

## Is There a Relationship between Phosphatidylinositol Trisphosphate and F-actin Polymerization in Human Neutrophils?\*

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Stimulation of human neutrophils with the chemoattractant *N*-formyl peptide caused rapid polymerization of F-actin as detected by right angle light scatter and 7-nitrobenz-2-oxa-1,3-diazol (NBD)-phalloidin staining of F-actin. After labeling neutrophils with  $^{32}\text{P}$ , exposure to *N*-formyl peptide induced a fast decrease of phosphatidylinositol 4-bisphosphate ( $\text{PIP}_2$ ), a slow increase of phosphatidic acid, and a rapid rise of phosphatidylinositol 4-trisphosphate ( $\text{PIP}_3$ ). Formation of  $\text{PIP}_3$  as well as actin polymerization was near maximal at 10 s after stimulation. Half-maximal response and  $\text{PIP}_3$  formation at early time points resulted from stimulation of neutrophils with 0.01 nM *N*-formyl peptide or occupation of about 200 receptors. Sustained elevation of  $\text{PIP}_3$ , prolonged right angle light scatter response, and F-actin formation required higher concentrations of *N*-formyl peptide, occupation of thousands of receptors, and high binding rates. When ligand binding was interrupted with an antagonist, F-actin rapidly depolymerized, transient light scatter response recovered immediately, and elevated [ $^{32}\text{P}$ ] $\text{PIP}_3$  levels decayed toward initial values. However, recovery of [ $^{32}\text{P}$ ] $\text{PIP}_2$  was not influenced by the antagonist. Based on the parallel time courses and dose response of [ $^{32}\text{P}$ ] $\text{PIP}_3$ , the right angle light scatter response, and F-actin polymerization,  $\text{PIP}_3$  is more likely than  $\text{PIP}_2$  to be involved in modulation of actin polymerization and depolymerization *in vivo*.

Specific ligand receptor interaction induces phospholipid turnover and actin polymerization (1, 2). In most cases the initiation of actin polymerization occurs close to the plasma membrane (3). Markey *et al.* (4) speculated that the G-actin molecules in this filament formation are derived from pro-

lactin complexes.  $\text{PI-4,5-P}_2$ <sup>1</sup> has been found to promote actin polymerization in two systems *in vitro*, dissociation of gelsolin-actin complexes, and dissociation of profilactin (5-11). Experiments *in vitro* revealed that profilin inhibits actin polymerization stoichiometrically by binding to G-actin monomers (5). Phosphoinositides have been found to promote dissociation of actin from the profilin-actin complex, leading to increased actin polymerization. At low calcium ion concentration,  $\text{PI-4,5-P}_2$  is more potent than  $\text{PI-4-P}$  or  $\text{PI}$  (6, 7). In addition, the severing activity of the  $\text{Ca}^{2+}$ -dependent actin-binding protein, gelsolin, is also inhibited by  $\text{PI-4,5-P}_2$  and  $\text{PI-4-P}$  (8, 9). These observations led Stossel (3) to propose that recovery of  $\text{PI-4,5-P}_2$  levels along with elevated  $\text{Ca}^{2+}$  following receptor-mediated hydrolysis are the signals promoting actin assembly in neutrophils stimulated with chemoattractants. Sklar *et al.* (12) and Bengtsson *et al.* (13) reported time courses of the changes in intracellular  $\text{Ca}^{2+}$  and  $\text{PI-4,5-P}_2$  which are slower than those required for F-actin polymerization *in vitro*. Furthermore, actin polymerization in neutrophils is observed when calcium ions are buffered by intracellular chelators and at very low ligand concentrations, below what is necessary to simulate many of the other responses such as calcium ion elevation, elastase release, or superoxide production.

