Biochimica et Biophysica Acta 969 (1988) 131–138
Elsevier

BBA 12232

Phenylmethylsulfonyl fluoride inhibits chemotactic peptide-induced actin polymerization and oxidative burst activity in human neutrophils by an effect unrelated to its anti-proteinase activity

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(Received 30 October 1987)

Key words: Phenylmethylsulfonyl fluoride; Actin polymerization; Proteinase; (Human neutrophil)

Stimulation of polymorphonuclear leukocytes with the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) causes conversion of monomeric actin to polymeric actin. We studied the role of proteinase inhibitors phenylmethylsulfonyl fluoride PMSF) and diisopropyl fluorophosphate in fMet-Leu-Phe-induced actin polymerization in polymorphonuclear leukocytes. Pre-incubation of cells with PMSF (2 mM) for 1 min caused inhibition of fMet-Leu-Phe-induced actin polymerization, as studied by 7-nitrobenz-2-oxa-1,3-diazole (NBD) -phallacidin labeling and flow cytometry. PMSF also inhibited fMet-Leu-Phe-induced hydrogen peroxide release, superoxide anion generation and chemiluminescence. In contrast, diisopropyl fluorophosphate (5 mM) was unable to inhibit fMet-Leu-Phe-induced actin polymerization and superoxide generation, but was effective in inhibiting hydrogen peroxide production and chemiluminescence. PMSF did not cause any change in membrane potential by itself and failed to inhibit the membrane potential changes induced by fMet-Leu-Phe, indicating that PMSF does not affect the binding of fMet-Leu-Phe to the receptors. The high concentration of PMSF required coupled with the fact that diisopropyl fluorophosphate was unable to inhibit fMet-Leu-Phe-induced actin polymerization suggested that this activity of PMSF might be unrelated to proteinase inhibitory activity. Polymyxin B, a membrane-active antibiotic, had an effect similar to PMSF on fMet-Leu-Phe-induced actin polymerization. This suggests that PMSF may also be acting via its membrane effect rather than its anti-proteinase effect.

Introduction

Stimulation of the polymorphonuclear leukocytes with the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) leads to rapid conversion of monomeric actin to polymeric actin [1–4]. The signaling event(s) required for induction of actin polymerization is not known. Studies indicate that none of the known second messengers seem to play a primary role in the induction of actin polymerization. Calcium fluxes have been shown to have no role in the initial stage of actin polymerization [5–7]. Although phorbol myristate acetate, a protein kinase C activator [8,9], causes actin polymerization [10] it does not seem to play a primary role in fMet-Leu-Phe-induced actin polymerization [11,12].

Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; NBD, 7-nitrobenz-2-oxa-1,3 diazole; PMSF, Phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; diS-C_3, 3,3′-dipropylthiadicarbocyanine iodide.

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Several surface proteinases have been described in different cell types including lymphocytes, monocytes, basophils and cultured cell lines from several tissue sources [13–15]. An important role for a cell surface proteinase has been proposed for many functions of phagocytic cells, including phagocytosis, chemotaxis, respiratory burst and inflammation [16–20]. Recently, the existence of a neutral serine proteinase in neutrophil membranes has been demonstrated [21,22].

In this study, we examined the role of cell-surface proteinases in actin polymerization and other neutrophil functions using two serine proteinase inhibitors, phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate. We discovered that PMSF inhibited fMet-Leu-Phe-induced actin polymerization, whereas diisopropyl fluorophosphate had no effect. The concentration of PMSF required to elicit this effect was very high (2 mM). This, combined with the fact that diisopropyl fluorophosphate was ineffective, suggested that the effect of PMSF might be due to a membrane effect rather than an anti-proteinase effect. Therefore, we tested the cell-surface membrane perturbing agent polymyxin B and found that it had a profound effect on the F-actin content in resting polymorphonuclear leukocytes and inhibited the fMet-Leu-Phe-induced actin polymerization. These observations suggest that the alterations in cell-surface membrane play an important role in the cytoskeletal reorganization in neutrophils following interaction with many stimulants.

