM to a level of 1.2 nmol/10^6 cells/10 min. The levels of superoxide stimulation by the chemoattractants FMLP and LTB_4 were substantially less than that noted for PMA and A23187. FMLP stimulated the oxidative burst to 0.5 nmol/10^6 cells/10 min and LTB_4 produced no appreciable O_{2} above that noted for HBSS alone (0.2 nmol/10^6 cells/10 min). These observations are similar to those noted previously by others (29, 30).

If the stimulation of PIP kinase by chemoattractants was related to their ability to activate the oxidative burst, then similar results should be expected with PMA and A23187. To evaluate this, isolated PMN were incubated with concentrations of PMA ranging from 1 nM to 0.5 μM for 3 min at 37 °C, following which the particulate membrane fractions were isolated and assayed for PI and PIP kinase activities. Fig. 6A shows that PMA produced no significant alterations in PI and PIP kinase activities even at concentrations which exceeded those that produced maximal stimulation of superoxide anion production. No significant stimulation of either enzyme was noted when the incubation time of 0.5 μM PMA with cells was varied from 30 s to 10 min (data not shown).

Incubation of PMN with concentrations of A23187 ranging from 0.1 to 10 μM for 3 min at 37 °C, similarly, did not stimulate PI or PIP kinase activities, but inhibited both enzymes by as much as 50% (Fig. 6B) at concentrations which exceeded those that produced maximal stimulation of superoxide anion production. These results are not surprising since calcium has been shown to inhibit both PI and PIP kinases derived from human PMN (15).

Effect of Cytochalasin B on Chemoattractant-induced Stimulation of PMN PIP Kinase—The anti-microfilament agent, cytochalasin B, augments the oxidative burst of PMN to soluble stimuli such as chemoattractants (31, 32). To determine whether this agent altered the stimulation of PMN PIP kinase in response to a submaximal stimulation by FMLP, isolated PMN were preincubated with HBSS alone or containing concentrations of cytochalasin B ranging from 0.1 to 5 μM for 10 min at 37 °C prior to the addition of HBSS or 10 nM FMLP. Incubations were terminated after 5 min and the particulate membrane fractions isolated and assayed for PIP kinase activity. Fig. 7 shows that cytochalasin B alone caused a significant (μ < 0.005) increase in PIP kinase activity at the highest concentration tested, 5 μM. In the presence of this concentration of cytochalasin B, PIP kinase activity was increased from 18.2 ± 0.6 pmol of 32PIP formed per min/mg protein ± S.D. when cells were incubated with HBSS alone to 33.8 ± 0.6 pmol of 32PIP formed per min/mg protein in the presence of 5 μM cytochalasin B. A suboptimal stimulatory concentration of FMLP (10 nM) alone increased PIP kinase activity to 35 ± 2.1 pmol of 32PIP formed per min/mg protein;
however, in the presence of cytochalasin B, there was a dose-dependent augmentation of cytochalasin B, FMLP increased the enzyme activity to 70.7 ± 2.7 pmol of \( ^{32} \text{PIP}_2 \) formed per min/mg protein, a value which is 3.8-fold that noted in cells incubated with HBSS alone.

**Effects of Exogenous \( \text{PIP}_2 \) and \( \text{PIP}_3 \) on the Stimulation of \( \text{PIP} \) Kinase by FMLP**—FMLP treatment of intact neutrophils results in a rapid decrease in both \( \text{PIP}_2 \) and \( \text{PIP}_3 \) levels with more dramatic effects occurring on the latter phospholipid. After the initial decrease, \( \text{PIP}_2 \) levels are noted to increase after the first minute and \( \text{PIP}_3 \) levels return to base line. In addition, \( \text{PIP}_2 \) has been shown to be an end product inhibitor of \( \text{PIP} \) kinase. Given these considerations, it was important to determine whether chemoattractant-induced stimulation of \( \text{PIP} \) kinase in human neutrophils might be due to resultant increased levels of \( \text{PIP} \) as substrate or decreased levels of \( \text{PIP}_2 \) as end product inhibitor.

