Platelet-Activating Factor Both Stimulates and “Primes” Human Polymorphonuclear Leukocyte Actin Filament Assembly

By Meir Shalit, Guissou A. Dabiri, and Frederick S. Southwick

The phospholipid inflammatory mediator, platelet-activating factor (PAF), can stimulate polymorphonuclear leukocyte (PMN) chemotaxis. Conversion of cytoplasmic actin from monomers to filaments is associated with PMN motile functions. Using the fluorescent actin filament stain nitrobenzoxazolone phallicidin, we have investigated PAF's effects on human PMN actin polymerization. Concentrations of PAF between 1 x 10^{-11} to 1 x 10^{-8} mol/L induced actin filament (F-actin) assembly. An optimal concentration of PAF (1-5 x 10^{-8} mol/L) induced a significantly lower rise in relative F-actin content (1.72 ± 0.07 SEM) than an optimal concentration (5 x 10^{-7} mol/L) of the chemotactic peptide FMLP (2.21 ± 0.06). Unlike FMLP (F-actin content: 1.25 ± 0.04 at five seconds), PAF stimulation was associated with a delay of more than five seconds (1.04 ± 0.01 at five seconds) before an increase in F-actin could be detected. F-actin concentration reached maximum levels by 30 to 60 seconds. Prolonged stimulation (20 minutes) with PAF was associated with two phases of polymerization and depolymerization. Like FMLP, the initiation of actin filament assembly by PAF required receptor occupancy, this reaction being totally blocked by the PAF receptor inhibitor, SKI 63-441. As evidenced by the lack of inhibition by nordihydroguaiaretic acid (5 to 20 µmol/L), the production of leukotriene B_4 was not required for the PAF-induced changes in F-actin. Like FMLP, PAF's ability to stimulate PMN actin polymerization was inhibited by pertussis toxin (0.05 to 2.5 µg/mL) but not impaired by the addition of EGTA and/or the calcium ionophore A23187. Preincubation with 1 x 10^{-10} to 1 x 10^{-8} mol/L PAF for 2 to 60 minutes enhanced the rise in F-actin content induced by low concentrations of FMLP (5 x 10^{-12} to 1 x 10^{-10} mol/L) indicating that this phospholipid was capable of “priming” the PMN actin polymerization response.

Materials and Methods

Aprotonin, benzamidine, dimethyl sulfoxide (DMSO), EDTA, EGTA, FMLP, gelatin, HEPES, leupeptin, lysophosphatidyl choline, phenylmethyl-sulfonyl fluoride (PMSF), and triton X-100 were purchased from Sigma Chemical Company, St Louis, L-α-phosphatidylycholine, β-acetyl-γ-ο-alkyl (PAF), DL-α-lysophosphatidylcholine, γ-Ο-hexadecyl (lyso-PAF), A23187 were obtained from Cal-Biochem, San Diego. Bovine serum albumin was purchased from Reheis Chemical Company, Phoenix; pertussis toxin from List Biological Laboratory, Campbell, CA. Nitrobenzoxazolone-phallacidin was obtained from Molecular Probes, Junction City, OR. The PAF antagonist SRI 63-441 was kindly supplied by C.W. Winslow of Sandoz, Hanover, NJ.

A modified Hank's buffer (138 mmol/L NaCl, 6 mmol/L KCl, 1.2 mmol/L MgSO_4, 5 mmol/L NaHCO_3, 0.64 mmol/L Na_2HPO_4 (pH 7.4), 0.66 mmol/L KH_2PO_4, 5.6 mmol/L glucose, 20 mmol/L HEPES) was used in all assays. CaCl_2, final concentration of 1 mmol/L, was added to cell suspensions ten minutes before cell stimulation unless otherwise noted in the text.

FMLP and A23187 (final concentrations 10 nmol/L) were dissolved in DMSO and kept frozen at -70°C until used. PAF and lyso-PAF were dissolved in modified Hank's solution containing NaCl, KCl, CaCl_2, and HEPES at concentrations of 138, 6, 1.2, and 20 mmol/L, respectively.