Materials and Methods

Reagents. Cytochrome *c* type III, cytochalasin B, fMet-Leu-Phe, horseradish peroxidase, scopoletin, PMSF, diisopropyl fluorophosphate, polymyxin B, luminol, bovine serum albumin (BSA) and L-α-lysophosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). 7-Nitrobenz-2-oxa-1,3 diazole (NBD) -phallacidin was from Molecular Probes, (Junction City, OR). PMSF was dissolved in dimethylsulfoxide (DMSO) and then added to Hepes buffer to give 4 mM concentration (a slight precipitate remained). Cytochalasin B was dissolved in DMSO to a concentration of 10 mM and final concentrations were made in Hepes buffer.

Isolation of polymorphonuclear leukocytes. Human peripheral blood was obtained by venipuncture and was added to a flask containing glass beads and shaken for 10–15 min to achieve defibrination and removal of platelets. The defibrinated blood was subjected to a Ficoll-Hypaque density gradient technique to remove mononuclear cells, and the red cell pellet was used to isolate polymorphonuclear leukocytes by dextran sedimentation technique. The red cells were lysed by either ammonium chloride (0.83% NH_4Cl, w/v; 0.05% $KHCO_3$, w/v; and 10^{-4} M EDTA) for 3 min or hypotonic shock for 40 s. The cells were washed twice in Hanks' balanced salt solution and suspended in a physiological buffer with the following composition: 138 mM NaCl/4.5 mM KCl/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/1.3 mM $CaCl_2$/5.6 mM glucose/5 mM $NaHCO_3$/20 mM Hepes (pH 7.4 at 37°C).

Determination of F-actin content. F-actin content was determined by NBD-phallacidin labeling as described by Wallace et al. [3]. Briefly, the cells were fixed with 3.2% paraformaldehyde in phosphate-buffered saline (0.01 M, pH 7.4) for 48–72 h. The cells were washed twice with phosphate-buffered saline/0.1% BSA and the cell button was stained with 100 μl of 0.6 μM NBD-phallacidin in phosphate-buffered saline/0.1% BSA for 30 min at room temperature. The cells were washed twice with phosphate-buffered saline/0.1% BSA and suspended in 0.7 ml of the same buffer for analysis by flow cytometry. The cells were analyzed in Ortho Spectrum III flow cytometer equipped with a 2140 computer. Histograms of cell number versus fluorescence intensity (linear mode) and forward angle light scatter were recorded for a minimum of 15 000 cells per sample.

F-actin content was quantitated by extracting the NBD-phallacidin into methanol as described by Howard and Oresajo [23]. Briefly, 100 μl of the cell suspension (20 million cells/ml) was incubated with various agents and fixed and stained in a single step by adding 100 μl of staining cocktail consisting of 6.4% paraformaldehyde/200 μg/ml lysophosphatidylcholine/0.6 μM NBD-phallacidin in phosphate-buffered saline for 30 min at room temperature. The cells were spun at

12000 × *g* for 1 min in a microfuge (Beckman) and the cell pellet was extracted with 1 ml of methanol for 10 min. The cells were spun, the supernate was removed and the procedure was repeated. The methanol extracts were pooled (2 ml) and the relative fluorescence intensity was measured in an LS-3 spectrofluorometer (Perkin Elmer Corp.), set at 465 nm excitation and 535 nm emission.

Hydrogen peroxide production. Hydrogen peroxide production was measured by monitoring the fluorescence of scopoletin [24]. Polymorphonuclear leukocytes (10 million cells) were suspended in 3 ml buffer containing 5 μl of 2 mM scopoletin and 10 μl of 10 mg/ml horseradish peroxidase. Resting hydrogen peroxide release was monitored at 37 °C with an excitation wavelength of 460 nm using fluorometer equipped with a stirrer (model MPE-3L, Perkin Elmer corp. Norwalk, CT, USA). Cells were activated with fMet-Leu-Phe, with and without pre-incubation with proteinase inhibitors, and fluorescence was monitored continuously with time.