Recently, Traynor-Kaplan *et al.* (14, 15) identified a novel phosphoinositide,  $\text{PIP}_3$ , in neutrophils. More recently, it has been identified in smooth muscle cells and platelets (16, 17). Generation of  $\text{PIP}_3$  in neutrophils was detected after stimulation with chemoattractants such as *N*-formyl peptide and leukotriene  $\text{B}_4$ . Since actin polymerization is one of the few responses which persists with low levels of stimulation it was of interest to study  $\text{PIP}_3$  formation under these circumstances. In this paper we demonstrate that  $\text{PIP}_3$  levels are elevated under stimulation with low concentrations of formyl peptide and that the time course of the appearance of  $\text{PIP}_3$  is consistent with its participation in actin polymerization.

### EXPERIMENTAL PROCEDURES

[ $^{32}\text{P}$ ]Orthophosphate (HCl-free) was obtained from Du Pont-New England Nuclear. Fluorescein-labeled *N*-formyl-Norleu-Leu-Phe-Norleu-Norleu-Tyr-Lys was prepared as described (18). The *N*-formyl peptide receptor blocker *t*-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe was purchased from Vega Biotechnologies, Inc. (Tucson, AZ). 7-Nitrobenz-2-oxa-1,3-diazol (NBD)-phalloidin was obtained from Molecular Probes, Inc. (Junction City, OR). Neutrophils were prepared by the elutriation protocol of Tolley *et al.* (19) and characterized as described.

**Lipid Analysis**—Lipid analysis was performed essentially as described (14, 15). Briefly,  $5 \times 10^7$  neutrophils/ml were labeled with [ $^{32}\text{P}$ ]orthophosphate (0.5 mCi/ml) for 90 min. Cells were washed three times, resuspended, and stimulated as indicated. The stimulated reaction of neutrophils with *N*-formyl peptide was stopped at the indicated times with chloroform/methanol (1:2, v/v) containing the antioxidant butylated hydroxytoluene (0.63 mg/ml) and 10  $\mu\text{g/ml}$  phosphoinositides (Sigma) as nonradioactive carrier. Phospholipids were extracted and analyzed as described previously (15).

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<sup>1</sup> The abbreviations used are:  $\text{PI-4,5-P}_2$ , phosphoinositol 4,5-bisphosphate;  $\text{PI-3,4,5-P}_3$ , phosphoinositol 3,4,5-trisphosphate;  $\text{PI-3-kinase}$ , phosphoinositol 3-kinase; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol;  $\text{PIP}$ , phosphatidylinositol 4-phosphate;  $\text{PIP}_3$ , phosphatidylinositoltrisphosphate; Norleu, norleucine;  $\text{PIP}_2$ , phosphatidylinositolbisphosphate; *t*-Boc, *t*-butoxycarbonyl pheleupheleuphe.

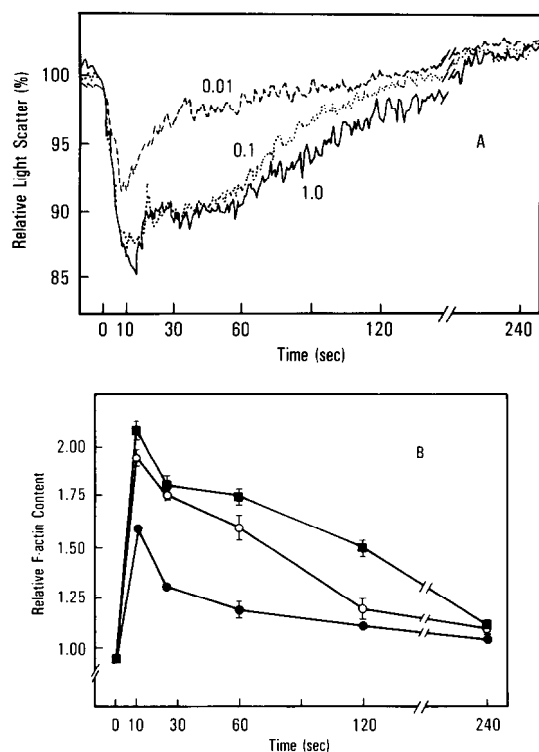
**F-actin and Light Scatter Measurements**—Measurements of F-actin formation and right angle light scatter were performed as described (12). Briefly, after stimulation, aliquots of cell suspension were withdrawn at the indicated times from the stirred sample compartment of the fluorometer (8000, SLM Instruments, Inc., Urbana, IL) during light scatter measurements, fixed with formaldehyde, and stained with NBD-phalloidin. The fluorescence intensity of F-actin was measured in a cytometer (FACScan, Becton-Dickinson, Sunnyvale, CA).