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Preparation of neutrophils. Six milliliters of venous blood were drawn from normal human volunteers into syringes containing 0.4 mL of 10% EDTA. RBCs were removed by hypotonic lysis, followed by centrifugation at 400 g for five minutes. Cells were resuspended in modified Hank's buffer and centrifuged at 400 g for five minutes. This procedure was repeated once and cells resuspended in modified Hank's to achieve a final concentration of 1.5 x 10^6 cells/mL. These preparations contained a mixture of 83 ± 1.4% (SEM) PMNs, 11 ± 1.7% lymphs and 5.1 ± 0.8% monocytes as determined by Wright stain (nine determinations). Platelets were not seen in any of our preparations. This method was developed to minimize handling of cells and reduce the likelihood of inadvertent PMN activation prior to chemotactic stimulation. On several occasions PMNs were purified by Ficoll/Hypaque which yielded >95% PMNs. Unstimulated F-actin content of PMNs tended to be higher in these preparations; however, the relative increase in PMN F-actin induced by PAF was comparable to cells purified by our other method. Cells were maintained at room temperature unless otherwise noted. All experiments were performed within three hours of venipuncture.

Neutrophil stimulation and fixation. Cell suspensions, 0.5 mL aliquots, (0.75 x 10^6 cells per sample) were preincubated for seven minutes at 25°C. Various concentrations of stimulants or antagonist, 0.5 mL, were added to each sample. The reaction was stopped by addition of 0.1 mL of 37% formaldehyde. Cells were incubated in fixative for 15 minutes at 25°C before staining. NBD-Phallacidin staining. Staining was performed according to the method of Howard and Oresajo. Briefly, 50 µL of modified Hank's buffer containing 3.46 x 10^-4 mol/L NBD-phallacidin and 2.1 mg/mL lysophosphatidylcholine were added to the fixed cell preparations; mixed and incubated for ten minutes at 37°C. Cells were then centrifuged at 12,000 g for one minute, supernatants removed, and pellets resuspended in 1.5 mL of modified Hank's solution.

Quantification of F-actin content by flow cytometry. Stained cells were filtered through nylon mesh and analyzed within two hours of staining in most cases. We have found that stained samples could be stored at 4°C in Hank's buffer for 16 hours without detectable differences in F-actin content as compared with the samples analyzed immediately after staining. The intracellular fluorescence was determined by an Ortho Spectrum II (Ortho Diagnostic Systems, Westwood, MA) flow cytometer equipped with an argon laser (488 emission, 500 mW output). Using the combination of low angle forward light scatter and 90° angle light scatter, lymphocytes, monocytes, remaining erythrocytes, and debris were excluded from analysis by appropriate gating. All intensities of cellular fluorescence were recorded on the linear scale ranging from 0 to 255 channels. Fluorescent histograms plotting cell number (vertical axis) vs fluorescence channel (horizontal axis) were recorded for each sample. In all instances histograms yielded a normal distribution (the peak fluorescence channel corresponded to the mean fluorescence). An average of 5,000 to 10,000 PMNs were analyzed per sample. Relative PMN F-actin content was expressed as the ratio of mean fluorescence intensity of stimulated cells to mean fluorescence intensity of neutrophils in buffer. Unstimulated values were comparable when cells were incubated in modified Hank's buffer or 0.01% DMSO (the highest concentrations used in our experiments) in modified Hank's buffer.