Superoxide anion generation. Superoxide generation was measured by monitoring cytochrome *c* reduction. Polymorphonuclear leukocytes (10 million cells) were suspended in 5 ml buffer containing 0.12 mM cytochrome *c*. The inhibitors and stimulants were added to the cell suspension and a 2 ml aliquot was removed and spun for 1 min at 2000 × *g* to pellet the cells. The absorbance of the supernate was measured spectrophotometrically at 550 nm. The remaining 3 ml of the suspension was incubated at 37 °C for 30 min, spun and the absorbance was measured. The difference between the first and second reading was taken as a measure of superoxide generation [25].

Membrane potential. Membrane potential was measured at 37 °C using the fluorescent probe DiS-C_3(5) [26] by a method described previously [27]. Polymorphonuclear leukocytes (23 million) were suspended in 3 ml buffer containing 0.66 μg/ml DiS-C_3(5). Fluorescence was measured at 37 °C with an excitation wavelength of 622 nm and an emission wavelength of 665 nm using the fluorometer set-up described above. After a resting level was attained, the drugs were added and the fluorescence response was monitored continuously with time. In this system, an increase in fluorescence indicates membrane depolarization.

Chemiluminescence. Chemiluminescence was measured by a liquid scintillation counter (Packard model 2002) set in out-of-coincidence mode as described previously [28]. Briefly polymorphonuclear leukocytes (10 million cells) were suspended in 3 ml buffer containing 0.02 μM luminol. The chemiluminescence of the resting cells was measured at zero time and the suspension was incubated at 37 °C. At various times, the vials were taken out briefly to measure counts and replaced in the incubator and thus readings were obtained vs. time.

Statistical analysis. The data were analyzed using paired *t*-test. In all experiments, control experiments were performed with the drugs alone and they did not have any effect, except where mentioned otherwise.

Results

Effect of PMSF and diisopropyl fluorophosphate on fMet-Leu-Phe-induced actin polymerization

Polymorphonuclear leukocytes were incubated at 37 °C with 2 mM PMSF for 1 min and then stimulated with fMet-Leu-Phe for 1 min. F-actin content was determined by the methanol extraction method, following NBD-phallacidin labeling.

TABLE I

EFFECT OF PMSF AND DIISOPROPYL FLUOROPHOSPHATE ON CHEMOTACTIC PEPTIDE-INDUCED ACTIN POLYMERIZATION IN POLYMORPHONUCLEAR LEUKOCYTES

F-actin content was measured by NBD-phallacidin labeling followed by methanol extraction as described in the text. The data are expressed as relative fluorescence intensity. F-actin content is directly proportional to fluorescence intensity.

	F-actin	*P* vs. control
Control cells	0.34 ± 0.04 [a]	–
Cells + fMet-Leu-Phe (10^{-7} M)	0.51 ± 0.14	< 0.05
Cells + PMSF ($2 \cdot 10^{-3}$ M) + fMet-Leu-Phe (10^{-7} M)	0.35 ± 0.07	> 0.2
Cells + diisopropyl fluorophosphate ($5 \cdot 10^{-3}$) + fMet-Leu-Phe (10^{-7} M)	0.51 ± 0.16	< 0.05

[a] Mean ± S.D. ($n = 4$)

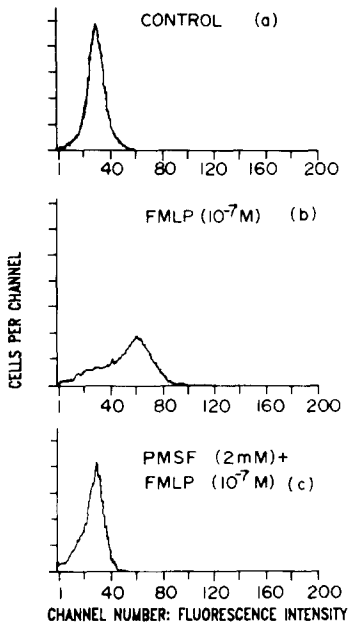

Fig. 1. Effect of PMSF on the fMet-Leu-Phe (FMLP)-induced actin polymerization in human polymorphonuclear leukocytes. F-actin contnet was determined by flow cytometry following labeling with NBD-phallacidin. The shift in fluorescence to higher channels represents an increase in the F-actin content (b). Pre-treatment of cells with PMSF for 1 min inhibited the fMet-Leu-Phe-induced actin polymerization (c).

