

Calcium Ionophore, Phorbol Ester, and Chemotactic Peptide-induced Cytoskeleton Reorganization in Human Neutrophils

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Abstract

Formyl-methionylleucylphenylalanine (fMLP) activation of neutrophils causes an increase in intracellular Ca^{2+} , activation of protein kinase C and an increase in F-actin content. To examine the role of Ca^{2+} and protein kinase C activation as determinants of change in F-actin content of neutrophils, we used the NBD-phalloidin extraction assay to compare the kinetics and extent of change in F-actin content of cells activated with fMLP, the calcium ionophore A23187 or phorbol myristate acetate (PMA). All stimuli increase the F-actin content in a dose-dependent manner; however, the rate of increase is slower and the maximum F-actin content is less for calcium ionophore and PMA than for fMLP-activated cells. The A23187-induced increase in F-actin content, but not that of fMLP, depends upon external free $[\text{Ca}^{2+}]_{\text{free}}$. In A23187-activated cells, F-actin content increases at $[\text{Ca}^{2+}]_{\text{free}} \geq 5 \mu\text{M}$, is maximal at $[\text{Ca}^{2+}]_{\text{free}} \geq 10 \mu\text{M}$ and is negligible at physiologic free $[\text{Ca}^{2+}]$ (10^{-7} – 10^{-6} M). Combinations of PMA with A23187 or fMLP inhibit the A23187, but not the fMLP, activated actin polymerization. Comparison and combination of these activators shows that neither Ca^{2+} -dependent activation with A23187 nor activation with PMA alone or in combination mimic the fMLP-induced changes in cytoskeleton organization of neutrophils.

Introduction

Chemotaxis, chemokinesis, and locomotion of human neutrophils require continuous assembly, disassembly and reorientation of the cytoskeleton. Dynamic modulation of the cytoskeleton is directed by the location and concentration of soluble chemotactic factors that include a variety of derivatized plasma and bacterial proteins (1, 2). The best characterized cytoskeletal alterations are those associated with binding of *formyl-methionylleucylphenylalanine* (fMLP),¹ a chemotactic peptide (3–5). Activation of neutrophils with fMLP results in rapid polymerization of actin followed by a slower depolymerization and redistribution

of F-actin within the cell (4–6). The exact mechanism whereby fMLP alters F-actin content and distribution in neutrophils is not understood. Like many receptor–ligand interactions, however, binding of fMLP to its receptor induces hydrolysis of polyphosphatidylinositides (7–10), release of intracellular calcium (11, 12), activation of protein kinase C (13–15), and metabolism of arachidonic acid (16). Both protein kinase C activation and a rapid rise in intracellular calcium to micromolar concentrations occur coincidentally with the initiation of actin polymerization and precede the time of maximal F-actin content in neutrophils (17).

Temporal interposition of the calcium rise and protein kinase C activation between receptor occupancy and maximal F-actin content suggests that one or both may be required for initiation and/or termination of the changes in F-actin content. This hypothesis is strengthened by the observation that neutrophils contain gelsolin, a calcium-dependent, actin regulatory protein, and that the kinetics of *in vitro* actin polymerization vary with free calcium concentration (18–24). To determine whether an increase in intracellular calcium concentration or protein kinase C activation alone can explain the fMLP-induced cytoskeletal reorganization, we compared the fMLP-induced changes in F-actin content to those induced by the calcium ionophore A23187 and a protein kinase C activator, phorbol myristate acetate (PMA) (25, 26). The studies suggest that neither protein kinase C activation nor increase in intracellular calcium alone or in combination are sufficient to explain the fMLP-induced changes in cytoskeletal organization. The A23187 activation of neutrophils causes significant changes in F-actin content; however, the required free calcium concentration is much higher than the free calcium concentrations reported in fMLP-activated cells. Activation of cells with PMA blocks the A23187-induced changes in F-actin content.

Methods

Materials. A23187 (Calbiochem Behring Corp., La Jolla, CA); phorbol myristate acetate; fMLP, MOPS (3-[*N*-morpholino]propanesulfonic acid), Hepes, EGTA, and CaCO_3 (Sigma Chemical Co., St. Louis, MO); NBD-phalloidin (Molecular Probes, Junction City, OR).