**Binding Assay**—Flow cytometric assay of *N*-formyl peptide binding was performed according to Sklar *et al.* (18) using as calibration standard fluorescent microbeads from Flow Cytometry Standard Corp. (Research Triangle Park, NC).

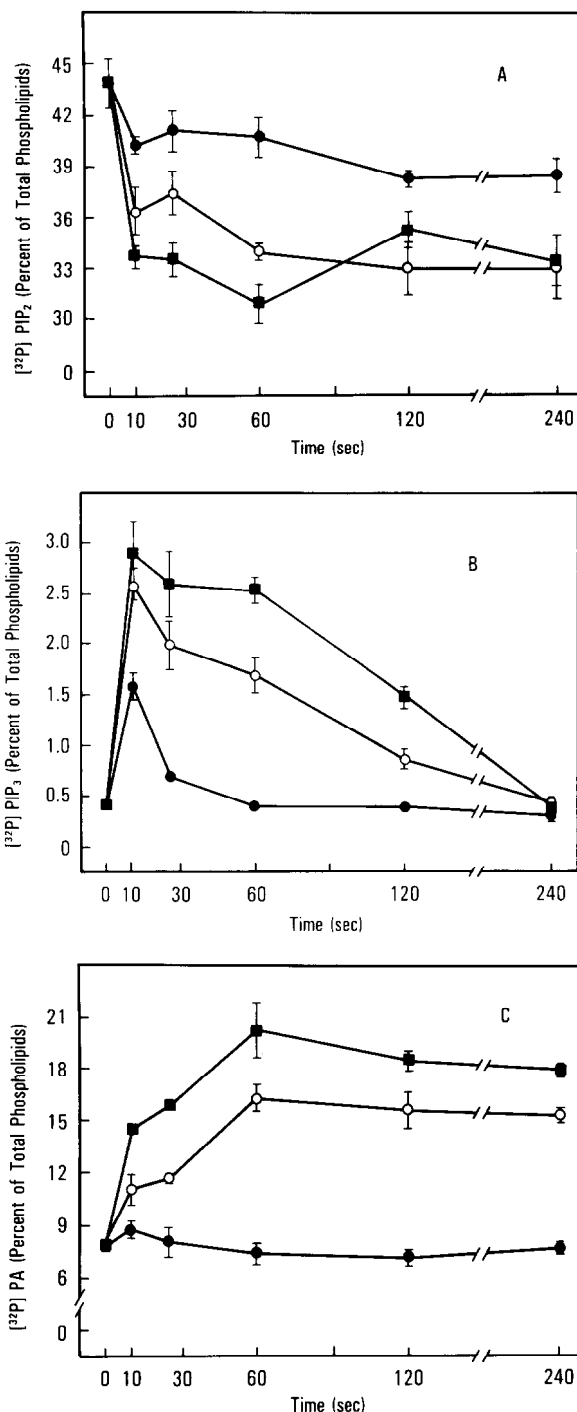
## RESULTS AND DISCUSSION

**Dose-Response Curves for Right Angle Light Scatter and Actin Polymerization in Human Neutrophils with *N*-Formyl Peptide**—As previously described by Sklar *et al.* (12) right angle light scatter (Fig. 1A) and actin polymerization (Fig. 1B) are tightly coupled events. The relative right angle light scatter decreased rapidly within 10 s after stimulation, and the rate of subsequent recovery to initial values depended on the concentration of *N*-formyl peptide. In parallel, the relative F-actin content, quantified by flow cytometry, exhibited a rapid early polymerization phase. Sustained polymerization could be detected at high *N*-formyl peptide concentrations.