fMet-Leu-Phe-induced actin polymerization is reflected as an increase in the relative fluorescence intensity. As shown in Table I, PMSF inhibited most of the actin polymerization induced by fMet-Leu-Phe. In contrast, diisopropyl fluorophosphate was unable to inhibit actin polymerization even at a concentration of 5 mM. The inhibitory activity of PMSF on actin polymerization was confirmed by meausring F-actin content by flow cytometry (Fig. 1).

Effect of PMSF and diisopropyl fluorophosphate on oxidative burst activity

The different effects of the two proteinases on fMet-Leu-Phe-induced actin polymerization, described above, was intriguing. The observations on actin polymerization prompted us to examine the effect of these two agents on other fMet-Leu-Phe-induced activities in polymorphonuclear leukocytes. fMet-Leu-Phe stimulation in these cells causes generation of superoxide, hydrogen peroxide production and chemiluminescence. The effect of PMSF and diisopropyl fluorophosphate on superoxide anion generation was studied by adding either PMSF (2 mM final concentration) or diisopropyl fluorophosphate (5 mM final concentration) immediately before the addition of fMet-Leu-Phe. The data in Table II demonstrate that PMSF was capable of inhibiting the superoxide anion generation but diisopropyl fluorophosphate was ineffective. In some experiments the superoxide production induced by fMet-Leu-Phe was enhanced by pretreating the cells with cytochalasin B for 15 min and the inhibitory effect of PMSF became even more evident. As expected from its effect on superoxide generation, PMSF was also effective in inhibiting the hydrogen peroxide production (Fig. 2). But with diisopropyl

TABLE II

EFFECT OF PMSF AND DIISOPROPYL FLUOROPHOSPHATE ON CHEMOTACTIC PEPTIDE-INDUCED SUPEROXIDE ANION GENERATION

	Superoxide release (nmol/10^6 cells per 30 min) [a]	P vs. control
Control cells	0.60 ± 0.45 [b]	–
Cells + PMSF ($2 \cdot 10^{-3}$ M) + fMet-Leu-Phe (10^{-7} M)	0.83 ± 0.29	> 0.2
Cells + diisopropyl fluorophosphate ($5 \cdot 10^{-3}$ M) + fMet-Leu-Phe (10^{-7} M)	1.41 ± 0.81	< 0.05
Experiment with cytochalasin B [c]		
Cells + cytochalasin B (10^{-5} M) + fMet-Leu-Phe (10^{-7} M)	13.8	
Cells + cytochalasin B (10^{-5} M) + PMSF ($2 \cdot 10^{-3}$ M) + fMet-Leu-Phe (10^{-7} M)	1.79	

[a] Superoxide anion release was converted from absorbance units to nmol using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$.
[b] Mean $\pm$ S.D. ($n = 4$).
[c] The cells were incubated with cytochalasin B for 15 min at 37°C and PMSF and fMet-Leu-Phe were added simultaneously.

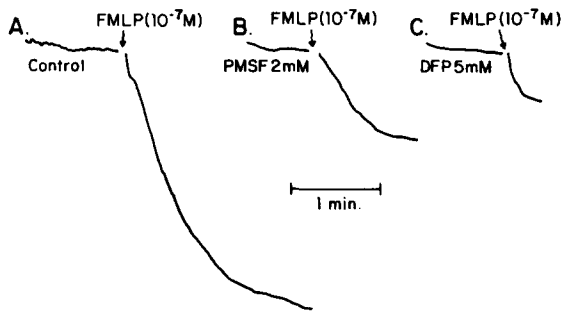

Fig. 2. Effect of PMSF and diisopropyl fluorophosphate on fMet-Leu-Phe-induced hydrogen peroxide production in human polymorphonuclear leukocytes. Cells were incubated with either PMSF or diisopropyl fluorophosphate for 1 min before adding fMet-Leu-Phe. This data is from one experiment representative of the four experiments performed.