Triton-insoluble cytoskeleton-associated actin. A modification of the method described by White et al was used. Human PMNs were purified by Ficoll/Hypaque and then suspended in modified Hank's buffer, centrifuged at 400 g and resuspended in the same buffer to achieve a final concentration of 1.5 x 10^6 cells per mL. Highly purified preparations of PMNs were required for this assay, since unlike FACS analysis that allows exclusive measurement of PMNs, the Triton-insoluble assay measures shifts in F-actin content of all cells in the solution. Five hundred microliters of the cell suspension was added to 55 µL of FMLP or PAF solution. The reaction was stopped by the addition of 65 µL of triton-stop solution containing 10% triton X-100, 7.5 mg% benzamide, 4 mg% aprotinin, 1 mg% leupeptin, 1 mmol/L PMSF, 54 mmol/L Pipes, 22.5 mmol/L Hepes, 9 mmol/L EGTA, 1.8 mmol/L MgCl2.

After mixing by two gentle inversions, the solution was allowed to sit for two minutes at 25°C, and then centrifuged for two minutes at 12,000 g in an Eppendorf table top centrifuge. The resulting pellet (the triton-insoluble fraction) was then solubilized in 100 µL of gel sample buffer containing 8 mol/L urea, 1 g% sodium dodecyl sulfate, 2 g% β-mercaptoethanol, 10 g% sucrose, 0.06 mol/L Tris-HCl, pH 6.8. The sample was next sonicated for 20 to 30 seconds using a Branson sonifier, model 200 with a tapered micro tip (Danbury, CT), output 3. Samples were then immediately boiled for five minutes and electrophoresed into 5% to 15% polyacrylamide slab gels. Triton-insoluble cytoskeleton-associated actin was quantified as previously described.

Statistics. The statistical significance of differences between means was determined using Student's t test.

RESULTS

The effects of varying concentrations of PAF on PMN F-actin content. Figure 1 demonstrates the effect of varying PAF concentration on neutrophil actin polymerization. A wide range of PAF concentrations 10^-11 to 10^-6 mol/L caused an increase in neutrophil F-actin content. A maximal relative F-actin concentration was observed at concentrations of 1.5 x 10^-8 mol/L PAF. Above this concentration a moderate decrease in F-actin content was observed.
Stimulation with the PAF analog lyso-PAF at concentrations of $5 \times 10^{-13}$ to $5 \times 10^{-4}$ mol/L failed to stimulate neutrophil actin assembly.

The kinetics of actin filament assembly following PAF stimulation. As shown in Fig 2A, exposure of human PMNs to an optimal concentration of PAF ($1 \times 10^{-8}$ mol/L) for various time periods resulted in a rise in F-actin content that reached a maximum value by 30 seconds. PAF's effects on PMN actin polymerization were delayed as compared with FMLP. After a five-second exposure to PAF there was no significant rise in F-actin content (F-actin content $1.04 \pm 0.01$ SEM, eight experiments) while FMLP ($5 \times 10^{-7}$ mol/L) induced a substantial increase in F-actin during the same period (relative F-actin concentration $1.25 \pm 0.04$ SEM, 13 experiments). Use of higher concentrations of PAF, up to $5 \times 10^{-7}$ mol/L failed to shorten this delay period (five seconds; F-actin content, 1.03). Concentrations of PAF of $10^{-6}$ mol/L or higher could not be used, since as observed by other investigators, these concentrations were associated with cell lysis. Addition of a low concentration of FMLP, $1 \times 10^{-10}$ mol/L, was not associated with a delay in actin filament assembly. The initial rise in F-actin content after five seconds, $1.22 \pm 0.05$ SEM (three determinations), was nearly identical to that induced by $5 \times 10^{-7}$ mol/L FMLP (Fig 2A inset).

Although the onset of actin filament formation was delayed, once this reaction began, the actin filament assembly rate associated with PAF was comparable to FMLP (4.4% increase in F-actin content/s v 4.8%/s for FMLP), and maximum F-actin concentrations were observed after a 30-second exposure to either stimulus. The peak concentration of F-actin induced by PAF was considerably lower than that induced by an optimal concentration of FMLP ($1.72 \pm 0.07$, 10 experiments v 2.21 ± 0.06, 13 experiments). At each time point, F-actin content was significantly lower following PAF stimulation as compared with FMLP ($P < .05$). F-actin content following 30 seconds stimulation with optimal concentrations of the two agents was also measured using the triton-insoluble-cytoskeleton assay. By this method PAF was also shown to induce a smaller rise in F-actin content as compared with FMLP (relative F-actin content: 1.6 ± 2.0).