While several actin-binding proteins are known to be involved in the control of F-actin formation (3, 6, 20, 21), the underlying mechanism for stimulating actin polymerization is not understood. Lassing and Lindberg (6) reported that PI-4,5-P<sub>2</sub> and PI-4-P can bind to profilin in profilactin complexes, causing a rapid and efficient liberation of G-actin for polymerization. In this case, the generation of PI-4,5-P<sub>2</sub> could



**FIG. 1. Dose response curves for analysis of right angle light scatter and F-actin formation.** Right angle light scatter response (A) and the relative F-actin content (B) were measured after exposure of neutrophils to 1 nM (■), 0.1 nM (○), and 0.01 nM (●) *N*-formyl peptide. Data are means with standard deviation of one representative experiment performed in duplicate repeated twice and similar to those reported earlier.



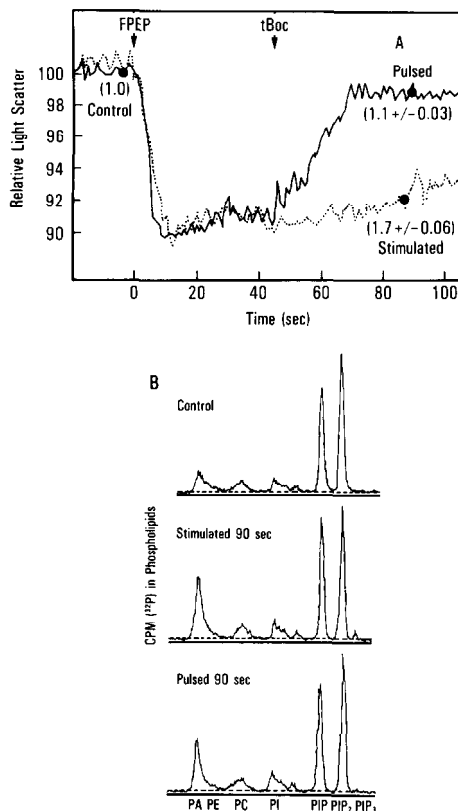
**FIG. 2. Dose response of <sup>32</sup>P-labeled phospholipids.** Changes in <sup>32</sup>P-labeled phospholipids after stimulation with 1 nM (■), 0.1 nM (○), and 0.01 nM (●) *N*-formyl peptide were analyzed. The individual data points represent the incorporation of <sup>32</sup>P in PIP<sub>2</sub> (A), PIP<sub>3</sub> (B), and PA (C) relative to the <sup>32</sup>P incorporation into total phospholipids plotted versus time. Data are means with standard deviation of one experiment repeated twice in duplicate.

induce the first morphological changes for F-actin formation (11).

**Analysis of <sup>32</sup>P-Labeled Phospholipids in Human Neutrophils**—Stimulation of neutrophils with *N*-formyl peptide induced a rapid decrease in [<sup>32</sup>P]PI-4,5-P<sub>2</sub> (Fig. 2A). The decrease of [<sup>32</sup>P]PIP<sub>2</sub> occurred within 10 s, and half-maximal breakdown was detected with 0.01 nM *N*-formyl peptide. The primary pathway decreasing [<sup>32</sup>P]PIP<sub>2</sub> is generally thought to involve the activation of phospholipase C which hydrolyzes PI-4,5-P<sub>2</sub> to inositol 1,4,5-trisphosphate and 1,2-diaclyglyc-

erol (22). An additional contribution to the loss of [<sup>32</sup>P]PIP<sub>2</sub> is a putative second pathway in which PI-3-kinase phosphorylates PI-4,5-P<sub>2</sub> in the D-3 position of the inositol ring to form PIP<sub>3</sub> (15). Since stimulation with *N*-formyl peptide lowers the level of PI-4,5-P<sub>2</sub> it is unlikely to be a direct second messenger for actin polymerization as has been suggested by Lassing and Lindberg (11) and Stossel (3).