fluorophosphate a curious dissociation was observed between its effect on superoxide generation and hydrogen peroxide production, induced by fMet-Leu-Phe. In contrast to its ineffectiveness in inhibiting superoxide generation, diisopropyl fluorophosphate exhibited good inhibitory activity on hydrogen peroxide production (Fig. 2). This suggests that diisopropyl fluorophopshate is capable of inhibiting a step between superoxide generation

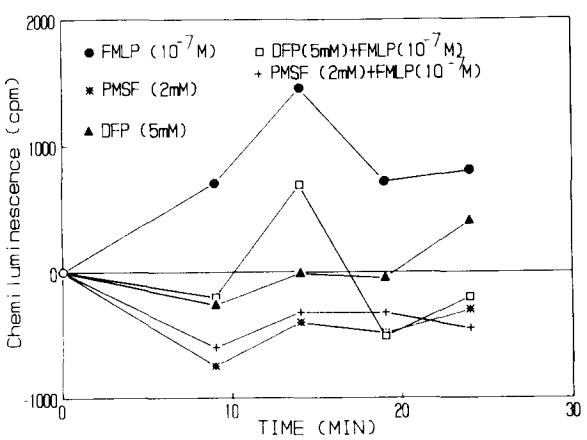

Fig. 3. Effects of PMSF and diisopropyl fluorophosphate (DFP) on fMet-Leu-Phe (FMLP)-induced chemiluminescence in human polymorphonulcear leukocytes. Chemiluminescence was measured as counts per min (cpm) in a liquid scintillation counter. Values plotted represent the cpm obtained by subtracting cpm of the control cells from cpm obtained by cells treated with various drugs as indicated, at each time interval. Data is from a single experiment representative of four experiments.

and H_2O_2 production, whereas PMSF inhibits an event prior to superoxide generation. Chemiluminescence, another event associated with oxidative burst activity, was inhibited by both PMSF and diisopropyl fluorophosphate (Fig. 3).

Effect of PMSF and diisopropyl fluorophosphate on membrane potential

FMLP binding to the receptor induces alterations in membrane potential. We reasoned that if the two proteinases we were studying cause alterations in binding of the chemotactic peptide to the receptors, this might be reflected in alterations in membrane potential patterns. As shown in Fig. 4, PMSF did not alter the resting membrane potential and did not affect the fMet-Leu-Phe-induced changes in membrane potential. This indicates that the binding of receptors by fMet-Leu-Phe is not affected by PMSF. In contrast to PMSF, diisopropyl fluorophosphate alone induced a depolarization response in the cells. However, the drug did not prevent subsequent depolarization upon addition of fMet-Leu-Phe, but altered its character in that the membrane potential did not return to the base line even after 5 min (Fig. 5).

Effect of polymyxin B on fMet-Leu-Phe-induced actin polymerization (Table III)

As discussed below, the effect of PMSF on

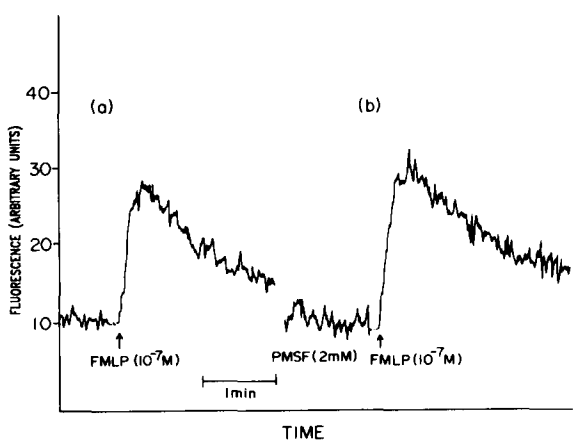

Fig. 4. Effect of PMSF on the changes in the membrane potential induced by fMet-Leu-Phe (FMLP) in human polymorphonuclear leukocytes. Membrane potential was measured by changes in the fluorescence of DiS-$C_3(5)$ (0.66 μg/ml). (a) fMet-Leu-Phe (b) cells preincubated for 1 min with PMSF before addition of fMet-Leu-Phe.