Preparation of neutrophils. Leukocytes were prepared from human peripheral blood in EDTA anticoagulant by Dextran 60 (Cutter Laboratories, Berkeley, CA) sedimentation. Contaminating erythrocytes were lysed with 0.83% ammonium chloride. Neutrophils were purified on Ficoll-Hypaque gradients, yielding 96–97% neutrophils, 2–3% eosinophils, 0–1% mononuclear cells (27). All experiments were done in Hanks' balanced salt solution or MOPS buffer within 5.5 h after the blood sample was obtained.

Quantification of F-actin content. F-actin content was determined by the NBD-phalloidin (nitrobenzo-oxadiazole phalloidin) extraction assay described by Howard et al. (6). Briefly, the relative fluorescence intensity (RFI) of methanol extracts from cells exposed to either control solvent dimethylsulfoxide (DMSO) (0.05 vol%) or activator in DMSO was determined by fluorometry (excite 465 nm; emit 535 nm). Relative F-actin content is expressed as RFI of test cells at specified time/RFI of DMSO

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1. *Abbreviations used in this paper:* H/HBSS, Hanks'/Hepes balanced salt solution; fMLP, *formylmethionylleucyl phenylalanine*; MOPS 3-[*N*-morpholino]propanesulfonic acid; NBD-phalloidin, nitrobenzo-oxadiazole phalloidin; PMA, phorbol myristate acetate; RFI, relative fluorescence intensity.

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sample at 0 s. All experiments were done at 25°C. All experiments were done in Hanks'/Hepes balanced salt solution (H/HBSS) (25 mM Hepes, 50 mM phosphate, 150 mM NaCl, 4 mM KCl, 1.0 mM MgCl₂) except those with controlled external free calcium concentration ([Ca²⁺]_{free}).

Preparation of [Ca²⁺]_{free} buffers. Buffers utilized to control external free [Ca²⁺] ([Ca²⁺]_{free}) were prepared according to a previously utilized (28) computer program that considers Fabiato's constants (29) for Ca²⁺ binding to EGTA, Mg²⁺ concentration, ionic strength, pH, EGTA concentration, Ca²⁺ stock concentration. The Ca²⁺ stock was prepared by reacting and solubilizing known dry weights of anhydrous CaCO₃ with concentrated HCl and diluting in deionized (< 2 μohms) doubly distilled water. Results did not vary with EGTA or Ca²⁺ stock since the minimal [Ca²⁺]_{free} necessary for any significant A23187-induced increase in F-actin content was ≥ 5 μM with each of four different stocks tested. Final composition of MOPS buffer was 2.0 mM MOPS, 4.5 mM KCl, 140 M NaCl, 1.2 mM phosphate, 1.2 mM MgCl₂, 1.0 mM EGTA at pH 7.15 ionic strength 150 and free calcium of 10⁻⁷-10⁻⁵ M. Varied [Ca²⁺]_{free} was controlled by addition of varied volumes of 0.1 M Ca²⁺ stock to MOPS buffer. The [Ca²⁺]_{free} buffers were prepared fresh daily and were tested initially, and subsequently at intervals, with a Ca²⁺ electrode to assure accuracy of [Ca²⁺]_{free}. For experiments in MOPS buffer, cells were prepared in buffer with no Ca²⁺ added and then resuspended in MOPS buffer with known [Ca²⁺]_{free}. Experiments were initiated by addition of indicated concentrations of A23187 at zero time. Presence or absence of MgCl₂ in MOPS buffer did not affect the rate or extent of actin polymerization since experiments in MOPS buffer with or without MgCl₂ yielded identical values for F-actin content.

Results

Effect of Ca²⁺ ionophore, PMA, and fMLP on F-actin content. Several neutrophil responses, including change in cytoskeleton-associated actin are induced by fMLP, A23187, and PMA (19, 20). The time course of changes in F-actin content are described in Fig. 1 and Table I. In these studies, Ficoll-Hypaque purified