F-actin content rapidly decreased when PMNs were exposed to PAF for greater than one minute, reaching a nadir between 2 and 6 minutes, then actin filament concentration again increased reaching a second peak after approximately ten minutes. This reaction was followed by a second decline in F-actin content. Figure 2B shows the effects of prolonged exposure of PAF on PMN actin filament content. PMNs were incubated at 25°C with $1 \times 10^{-10}$ mol/L PAF and F-actin content measured at the times indicated. Bars represent the SEM, the numbers above the bars, the number of separate determinations.

Effects of a PAF receptor antagonist (SRI 63-441), pertussis toxin, and NDGA on PAF-induced PMN actin filament assembly. The PAF receptor inhibitor (SRI 63-441) blocked neutrophil actin filament assembly induced by PAF, complete inhibition being observed at $1 \times 10^{-6}$ mol/L, 50% inhibition at $1 \times 10^{-7}$ mol/L, and no inhibition at $1 \times 10^{-8}$ mol/L (Fig 3).
after being washed once in buffer with increasing concentrations of PT for 90 minutes at 37°C. Cells stimulation of PMN actin polymerization. PMNs were incubated in the same concentration of pertussis toxin for two hours resulted in 400 ng/mL of this agent caused a 50% to 60% inhibition of observed with FMLP, incubation of cells for 90 minutes with a reagent inhibited both FMLP and PAF stimulation of actin polymerization and was associated with blebbing of the PMN peripheral cytoplasm, suggesting a more generalized toxic effect.

The effects of EGTA and the ionophore A23187 on PAF- and FMLP-induced neutrophil actin filament assembly. To assess the calcium requirement of PAF-induced neutrophil actin assembly, EGTA and the calcium ionophore A23187 were added to the incubation media. As shown in Fig 5, EGTA in the presence or absence of A23187 failed to inhibit PAF's effects on actin filament assembly. Addition of 1 mmol/L EGTA to the media also failed to inhibit the second rise in F-actin content observed after 20 minutes' exposure to PAF (Fig 2B). Similarly FMLP-stimulated PMN actin filament formation was not inhibited by addition of EGTA and/or A23187 to the buffer. In an additional experiment not depicted in Fig 5, PMNs were preincubated with EGTA for 20 minutes followed by exposure to A23187 for four rather than two minutes to further deplete intracellular calcium stores. This condition also failed to inhibit PAF- and FMLP-induced rises in F-actin content. Values were comparable to those depicted in column d of Fig 5. As observed by other investigators, A23187 in the presence or absence of EGTA caused moderate increases in F-actin concentration (1.33 ± 0.14 and 1.59 ± 0.06, respectively, after two minutes exposure, three determinations). A23187 alone, however, had no significant effect on

Filament assembly was examined using pertussis toxin. As observed with FMLP, incubation of cells for 90 minutes with 400 ng/mL of this agent caused a 50% to 60% inhibition of PAF-induced neutrophil actin assembly. Incubation with the same concentration of pertussis toxin for two hours resulted in 80% inhibition of both the PAF- and FMLP-induced rise in F-actin concentration. Higher concentrations of pertussis toxin (2.5 μg/mL) also resulted in marked inhibition of FMLP- and PAF-induced actin polymerization (Fig 4).