Therefore, it is of interest to study the products of PI-4,5-P<sub>2</sub> metabolism and their relation to cytoskeletal changes. Bengtsson *et al.* (13) already demonstrated that breakdown products of PIP<sub>2</sub>, inositol 1,4,5-trisphosphate, and diacylglycerol are not involved in actin polymerization. We analyzed the kinetics of [<sup>32</sup>P]PIP<sub>3</sub> formation. <sup>32</sup>P could become elevated either by the stimulation of the PI-3-kinase or the inhibition of a degradative enzyme. A rapid and transient increase of [<sup>32</sup>P]PIP<sub>3</sub> (within 10 s) from about 0.4% of the total phospholipids to a maximum of 2.8% was detected (Fig. 2B) following



**FIG. 3. Pulse protocol of right angle light scatter, actin polymerization, and phospholipids after interruption of formyl peptide binding with the antagonist *t*-Boc-Phe-Leu-Phe-Leu-Phe.** A, right angle light scatter response of 1 nM *N*-formyl peptide (*FPEP*)-stimulated neutrophils with (—) and without (····) 20 μM antagonist added at 45 s. The relative F-actin content after 90 s with and without added antagonist is indicated in parentheses. B, the profile of incorporated <sup>32</sup>P radioactivity in phospholipids separated by TLC in control neutrophils and *N*-formyl peptide-stimulated neutrophils with and without added antagonist. Relative incorporation <sup>32</sup>P radioactivity into the phospholipids was calculated using the AMBIS scanning system. The relative distribution of incorporated <sup>32</sup>P into phospholipids by control cells was 12.75% (±0.21) PA, 1.5% (±0.28) PE, 7.7% (±0.14) PC, 7.55% (±0.78) PI, 30.35% (±1.34) PIP, 39.75% (±1.77) PIP<sub>2</sub> and 0.25% (±0.06) PIP<sub>3</sub>; by *N*-formyl peptide-stimulated cells after 90 s 25.35% (±0.21) PA, 2.55% (±0.71) PE, 6.3% (±0.0) PC, 7.45% (±0.35) PI, 26.20% (±2.19) PIP, 30.35% (±2.33) PIP<sub>2</sub>, and 1.8% (±0.07) PIP<sub>3</sub>; and by stimulated cells with added *t*-Boc 20.65% (±0.78) PA, 3.05% (±0.92) PE, 8.1% (±1.27) PC, 10.25% (±1.2) PI, 27.7% (±0.57) PIP, 29.9% (±2.4) PIP<sub>2</sub>, and 0.35% (±0.07) PIP<sub>3</sub>. The data reflect the means of duplicates with standard deviation of a single experiment representative of an experiment repeated three times.

TABLE I

Receptor occupancy versus time and ligand concentration

The number of occupied *N*-formyl peptide receptors was calculated using flow cytometric binding measurements as described under "Experimental Procedures." Data are means of duplicates with standard deviation of a single experiment, representative of an experiment repeated twice.

Length of binding period	Receptor occupancy		
	1 nM <sup>a</sup>	0.1 nM <sup>a</sup>	0.01 nM <sup>a</sup>
<i>s</i>			
10	12,674 ± 2533	2,532 ± 850	210 ± 20
25	20,002 ± 1872	4,198 ± 239	409 ± 13
60	25,660 ± 1525	6,920 ± 735	767 ± 37
120	33,605 ± 45	10,551 ± 1667	1231 ± 171
240	37,600 ± 800	12,845 ± 2325	1441 ± 192

<sup>a</sup> Ligand concentration.

TABLE II

Binding rates

Binding rates *db/dt* are estimates which reflect the velocity of binding versus time. *db/dt* is calculated as  $((Nx + 1) - Nx) / ((tx + 1) - tx)$ , where *Nx* and *Nx + 1* reflect the number of occupied receptors at the time points *tx* and *tx + 1*. Calculations are based on binding data of Table I.