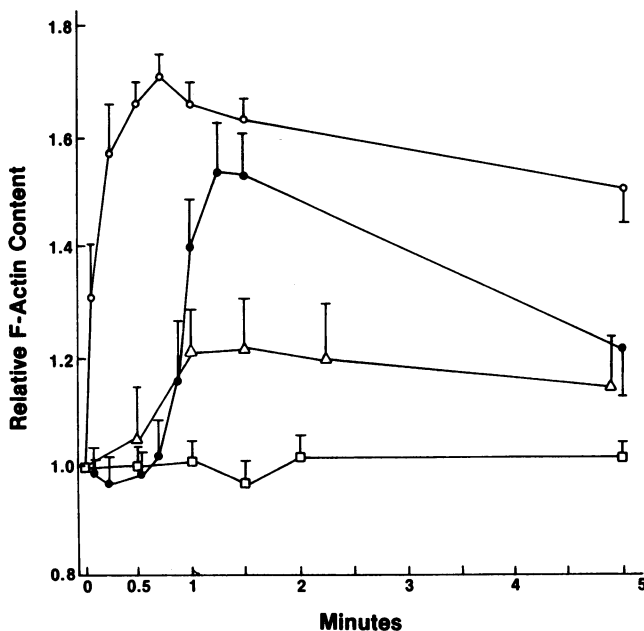


Figure 1. Time course of F-actin content in neutrophils activated with fMLP, A23187, and PMA. At time zero neutrophils in H/HBSS (total [Ca²⁺] = 1.0 mM, free [Ca²⁺] > 10⁻⁵ M) were exposed to control solvent DMSO (0.05 vol%) (—□—), 0.5 μM fMLP (—○—), 1 μM A23187 (—●—), or 1 μM PMA (—△—) at 25°C and relative F-actin content was determined at indicated times. Results shown are mean±SD from five trials at each time.

Table I. Comparison of Kinetics and Extent of Change in F-actin after fMLP, Ca²⁺ Ionophore, or PMA Activation

	Rate of polymerization (% F-actin)/s*	Maximum relative F-actin
0.5 μM fMLP	3.80±0.60	1.72±0.05
1.0 μM A23187†	1.50±0.10‡	1.54±0.08‡
1.0 μM PMA	0.37±0.19‡	1.22±0.11‡

* In Ca²⁺ ionophore experiments, the total calcium concentration of the buffer was 1.0 mM.

† Rate of polymerization is expressed as percent increase in F-actin content/second determined between 0 and 20 s for fMLP; 40–75 s for A23187; 0–60 s for PMA. Values are ± 1 SD from five trials.

‡ P < 0.01 when compared with 0.5 μM fMLP.

cells in Hanks'/Hepes buffer containing 1 mM total calcium concentration were activated at the zero time with 0.5 μM fMLP, 1 μM A23187 or 1 μM PMA and the F-actin content was determined at the indicated times after activation. All activators cause an increase followed by a decrease in F-actin content. The maximum extent of fMLP-induced change in F-actin content is significantly greater than that caused by A23187 or PMA (P > 0.01). The time to maximal F-actin content with fMLP is shorter than that with calcium ionophore or PMA.

The rate of actin polymerization in fMLP-, calcium ionophore-, and PMA-activated cells is also different. As shown in Table I, at 25°C the mean rate of fMLP-induced actin polymerization (3.80% increase in F-actin content/s) is greater than the mean rate of polymerization in A23187-activated (1.50%) or PMA-activated (0.37%) cells. The rate of depolymerization is similar for A23187 and fMLP-activated neutrophils (9–12% and 6–10% decrease in F-actin content per minute, respectively).

Effect of activator dose on F-actin content. The dose response for fMLP-induced F-actin content is known (3–5). F-actin content is maximal for fMLP concentrations > 0.01 μM. Fig. 2 shows the effect of increasing concentrations of A23187 and

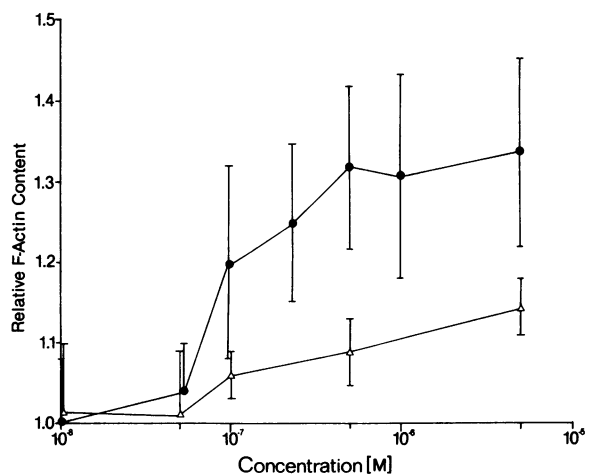


Figure 2. Effect of concentration of A23187 and PMA on maximum F-actin content of neutrophils. Neutrophils in H/HBSS ([Ca²⁺]_{total} = 1.0 mM, [Ca²⁺]_{free} > 10⁻⁵ M) were exposed to increasing concentrations of A23187 (—●—) or PMA (—△—) at 25°C for 1.5 min. The relative F-actin content was determined at 1.5 min. Shown are mean±SD from five trials.