It has been suggested by some investigators that PAF activates PMNs by inducing endogenous production of leukotriene B4. To investigate whether production of this agent might be responsible for PMN actin polymerization, cells were preincubated with the lipoxigenase inhibitor, nordihydroguaiaretic acid (NDGA) before stimulation. Preincubation with 5, 10, and 20 μmol/L NDGA for five minutes at 37°C (concentrations previously shown to cause near total inhibition of leukotriene production by PAF21-23) failed to significantly inhibit the rise in F-actin content induced by PAF. A higher concentration (50 μmol/L) of this reagent inhibited both FMLP and PAF stimulation of actin polymerization and was associated with blebbing of the PMN peripheral cytoplasm, suggesting a more generalized toxic effect.

Fig 3. The effects of the PAF receptor antagonist SRI 63-441 on PAF stimulation of PMN actin polymerization. PAF 1 × 10⁻⁹ mol/L, was added simultaneously with varying concentrations of SRI 63-441. Samples were fixed, lysed, and stained after incubation at 25°C for 60 seconds. Values represent the mean of two determinations performed on separate days. The far left hand value (1.6) represents the F-actin content of PMNs stimulated in buffer.

Fig 4. The effects of pertussis toxin (PT), on PAF and FMLP stimulation of PMN actin polymerization. PMNs were incubated with increasing concentrations of PT for 90 minutes at 37°C. Cells after being washed once in buffer were stimulated with PAF 1 × 10⁻⁹ mol/L or FMLP 5 × 10⁻⁷ mol/L for 60 seconds.

Fig 5. The effects of EGTA and the calcium ionophore A23187 on PAF- and FMLP stimulation of PMN actin polymerization. PMNs were incubated at 25°C (a) in buffer containing 1 mmol/L CaCl₂, (b) in buffer containing 1 mmol/L CaCl₂ and 5 × 10⁻⁷ mol/L A23187 for two minutes, (c) in buffer without added calcium plus 1 mmol/L EGTA for 20 minutes, or (d) in EGTA buffer for 20 minutes followed by the addition of A23187 for two minutes. PMNs were then stimulated with 2.5 × 10⁻⁷ mol/L PAF or 5 × 10⁻⁷ mol/L FMLP for 60 seconds at 25°C. Samples were analyzed as described in Fig 1. Values represent the mean ± SEM of experiments performed on three different days. The differences in F-actin content induced by PAF or FMLP under these four conditions were not statistically significant, with the exception of condition (d). The FMLP induced rise in F-actin content was significantly higher under these conditions when compared with PMNs stimulated with FMLP in buffer (a) (P = .014).

Fig 6. The effects of EGTA and the calcium ionophore A23187 on PAF and FMLP stimulation of PMN actin polymerization. PMNs were incubated at 25°C (a) in buffer containing 1 mmol/L CaCl₂, (b) in buffer containing 1 mmol/L CaCl₂ and 5 × 10⁻⁷ mol/L A23187 for two minutes, (c) in buffer without added calcium plus 1 mmol/L EGTA for 20 minutes, or (d) in EGTA buffer for 20 minutes followed by the addition of A23187 for two minutes. PMNs were then stimulated with 2.5 × 10⁻⁷ mol/L PAF or 5 × 10⁻⁷ mol/L FMLP for 60 seconds at 25°C. Samples were analyzed as described in Fig 1. Values represent the mean ± SEM of experiments performed on three different days. The differences in F-actin content induced by PAF or FMLP under these four conditions were not statistically significant, with the exception of condition (d). The FMLP induced rise in F-actin content was significantly higher under these conditions when compared with PMNs stimulated with FMLP in buffer (a) (P = .014).
PAF STIMULATES AND PRIMES ACTIN ASSEMBLY

Table 1. Percent Increase in Relative F-Actin Content After 60 Second Stimulation With Various Concentrations of PAF or FMLP Alone and in Combination