To address these possibilities, intact cells were incubated with HBSS alone, or containing 50 nM FMLP for 3 min at 37 °C following which particulate membrane fractions were prepared and assayed for \( \text{PIP} \) kinase in the presence of 0.1 mM \( [\gamma-\text{ATP}] \) and 75 μg/ml \( \text{PIP} \). The experiment is representative of three performed.

The effects of exogenous \( \text{PIP}_2 \) addition were similarly evaluated. Concentrations of \( \text{PI} \) ranging from 0 to 50 μg/assay (0–500 μg/ml) did not significantly alter the relative stimulation of \( \text{PIP} \) kinase by FMLP, though the overall enzyme activity in both HBSS- and FMLP-treated membranes was inhibited by concentrations of \( \text{PI} \) ≥ 10 μg/assay (data not shown).

**Effect of FMLP on the Hydrolysis of ATP**—To determine if the stimulating effects of FMLP on \( \text{PIP} \) kinase could be explained by protection of ATP from hydrolysis, the rate of ATP degradation was measured in the presence of particulate membrane fractions which were prepared from PMN incubated with 50 nM FMLP. \([\text{H}]\text{ATP} (0.1 \text{ mM}, 5 \mu \text{Ci}) \) was incubated with 50 nM Tris, 10 mM MgCl\(_2\), pH 7.4, and 7.5 μg of \( \text{PI} \) and particulate membrane fractions derived from HBSS- or FMLP-treated cells for 1–5 min at 25 °C. Reactions were stopped by the addition of methanol and rapid centrifugation, and adenosine nucleotides were separated by thin layer chromatography as described under “Experimental Procedures.” Fig. 8 shows that there were no significant differences at any time tested in the amount of \([\text{H}]\text{ATP} \) remaining in incubations containing membranes from HBSS- versus FMLP-treated cells. In addition, the overall degradation of \([\text{H}]\text{ATP} \) measured during the 5-min incubation period was minimal under both conditions (96.2 ± 6.5% \([\text{H}]\text{ATP} \) remaining in the presence of HBSS membranes; 85.5 ± 7.3% \([\text{H}]\text{ATP} \) remaining in the


**FIG. 8. Effects of FMLP on the hydrolysis of [3H]ATP by PMN membranes.** Particulate membrane fractions were isolated from cells which had been treated with HBSS alone (○) or 50 nM FMLP (□) and incubated with [3H]ATP (5 μCi, 0.1 mM) and 7.5 μg of PIP for the indicated times at 25 °C. Reactions were stopped and adenosine nucleotides were separated by TLC as described under "Experimental Procedures." The residual [3H]ATP was measured spectrophotometrically and is expressed as the residual counts/min ± S.D. of duplicate samples. Time 0 represents [3H]ATP incubated in the absence of membranes. The results are representative of two experiments performed.

**Discussion**

Many hormones and neuropeptides as well as chemoattractants institute receptor-mediated transmembrane signaling via activation of phospholipase C with subsequent degradation of PIP₂ and generation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (4, 6, 8, 10).

Many of these receptor systems including those for N-formyl peptide chemoattractants require intermediate coupling with guanine nucleotide regulatory proteins (6, 33-35). The phosphorylated derivative of PI, PIP₂, is the necessary substrate for phospholipase C in this reaction but is present in very small amounts within eukaryotic cells, suggesting that its synthesis is under stringent regulation. We report here that the synthesis of PIP₂ is directly influenced by chemoattractant-receptor occupancy in human neutrophils. The incubation of intact cells with N-formylated chemoattractants or leukotriene B₄ caused a rapid (within 30 s) stimulation of PIP kinase with a maximal effect occurring at 3 min at 37 °C. The increase in the enzyme activity was dose-dependent over the range of concentrations of these substances which have been shown to stimulate chemotaxis maximally in Boyden chamber assays in vitro (20, 26, 27). No stimulation of PI kinase activity was noted in identical membrane preparations derived from chemoattractant-stimulated cells, indicating that these agents do not globally and nonspecifically alter enzymes involved in phospholipid synthesis.