PMA on F-actin content of neutrophils. F-actin content is maximal for A23187 concentrations $\geq 0.5 \mu\text{M}$ and for PMA concentrations $\geq 0.1 \mu\text{M}$. Unlike *f*MLP and A23187 the PMA effect does not reach saturation and the concentrations required for even minimal change in F-actin content are massive when compared to PMA concentrations required for functional responses such as superoxide production (30).

Effect of Ca^{2+} on A23187 and *f*MLP-induced change in F-actin content. The actin polymerization observed with A23187 activation requires the presence of external calcium; *f*MLP-induced increase in F-actin content is independent of external calcium concentration. As shown in Fig. 3, neutrophils exposed to $1 \mu\text{M}$ A23187 or $0.5 \mu\text{M}$ *f*MLP in the presence of 1 mM $[\text{Ca}^{2+}]_{\text{total}}$ increase F-actin content 1.5 min after addition of activator. Addition of 3 mM EGTA prior to addition of A23187, inhibits the A23187-induced but not the *f*MLP-induced increase in F-actin content. DMSO (0.05 vol%), the solvent for both activators, does not alter F-actin content of cells after 1.5 min in the presence or absence of free calcium. Cells incubated in buffer with 3 mM EGTA for as long as 3 h, retain the ability to polymerize actin in response to *f*MLP activation (data not shown). These results show that external calcium is required for A23187-induced actin polymerization and raise the question of whether the external calcium concentration required for A23187-induced actin polymerization is physiologically relevant.

To study the effect of $[\text{Ca}^{2+}]$ on F-actin content in A23187 activated cells we determined the effect of varied external $[\text{Ca}^{2+}]_{\text{free}}$ on the F-actin content of A23187-activated neutrophils. Cells in MOPS buffer with varied external $[\text{Ca}^{2+}]_{\text{free}}$ were activated by addition of $1 \mu\text{M}$ A23187 and F-actin content was determined at increasing times after A23187 addition. As shown in Fig. 4, no significant increase in F-actin content is observed when $[\text{Ca}^{2+}]_{\text{free}}$ is $< 5 \mu\text{M}$. A minimal increase is observed in the presence of $5 \mu\text{M}$ $[\text{Ca}^{2+}]_{\text{free}}$. An initial decline in F-actin content at 5 s is noted and is maximal for $10 \mu\text{M}$ $[\text{Ca}^{2+}]_{\text{free}}$. At all $[\text{Ca}^{2+}]_{\text{free}}$, the F-actin content was maximal at 1 or 1.5 min after A23187 addition.

Fig. 5 shows the $[\text{Ca}^{2+}]_{\text{free}}$ dose response of F-actin content in A23187-activated cells at 5 s, 1 min, and 1.5 min. The modest decrease in F-actin content at 5 s is statistically insignificant. The minimum $[\text{Ca}^{2+}]_{\text{free}}$ that causes a modest, significant increase in F-actin content is $5 \mu\text{M}$. This free calcium concentration is greater than the reported intracellular $[\text{Ca}^{2+}]_{\text{free}}$ in *f*MLP-activated cells (11, 12, 21) and the maximum extent of change in

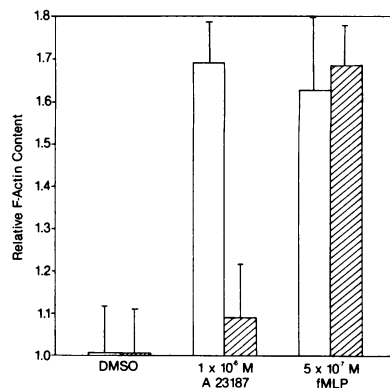


Figure 3. Effect of external calcium concentration on F-actin content of A23187 and *f*MLP-activated neutrophils. Cells were exposed to DMSO (0.05 vol%), $1 \mu\text{M}$ A23187, or $0.5 \mu\text{M}$ *f*MLP for 1.5 min at 25°C in the presence of 1 mM $[\text{Ca}^{2+}]_{\text{total}}$ or 1 mM $[\text{Ca}^{2+}]_{\text{total}}$ plus 3 mM EGTA. The relative F-actin content was determined. Results shown are mean \pm SD for five trials.