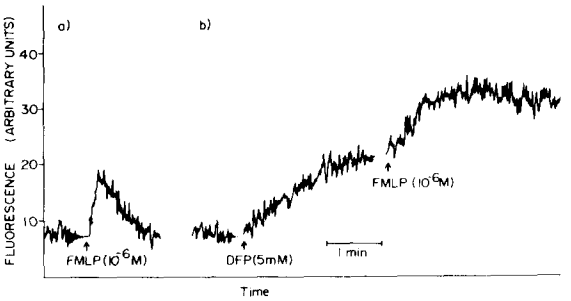

Fig. 5. Effect of diisopropyl fluorophosphate (DFP) on the membrane potential in human polymorphonuclear leukocytes. Membrane potential was measured by changes in the fluorescence of DiS-$C_3(5)$ (0.66 μg/ml). (a) fMet-Leu-Phe (FMLP), (b) diisopropyl fluorophosphate followed by fMet-Leu-Phe.

actin polymerization appeared to be independent of its anti-proteinase activity. One possibility is that it might be acting by perturbing the cells surface membrane. We tested this hypothesis indirectly by studying the effect of polymyxin B on the actin state in polymorphonuclear leukocytes. Incubation of cells with polymyxin B alone, for 30 min, resulted in a decrease in the F-actin content. A bimodal response was observed with a maximal decrease at 100 μg/ml and lesser effect at 500 μg/ml. Shorter incubation times up to 10 min had no significant effect. fMet-Leu-Phe failed to induce actin polymerization in polymorphonuclear leukocytes treated with polymyxin B for 30 min. Incubation of cells with 10 μg/ml of polymyxin B had no effect on either the basal level of actin or on the fMet-Leu-Phe-induced actin polymerization (data not shown).

TABLE III

EFFECT OF POLYMYXIN B ON THE F-ACTIN CONTENT IN UNSTIMULATED AND fMet-Leu-Phe-STIMULATED HUMAN POLYMORPHONUCLEAR LEUKOCYTES

F-actin content was determined by labeling the cells with NBD-phallacidin and extracting the dye into methanol as described in the text. Percent change was calculated as follows (RFI, relative fluorescence intensity): [(RFI of control cells − RFI of treated cells)/RFI of control cells)] · 100

	Change in F-actin (%)
Cells + fMet-Leu-Phe	48 ± 3 [a]
Cells + polymyxin B (500 μg)	−9 ± 9
Cells + polymyxin B (200 μg)	−32 ± 7 [b]
Cells + polymyxin B (100 μg)	−38 ± 6 [b]
Cells + polymyxin B (200 μg) + fMet-Leu-Phe (10^{-7} M)	−22 ± 8 [c]
Cells + polymyxin B (100 μg) + fMet-Leu-Phe (10^{-7} M)	−29 ± 3 [c]

[a] Mean ± S.E. of 4–7 different experiments.
[b] The values are statistically different from F-actin content in untreated control cells.
[c] These values are not statistically different from cells treated with corresponding concentrations of polymyxin B alone.

Discussion

Stimulation of polymorphonuclear leukocytes with the chemotactic peptide fMet-Leu-Phe leads to rapid conversion of monomeric actin to polymeric actin [1–4]. The signal(s) necessary for this conversion is not known at present. It appears that none of the known second messengers seem to have a primary role in the induction of actin polymerization. Calcium and protein kinase C seem to play a secondary role [5–7,11,12]. Although it has been claimed that cyclic AMP levels may alter the G-actin content in macrophages [29] the concentrations of dibutyryl cAMP used (200 μM) were so high one cannot exclude nonspecific effects on the cell surface membrane. In our laboratory, dibutyryl cAMP at concentrations of up to 0.1 mM had no effect on the G-actin or F-actin content of resting polymorphonuclear leukocytes and failed to modify the fMet-Leu-Phe-induced actin polymerization (unpublished observations). These observations prompted us to investigate whether any cell-surface proteinases might be involved in the induction of actin polymerization by the chemotactic peptide fMet-Leu-Phe. We found that PMSF at very high concentrations (2 mM) can inhibit fMet-Leu-Phe-induced actin polymerization. Diisopropyl fluorophosphate on the other hand was ineffective even at a concentration of 5 mM. A similar dissociation between the effect of PMSF and diisopropyl fluorophosphate was noted with respect to the superoxide anion generation. But hydrogen peroxide production and chemiluminescence were inhibited by both PMSF and diisopropyl fluorophosphate.