<table>
<thead>
<tr>
<th>[FMLP] (mol/L)</th>
<th>10⁻⁸</th>
<th>10⁻⁹</th>
<th>10⁻¹⁰</th>
<th>10⁻¹¹</th>
<th>None</th>
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<tr>
<td>[PAF] (mol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10⁻⁸</td>
<td>117 ± 23 (2)</td>
<td>—</td>
<td>98</td>
<td>—</td>
<td>70 ± 10 (2)</td>
</tr>
<tr>
<td>5 x 10⁻¹⁰</td>
<td>—</td>
<td>108</td>
<td>—</td>
<td>—</td>
<td>62</td>
</tr>
<tr>
<td>5-10 x 10⁻¹¹</td>
<td>—</td>
<td>81 ± 14 (2)</td>
<td>52 ± 11 (4)</td>
<td>—</td>
<td>28 ± 3 (6)</td>
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<tr>
<td>1-10 x 10⁻¹²</td>
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<td>31</td>
<td>37 ± 21 (4)</td>
<td>21 ± 9 (3)</td>
<td>3 ± 1 (8)</td>
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<tr>
<td>None</td>
<td>119 ± 11 (2)</td>
<td>36 ± 5 (4)</td>
<td>11 ± 3 (9)</td>
<td>9 ± 3 (3)</td>
<td>0 (17)</td>
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</table>

PAF and FMLP were added simultaneously to 7.5 x 10⁶ PMNs and cells fixed after 60 seconds. F-actin content was measured as described in Fig 1 and Materials and Methods. Numbers in parentheses represent the number of separate determinations. Values are percentages ± SEM.

Combined stimulation with PAF and FMLP As shown in Table 1, when PMNs were exposed to low concentrations of PAF and FMLP simultaneously, the F-actin content increased to levels higher than that induced by either stimulus alone. The resulting rise in F-actin content was generally equivalent to or above the sum of the rise in F-actin content induced by the individual chemotactic stimuli. This additive effect was seen when concentrations of FMLP of 7.5 x 10⁻¹⁰ to 1 x 10⁻¹¹ mol/L were combined with 1 x 10⁻⁸ to 1 x 10⁻¹² mol/L concentrations of PAF. Use of optimal concentrations of FMLP as well as PAF failed to stimulate increases in F-actin content above that induced by FMLP alone.

Preincubation of PMNs with PAF (1 x 10⁻¹⁰ mol/L) for 2, 5, 10, 20, and 60 minutes also enhanced F-actin production in response to a low concentration of FMLP (5 x 10⁻¹⁰ mol/L). At each time point the rise in F-actin was greater than the sum of the two individual stimuli (data not shown). As shown in Table 2, preincubation of PMNs with varying concentrations of PAF (1 x 10⁻¹¹ to 1 x 10⁻⁸ mol/L) for 60 minutes resulted in an enhanced F-actin response to low concentrations of FMLP (5 x 10⁻¹² to 1 x 10⁻¹⁰ mol/L). F-actin content was 20% to 200% greater than the sum of rises in F-actin content induced by the individual stimuli. Monocyte contamination did not appear to play a role in PAF- or FMLP-induced neutrophil actin polymerization (Fig 5, column b).

Table 2. Percent Increase in Relative F-Actin Content After 60 Minutes Stimulation With Varying Concentrations of PAF Alone, After 60 Seconds Stimulation With Varying Concentrations of FMLP Alone and After 60 Minutes Stimulation With PAF Followed by 60 Seconds Stimulation With FMLP

<table>
<thead>
<tr>
<th>[FMLP] (mol/L)</th>
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<th>5 x 10⁻¹²</th>
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</tr>
</thead>
<tbody>
<tr>
<td>[PAF] (mol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>54 ± 8 (+50)</td>
<td>42 ± 1 (+27)</td>
<td>24 ± 2 (+20)</td>
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<td>15 ± 3</td>
<td>2 ± 1</td>
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</table>

Numbers in parentheses represent the percent rise above the sum of the two individual stimuli: % F-actin content above additive = 100 x [F-actin content of PMN receiving combined stimuli - (F-actin content of PMN stimulated with FMLP x 60 + F-actin content after PAF x 60 min)/F-actin content after FMLP + F-actin content after PAF]. Values are percentages ± SEM of three to seven separate determinations.