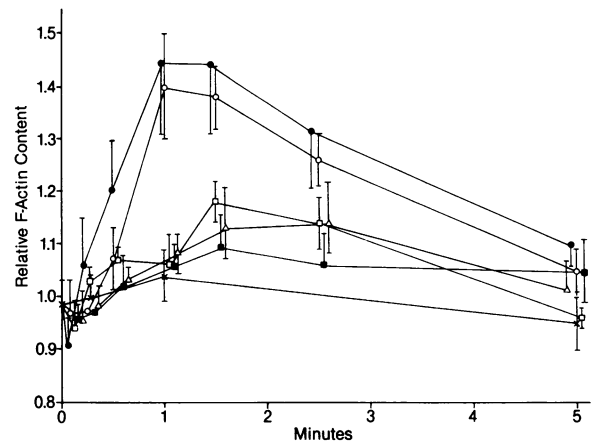


Figure 4. Time course of F-actin content in A23187-activated neutrophils at varied $[\text{Ca}^{2+}]_{\text{free}}$. At zero time, cells were exposed to DMSO (0.05 vol%) (\times) or $1 \mu\text{M}$ A23187 in MOPS buffer with $[\text{Ca}^{2+}]_{\text{free}}$ of $0.1 \mu\text{M}$ (\square), $1 \mu\text{M}$ (\triangle), $5 \mu\text{M}$ (\square), $10 \mu\text{M}$ (\bullet) or $> 10 \mu\text{M}$ (\circ) and relative F-actin content was determined at indicated times. Results are mean \pm SD from five trials.

F-actin content of *f*MLP-activated cells is in excess of that induced by any $[\text{Ca}^{2+}]_{\text{free}} < 10 \mu\text{M}$.

Effect of combining A23187 and PMA on F-actin content of neutrophils. Since neither A23187 nor PMA mimic the effect of *f*MLP on F-actin content of neutrophils and since these activators can modulate the effect of each other on superoxide production (35, 36), we investigated the effect of combining A23187

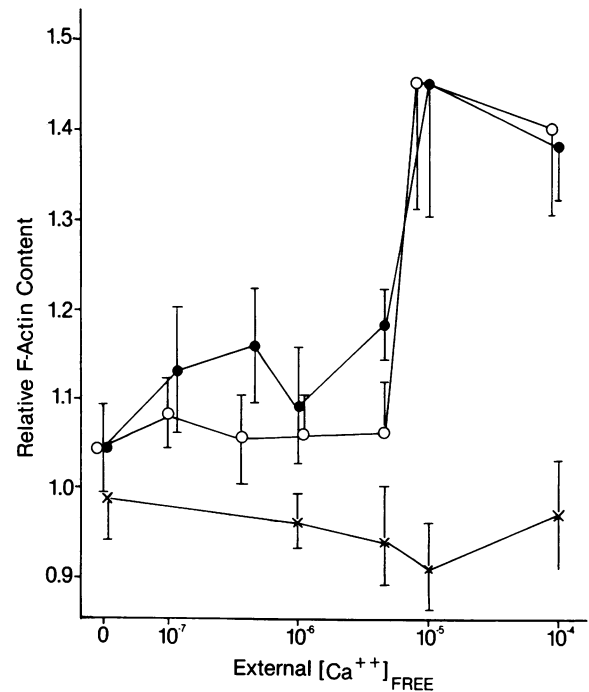


Figure 5. Effect of $[\text{Ca}^{2+}]_{\text{free}}$ on F-actin content of A23187-activated neutrophils. Cells were exposed to $1 \mu\text{M}$ A23187 as described in Fig. 4. The values plotted are the mean \pm SD for five trials at 5 s (\times), 1.0 min (\bullet) and 1.5 min (\circ) after addition of A23187 in the indicated $[\text{Ca}^{2+}]_{\text{free}}$. The 0 point refers to DMSO (0.05 vol%) value at the indicated time.