Although PMSF is generally used as a proteinase inhibitor several considerations lead us to believe that the effect of PMSF in this instance is exerted through a mechanism other than pro-

teinase inhibition. First, PMSF was effective at very high concentration (2 mM) and no effect was observed at 100 μM. Second, diisopropyl fluorophosphate did not have any effect even at a concentration of 5 mM. Third, PMSF was effective even when stored in aqueous buffers for several days at 4°C. It has been shown that the half-life of the inhibitor is only a couple of hours, at best, in aqueous buffers [30].

It is becoming clear that subtle cell-surface membrane perturbations might be enough to trigger reorganization of actin within the cell. In *Entamoeba histolytica* actin polymerization can be induced by incubating the cells with protein liposomes of lipid extracts of erythrocytes [31]. In order to explore the possibility that cell membrane perturbation by PMSF might be responsible for the effects noted in this study we used the antibiotic polymyxin B. This antibiotic has been shown to penetrate the lipid bilayers and is believed to exert its effect by affecting the cell-surface membrane [32,33]. Polymyxin B has been shown to modify certain stimulus-induced responses in neutrophils [34]. We found that this drug decreased resting F-actin content significantly, and inhibited the fMet-Leu-Phe-induced actin polymerization. These observations suggest that certain membrane-active substances might be able to alter actin reorganization.

Several observations indicate that PMSF is not capable of penetrating the cell membrane. Amrein and Stossel showed that PMSF is incapable of preventing degradation of neutrophil proteins when added to intact cells, but was effective in preventing protein degradation when added to cell lysates [35]. In contrast, they found that diisopropyl fluorophosphate was very effective even when added to intact cells. On the basis of these observations, they concluded that diisopropyl fluorophosphate was capable of crossing the cell membrane, whereas PMSF was only active at the cell surface. Our studies on membrane potential changes seem to confirm such conclusion. PMSF did not induce any change in resting membrane potential and failed to modify fMet-Leu-Phe-induced membrane potentials. The failure of PMSF to induce changes in membrane potential suggests that it is not capable of entering the cell and its action is probably at the cell surface. Secondly, the lack of any effect on the fMet-Leu-Phe-induced membrane depolarization indicates that PMSF does not inhibit the binding of the chemotactic peptide to the cell surface receptors.

In contrast to the effect of PMSF, diisopropyl fluorophosphate by itself induced a sustained change in the membrane potential which was further enhanced by the addition of fMet-Leu-Phe. In addition, the fMet-Leu-Phe-induced change was also prolonged. These observations suggest that diisopropyl fluorophosphate might cause significant alterations in the membrane ionic channels.

This study was undertaken to study the effect of proteinase inhibitors on stimulus-induced actin polymerization and led to several unanticipated findings which might contribute to a better understanding of the molecular events associated with the stimulation of polymorphonuclear leukocytes with fMet-Leu-Phe. First, the data presented here suggest that subtle alterations in the cell-surface membrane by agents such as polymyxin B can cause profound changes in the actin state in polymorphonuclear leukocytes. Second, the inhibitory activity of PMSF on fMet-Leu-Phe-induced actin polymerization is not due to its anti-proteinase activity. Third, a step involving a proteinase may be required in the conversion of superoxide generated into hydrogen peroxide. Similarly, a proteinase may be involved in producing chemiluminescence. Finally, it appears that cell-surface membrane modulating agents may serve as useful tools for studying the interaction between the cell membrane and the cytoskeleton, and the molecular mechanisms involved in other fMet-Leu-Phe-induced events.

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