Length of binding period	Binding rates		
	1 nM <sup>a</sup>	0.1 nM <sup>a</sup>	0.01 nM <sup>a</sup>
<i>s</i>			
		receptor/s	
10	1267	253	21
25	489	111	13
60	162	78	10
120	132	61	8
240	34	19	2

<sup>a</sup> Ligand concentration.

the addition of 1 nM *N*-formyl peptide. Half-maximal elevation of [<sup>32</sup>P]PIP<sub>3</sub> at early time points (10 s) followed by a rapid decay was observed after 0.01 nM *N*-formyl peptide. Higher concentrations of *N*-formyl peptide resulted in prolonged elevation of [<sup>32</sup>P]PIP<sub>3</sub> as described earlier (15). Interestingly, the time courses of [<sup>32</sup>P]PIP<sub>3</sub> were similar to the kinetics of actin polymerization and light scatter response. This rapid rise in [<sup>32</sup>P]PIP<sub>3</sub> following stimulation appears to be dependent on a pertussis toxin-sensitive G-protein, is independent of the rise in intracellular Ca<sup>2+</sup>, and does not seem to be a product of protein kinase C activity (15).

The elevation of [<sup>32</sup>P]phosphatidic acid did not correlate with actin polymerization. Its increase was slower and monotonic (Fig. 2C). The half-maximal response was observed at 0.1 nM *N*-formyl peptide, and no significant increase of [<sup>32</sup>P]phosphatidic acid could be detected after 0.01 nM *N*-formyl peptide stimulation.

*Comparison of F-actin Formation and Phospholipid Turnover after Exposure to a Pulse Protocol with the N-Formyl Peptide Antagonist t-Boc-Sklar et al.* (12) reported that stimulated right angle light scatter changes which are associated with actin polymerization require continuing occupation of receptors by the ligand. After interrupting *N*-formyl peptide binding with the specific competitive antagonist *t*-Boc-Phe-Leu-Phe-Leu-Phe, a fast decline of F-actin and recovery of the light scatter response to initial values can be detected (Fig. 3A). We studied the influence of the antagonist on the phospholipid metabolism. The highly phosphorylated phospholipid [<sup>32</sup>P]PIP<sub>3</sub> decayed rapidly toward basal levels after interruption of binding with the antagonist (Fig. 3B). However, interruption of continuous binding by the antagonist did not influence the recovery of [<sup>32</sup>P]PIP<sub>2</sub>. These results suggest that [<sup>32</sup>P]PIP<sub>3</sub> levels are tightly coupled to receptor

occupation either as a result of linkage to a PI-3-kinase or a degradative pathway. [<sup>32</sup>P]PIP<sub>2</sub> levels are less tightly controlled. Therefore, changes in [<sup>32</sup>P]PIP<sub>3</sub> parallel actin polymerization.

*Real Time Formyl Peptide Ligand Receptor Binding Studies*—Preliminary reports based on calculations suggested that actin polymerization requires low ligand binding rates or the occupation of only a few receptors (12). To quantify the requirements of occupied receptors for stimulated F-actin formation and PIP<sub>3</sub> generation we performed binding studies by flow cytometry (Table I). These binding studies revealed decreasing binding rates with prolonged exposure of neutrophils to the ligand (Table II). The highest binding rates were measured at early time points. Under these conditions the occupation of about 200 receptors within 10 s or a binding of about 20 receptors/s was sufficient to induce half-maximal actin polymerization, light scatter response, and formation of PIP<sub>3</sub>. At later time points (60 and 120 s) 3–7-fold higher *N*-formyl peptide binding rates and occupation of thousands of receptors were required to elicit similar enhancement of PIP<sub>3</sub>, actin polymerization, and light scatter reduction. This fact might be associated with a phenomenon called signal adaptation in which an unknown inhibitory pathway might alter the affinity of the receptor (23) and/or the efficiency of signal transduction.

In summary, it is generally believed that occupation of few receptors and subsequent polymerization of F-actin are required for chemotaxis. Previous reports speculated that stimulated generation of PIP<sub>2</sub> might modulate F-actin polymerization (3, 11), and a recent report provides evidence for modulation of phosphoinositide metabolism by the actin-binding protein profilin (24). We provide evidence that the highly charged phospholipid PIP<sub>3</sub>, putatively PI-3,4,5-P<sub>3</sub>, rather than PI-4,5-P<sub>2</sub>, might be involved in regulating this cell response. To study the interactions of PIP<sub>3</sub> with profilactin and gelsolin will require the synthesis or isolation of quantities of PI-3,4,5-P<sub>3</sub> which have not previously been available.

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