and PMA simultaneously or serially on F-actin content of neutrophils. We determined the effect of exposing neutrophils in $> 10^{-5}$ M $[Ca^{2+}]_{free}$ or 10^{-7} M $[Ca^{2+}]_{free}$ to either PMA or A23187 or a combination of PMA and A23187 simultaneously. The results are shown in Table II. F-actin content was determined at the time of expected maximum F-actin content. The results suggest that PMA blocks the A23187-activated increase in F-actin content observed at $\geq 10^{-5}$ M $[Ca^{2+}]_{free}$. The F-actin content observed following simultaneous A23187 and PMA addition is much less than that observed with *f*MLP. Simultaneous activation with PMA and A23187 in the presence of 10^{-7} M $[Ca^{2+}]_{free}$ does not cause a change in F-actin content that is significantly different from that induced by A23187 or PMA alone. Table III shows the effect of pre-activation of neutrophils with A23187 or PMA on the subsequent A23187- or PMA- and *f*MLP-induced changes in F-actin content. In these experiments, neutrophils in $\geq 10^{-5}$ M $[Ca^{2+}]_{free}$ were preincubated in 0.1 μ M PMA or 1 μ M A23187 for 10 min and then activated with A23187 or PMA or *f*MLP. F-actin content was determined at the time of expected maximum F-actin content for the second stimulant. As shown in Table III, preincubation of cells with PMA limited the subsequent A23187-induced, but not the *f*MLP-induced, increase in F-actin content. The final F-actin content with A23187 activation resembles that with PMA alone. Also, as shown in Table III reversal of the sequence of addition, i.e., PMA activation following A23187 preincubation, did not alter F-actin content above that expected with PMA alone and the combined effects of A23187 and PMA on F-actin content were not additive. *f*MLP-induced polymerization in cells preincubated with A23187 was not changed. Since *f*MLP induces significant actin polymerization in cells preincubated with either PMA or A23187, this indicates that the actin is able to polymerize. Since either combining PMA with A23187 or PMA preincubation limits the expected A23187 induced polymerization, this suggests that either the extent or the kinetics of A23187-induced polymerization are altered by PMA. To examine this point, we determined the time course of F-actin content in cells preincubated with PMA and then activated with A23187. As shown in Fig. 6, preincubation of cells with PMA inhibits the A23187-induced actin polymerization in neutrophils. These results show that combinations of A23187 with PMA do not mimic *f*MLP-induced polymerization and PMA blocks A23187-induced polymerization in neutrophils.

Table II. Simultaneous Combination of A23187, PMA, and *f*MLP at Varied $[Ca^{2+}]_{free}$

Activator(s)	$\geq 10^{-5}$ M $[Ca^{2+}]_{free}$	10^{-7} M $[Ca^{2+}]_{free}$
DMSO (0.02%)	0.98 \pm 0.04	0.99 \pm 0.03
0.5 μ M <i>f</i> MLP	1.75 \pm 0.10	1.68 \pm 0.10
1.0 μ M A23187	1.51 \pm 0.12	1.06 \pm 0.03
0.1 μ M PMA	1.15 \pm 0.08	1.20 \pm 0.10
0.1 μ M PMA + 0.5 μ M <i>f</i> MLP	1.76 \pm 0.09	ND
0.1 μ M PMA + 1.0 μ M A23187	1.18 \pm 0.07	1.12 \pm 0.08

Values are F-actin content \pm 1 SD from five trials and all values expressed relative to cells plus buffer for 60 s. The DMSO is the solvent for all three activators. Time F-actin assayed after PMA 90 s, *f*MLP 60 s, A23187 75 s, DMSO 60 s, PMA + *f*MLP = 75 s, PMA + A23187 = 75 s.

Discussion

The studies presented here compare the changes in F-actin content of neutrophils caused by activation with *f*MLP, a calcium ionophore and PMA. The studies show that neither PMA or ionophore alone or in combination induce changes in F-actin content that mimic those caused by *f*MLP. Specifically, the data demonstrates the following points: (a) Under conditions that maximize the effects of all three activators, the maximum extent of actin polymerization and the rate of polymerization are greater with *f*MLP than with PMA or the Ca^{2+} ionophore; (b) The effects of *f*MLP and the Ca^{2+} ionophores are dose dependent and PMA effects occur at massive PMA concentrations; (c) The A23187-induced changes in F-actin content, but not those caused by *f*MLP, require external $[Ca^{2+}]_{free}$ of at least 5 μ M; (d) Combined activation with A23187 and PMA does not mimic *f*MLP-induced polymerization; (e) Preactivation of neutrophils inhibits A23187-induced but not *f*MLP-induced actin polymerization in neutrophils. Since protein kinase C activation is the major, though not the only effect of PMA on neutrophils, the results suggest that although protein kinase C activation and increase of intracellular $[Ca^{2+}]_{free}$ are early events in *f*MLP-activated cells, neither event alone causes the *f*MLP-induced polymerization or depolymerization of actin.