DISCUSSION

Human and rabbit PMNs rapidly increase their F-actin content after being stimulated with FMLP. Under optimal conditions this chemotactic stimulus increases F-actin content by approximately twofold within 30 seconds. Other agents have also been shown to stimulate PMN actin polymerization including leukotriene B₄, phorbol myristate acetate, and arachidonic acid.¹⁶ PAF has also been shown by the triton-insoluble cytoskeleton assay to induce a rise in the F-actin content of rabbit PMNs.¹⁶,¹⁷ The fluorescent F-actin stain, NBD-phallicidin, has recently been used to measure F-actin content in PMNs.³ Using this assay we have systematically compared the individual effects of PAF and FMLP on human PMN actin filament assembly and have also investigated how these two agents in combination may affect in vivo PMN actin polymerization. The NBD-phallicidin assay makes possible careful kinetic analysis of PMN actin filament formation and also allows reproducible quantitation of relative F-actin concentration.³ These investigations have demonstrated several differences between FMLP and PAF.

First, on examining the kinetics of the PAF-induced rise in F-actin content, we found a brief but reproducible lag period of five seconds between the time of stimulation and the initial increase in F-actin content. This delay in actin polymerization was not observed after stimulation with FMLP even by addition of a higher concentration of FMLP for 60 seconds (1 x 10⁻¹⁰ to 1 x 10⁻⁹ mol/L) was associated with a less than additive rise in F-actin content in all instances (Table 3). The combined stimulus resulted in an F-actin content that was 10% to 62% below the sum of the individual stimuli.
when low concentrations of FMLP were used to stimulate actin filament assembly. Stimulation with higher concentrations of PAF did not shorten the time of initial actin filament assembly, therefore a delay in binding of PAF to PMN membrane receptors seemed an unlikely explanation for our findings. Such a lag period might be present if PAF’s effects on actin filament assembly were mediated by the production of a second compound, such as leukotriene B₄. However, preincubation of PMNs with the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), a condition that should block leukotriene production, did not affect PAF-induced actin polymerization. When human PMNs bind PAF they rapidly metabolize this agent. We cannot exclude the possibility that the observed lag period represents the time required to metabolize PAF to a more active byproduct.

Not only the early kinetics of polymerization, but also the time course of depolymerization may differ from FMLP. Prolonged incubation with PAF was associated with two phases of polymerization and depolymerization. The initial rapid rise in F-actin content was followed by a decrease in F-actin over the ensuing two to six minutes. Depolymerization was then followed by a moderate second rise in F-actin content that in turn was followed by a second depolymerization phase. With the exception of Sklar et al., who observed two phases of polymerization and depolymerization during the first two minutes of oligopeptide stimulation, other investigators have described a single abrupt rise in F-actin content followed by a single phase of depolymerization following FMLP stimulation. The cause of this second rise in F-actin content is not clear, but could be the result of PMN aggregation or the production of second mediator that in turn induces a second actin polymerization reaction.

There are also a number of similarities between these two chemotactic agents. Receptor inhibitor studies indicate that PAF, like FMLP, requires receptor occupancy to induce PMN actin polymerization. The marked inhibitory effects of pertussis toxin on human PMNs suggest that as observed in rabbit PMNs, both chemotactic agents require functioning G-proteins to stimulate actin polymerization.

Neither PAF nor FMLP required extracellular calcium to induce increases in F-actin concentration. This finding suggests that the large influx of ionized calcium generally associated with PAF and FMLP stimulation is not required for actin filament assembly. The effects of combined treatment of PMNs with EGTA and A23187 are complex and variable; therefore, no firm conclusions can be drawn from our experiments concerning the importance of intracellular calcium in mediating PAF- and FMLP-induced actin filament assembly. These experiments do suggest that these two classes of chemotactic agent may function similarly, since this treatment failed to inhibit actin filament assembly induced by either reagent.