The chemoattractant-induced increase in PIP kinase activity was not a nonspecific consequence of superoxide anion generation in PMN, since cellular activators such as PMA and A23187 did not significantly increase PIP kinase activity at concentrations which stimulated superoxide production.
more effectively than the chemoattractants, FMLP and LTB4 (Fig. 5). In addition, a concentration of superoxide dismutase (50 μg/ml) which completely inhibited detection of superoxide production by various cellular activators did not interfere with the ability of FMLP to stimulate PMN PIP kinase activity (data not shown).

Interestingly, cytochalasin B, which binds to actin and disrupts microfilaments, markedly increased the ability of FMLP to stimulate PIP kinase activity (data not shown). We have previously demonstrated that antimicrofilament agents increase the extractability of PMN PIP kinase in nonionic detergents (36). The explanation for the augmentation of FMLP-induced stimulation of PIP kinase by cytochalasin B is unknown at this time; however, it has been repeatedly demonstrated that antimicrofilament agents greatly increase receptor-mediated stimulation of the oxidative burst by chemoattractants in leukocytes (31, 32).

Chemoattractant-stimulated PIP kinase activity was characterized by as much as a two-fold increase in the $V_{max}$ of the enzyme without a significant effect on the apparent $K_m$ for ATP determined in the presence of saturating concentrations of PIP. The increased production of $^{32}$P-PIP by FMLP could not be explained by an inhibition of ATP hydrolysis nor by inhibition of PIP$_2$ phosphomonoesterase activity or phospholipase C activity.

The mechanism of the increase in the $V_{max}$ of PIP kinase by chemoattractants is unknown at this time but does not appear to be due to changes in the Mg$^{2+}$ requirements of the enzyme or in its sensitivity to inhibition by calcium. In addition, new protein synthesis did not appear to be required for the stimulation of PIP kinase by chemoattractants. Treatment of cells with chemoattractants may result in either removal of a tonic inhibitor of PIP kinase activity or in generation or stimulation of an activator of the enzyme. We cannot explain the stimulation of the enzyme by FMLP, however, on the basis of increases in PIP levels or decreases in PIP$_2$, a known inhibitor of PIP kinase in human PMN, since the addition of large excesses of both these phospholipids did not alter the level of stimulation of PIP kinase in membranes isolated from FMLP-treated cells. Since guanine nucleotide regulatory proteins are required for coupling of chemoattractant receptor-mediated signals in leukocytes through phospholipase C activation (6), it is possible that these membrane elements also mediate the stimulation of PIP kinase. Indeed, Smith and Chang (37) have shown that non-hydrolyzable analogues of GTP directly stimulate rat brain PIP kinase activity. We are actively investigating this association at present.

The chemoattractant-stimulated PIP kinase activity may be similar to that observed in immunoprecipitates of platelet-derived growth factor-stimulated smooth muscle cells (19). Our method of separation of the phosphoinositides does not distinguish between isomers of PIP$_2$ such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 4,5-bisphosphate. Further studies are needed to evaluate this potential association. Traynor-Kaplan et al. (38) have demonstrated the appearance of an inositol tetrakisphosphate-containing phospholipid (phosphatidylinositol trisphosphate) in intact human neutrophils treated with chemoattractants. Increased levels of phosphatidylinositol trisphosphate have also been demonstrated in platelet-derived growth factor-stimulated smooth muscle cells (19). From the present data, we cannot eliminate the possibility that a novel enzyme, PIP$_3$ kinase, is also stimulated in human neutrophils by chemoattractants. We are actively investigating this at present.

Whatever the mechanism, these findings demonstrate direct, chemoattractant-mediated, stimulation of PIP kinase, the enzyme which forms the reservoir for second messenger substances. PIP$_3$. Increased synthesis of PIP$_3$ by chemoattractant-stimulated PIP kinase may serve to replenish this important phospholipid in the membrane following its hydrolysis by phospholipase C.

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