Several lines of experimental data suggest that either an increase in intracellular $[Ca^{2+}]_{free}$ or activation of protein kinase C may cause the dramatic cytoskeletal reorganization that follows *f*MLP binding to neutrophils. These include observations on the effect of $[Ca^{2+}]_{free}$ on actin polymerization in vitro (22, 23), demonstration of the Ca^{2+} -sensitive, actin regulatory protein, gelsolin, in neutrophils (18) and descriptions of the temporal relationship of protein kinase C activation and increase in intracellular $[Ca^{2+}]_{free}$ to actin polymerization in *f*MLP-stimulated neutrophils (11–13).

The $[Ca^{2+}]_{free}$ dramatically affects the polymerization of actin in vitro. Kasai (22) demonstrated that nucleation of purified muscle actin is sensitive to $[Ca^{2+}]_{free}$. Free $[Ca^{2+}]$ in the nanomolar to millimolar range exhibit a triphasic effect on in vitro actin polymerization. Micromolar $[Ca^{2+}]_{free}$ inhibits nucleation of actin (22) and decreases rate of polymerization while both nanomolar and millimolar $[Ca^{2+}]_{free}$ promote nucleation and increase the rate of actin polymerization (23). Logically, therefore, a change in intracellular $[Ca^{2+}]_{free}$ may be an important determinant of the state of actin polymerization in neutrophils. An effect of $[Ca^{2+}]_{free}$ on polymerization could reflect a direct effect of Ca^{2+} on actin or an effect of Ca^{2+} mediated by gelsolin.

Gelsolin is a 92-kD, Ca^{2+} -sensitive, actin regulatory protein described by Yin et al. (19) that in the presence of 1 μ M $[Ca^{2+}]_{free}$ has several effects on actin polymerization in vitro (19–21). This protein could mediate calcium-dependent changes in cytoskeletal organization in the cell. Based upon current data, it is unlikely that *f*MLP-induced actin polymerization is caused by Ca^{2+} -activation of gelsolin because *f*MLP-induced polymerization occurs at the barbed end of filaments and is cytochalasin sensitive (4, 5), while in vitro, gelsolin-induced polymerization occurs at the pointed end of actin filaments (21). In spite of this theoretical argument, recent studies of gelsolin-actin interactions in *f*MLP-activated macrophages show that in vitro interactions of gelsolin and actin may differ from their in vivo interactions (32). These studies allow a possible role for gelsolin in actin polymerization or depolymerization in neutrophils. Specifically, Kurth et al. (33, 34) showed that in vitro, in the presence of 1 μ M $[Ca^{2+}]_{free}$,

Table III. Effect of Serial Combination of A23187, PMA, and *f*MLP on F-actin Content

	DMSO Control	0.1 μ M PMA	1.0 μ M A23187	0.5 μ M <i>f</i> MLP
Activator added after 10-min preincubation with DMSO (0.01 vol %)	1.00 \pm 0.04	—	1.48 \pm 0.06	1.83 \pm 0.09
0.1 μ M PMA	1.20 \pm 0.07	—	1.19 \pm 0.07	1.62 \pm 0.11
Activator added after 10-min preincubation with DMSO (0.01 vol %)	0.98 \pm 0.03	1.12 \pm 0.04	—	1.75 \pm 0.09
1 μ M A23187	1.16 \pm 0.05	1.20 \pm 0.06	—	1.75 \pm 0.10

Values are F-actin content \pm 1 SD from five trials and all values are expressed relative to cells plus buffer incubated for 10 min. Time F-actin assayed after PMA = 90 s, A23187 = 75 s, *f*MLP = 60 s, DMSO = 60 s.

purified gelsolin forms an irreversible 130-kD complex with actin; however, more recently Chaponnier et al. (32) showed that in macrophages the 130-kD complex is reversible despite the presence of $\geq 1 \mu\text{M}$ $[\text{Ca}^{2+}]_{\text{free}}$. Therefore, modification of gelsolin-actin interactions via calcium or as yet undefined mechanisms may allow a role for gelsolin in polymerization or depolymerization of actin in *f*MLP-activated neutrophils.