In addition to acting directly to induce neutrophil aggregation, degranulation, superoxide production, chemotaxis, and actin polymerization, PAF can regulate neutrophil responses to other stimuli. PAF can desensitize the PMN aggregation response to leukotriene B₄. When combined with opsonized zymosan, PAF induces an additive rise in superoxide production. When combined with FMLP, PAF synergistically enhances the oxidative response. Maximum enhancement is observed when optimal oxidative concentrations of both chemotactic agents are used (10⁻⁷ mol/L FMLP and 10⁻⁸ mol/L PAF).

Our studies demonstrated that PAF also is able to modulate PMN actin responsiveness to FMLP. However, unlike the oxidative response, PAF only enhanced the effect of suboptimal FMLP concentrations. Simultaneous addition of or preincubation for up to 60 minutes with PAF caused an enhanced response to FMLP. Particularly after prolonged preincubation with PAF, the combined effect of these two chemotactic agents on F-actin content was consistently greater than the sum of the effects of two individual stimuli. Preincubation with FMLP followed by exposure to a second higher concentration of FMLP, on the other hand, resulted in a less than additive response. These findings suggest as observed for oxidative metabolism, that PAF may act as a "priming" stimulus for FMLP. Although these two chemotactic agents bind to separate receptors, they may have common intermediate biochemical pathways serving to induce PMN actin filament assembly. PAF could serve to activate one or more proteins responsible for this reaction, a condition that might augment PMN actin assembly in response to a second stimulus. It is also possible that PAF stimulation may increase the number and/or affinity of FMLP receptors.

Understanding how these two chemotactic agents affect actin filament assembly alone and in combination are of importance in understanding the in vivo inflammatory responses to FMLP and PAF.

### Table 3. Percent Increase in Relative F-Actin Content After 60 Minutes Stimulation With Varying Concentrations of FMLP Alone, After 60 Seconds Stimulation With Varying Concentrations of FMLP Alone and After 60 Minutes Stimulation with FMLP Followed by 60 Seconds Stimulation With a Second Higher Concentration of FMLP

<table>
<thead>
<tr>
<th>[FMLP] (mol/L; 60 min)</th>
<th>10⁻⁹</th>
<th>5 x 10⁻¹⁰</th>
<th>1 x 10⁻¹⁰</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10⁻¹⁰</td>
<td>66 (-31)</td>
<td>—</td>
<td>—</td>
<td>38</td>
</tr>
<tr>
<td>1 x 10⁻¹⁰</td>
<td>61 (-12)</td>
<td>47 ± 9* (-10)</td>
<td>—</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>5 x 10⁻¹¹</td>
<td>57 (-20)</td>
<td>31 ± 7 (-42)</td>
<td>13 ± 6 (-62)</td>
<td>13</td>
</tr>
<tr>
<td>None</td>
<td>58</td>
<td>41 ± 2</td>
<td>21 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses represents the % below the sum of the two individual stimuli: % F-actin content below additive = 100 x [F-actin content of PMN receiving combined stimuli - (F-actin content of PMN stimulated with FMLP x 60 s + F-actin content after PAF x 60 min)]/[F-actin content after FMLP + F-actin content after PAF].

*Values are percentages ± SEM of three separate determinations; other experiments represent the mean of two separate determinations.
response, since both of these chemotactic agents may be present in inflammatory exudates and PAF may be synthesized and retained on the membrane of stimulated PMNs. The present findings suggest that the lipid chemotactic agent, PAF, not only can induce PMN actin filament assembly, but may also serve to make the PMN actin response more sensitive to low concentrations of peptide chemotactic agents like FMLP.

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REFERENCES

Platelet-activating factor both stimulates and "primes" human polymorphonuclear leukocyte actin filament assembly

M Shalit, GA Dabiri and FS Southwick