Finally, since protein kinase C activation and increase in intracellular $[\text{Ca}^{2+}]_{\text{free}}$ are among the earliest events that occur during signal transduction in *f*MLP-activated neutrophils, their interposition between ligand binding and maximal F-actin content suggest that either may be the cause of actin polymerization (11–13). Change in cytosolic free calcium concentration may affect actin polymerization independently or by an effect on gelsolin as noted above. The mechanism whereby phosphorylations of substrates by protein kinase C might alter F-actin content is more speculative. However, it is clear that protein kinase C mediated phosphorylations are pivotal in regulating several other neutrophil functions (30).

Despite the logical argument that change in intracellular $[\text{Ca}^{2+}]_{\text{free}}$ or protein kinase C activation may cause *f*MLP-induced actin polymerization/depolymerization, the studies presented here show that stimulation of neutrophils with Ca^{2+} ionophore in the presence of Ca^{2+} , with PMA concentrations that activate protein kinase C or with A23187 and PMA in combination do not mimic *f*MLP-induced cytoskeletal reorganization. The studies show that a minimal, nonsaturable effect of PMA on F-actin content is observed and that the $[\text{Ca}^{2+}]_{\text{free}}$ required for A23187-induced change in F-actin content exceeds the max-

imum intracellular $[\text{Ca}^{2+}]_{\text{free}}$ reported with Quin 2, Indo 1, or Fura 2 in *f*MLP-activated neutrophils (11, 12, 31). Our studies with fixed, NBD-phalloidin stained neutrophils presented here are in agreement with the studies of Sklar et al. (17), Yassin et al. (24), and Sha'afi (34). In studies with Quin 2 buffering of calcium transients and measurement of cytoskeleton associated actin, these investigators suggest that an increase in intracellular calcium is neither necessary or sufficient to explain *f*MLP-induced actin polymerization.

The studies presented here and earlier studies by Sha'afi (34) clearly suggest that PMA or Ca^{2+} -dependent, A23187 activation of neutrophils alone or in combination do not mimic *f*MLP-induced changes in F-actin content. However, significant differences in our observations with A23187 and those of Sha'afi (34) do exist. Specifically, while Sha'afi reports little effect of $[\text{Ca}^{2+}]$ on A23187-induced change in the cytoskeleton-associated actin of operationally defined Triton insoluble cytoskeletons, the A23187-induced effects we observe clearly depend on external Ca^{2+} concentration. Previous work from our laboratory (6) suggests that cytoskeleton-associated actin measured by gels and F-actin content as determined by NBD-phalloidin extraction may not be identical. The reason for the differences in Ca^{2+} dependence is not clear; however, cytoskeleton-associated actin in operationally defined Triton insoluble cytoskeletons is clearly affected by Ca^{2+} -dependent proteolysis in the system used by Sha'afi (34). Further studies are necessary to resolve these differences. However, regardless of these differences, both investigators' results clearly show that PMA activation or Ca^{2+} -dependent, A23187 activation of neutrophils alone or in combination do not mimic the *f*MLP-induced changes in the state of actin polymerization. Furthermore, the results show that PMA preactivation blocks A23187-induced but not *f*MLP-induced polymerization. These results suggest that neither protein kinase C activation alone nor increase in cytosolic $[\text{Ca}^{2+}]_{\text{free}}$ are responsible for *f*MLP-induced actin polymerization.

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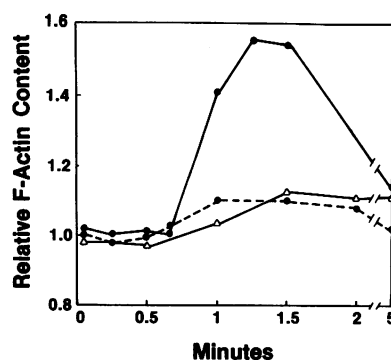


Figure 6. Effect of PMA preincubation on A23187-induced polymerization. Cells were exposed to 0.1 μM PMA or control solvent (DMSO 0.01 vol %) for 10 min at 25°C. Then, 1 μM A23187 was added to DMSO preincubated (—●—) or PMA preincubated cells (---○---) or 0.1 μM PMA was added to DMSO

preincubated cells (—△—) and F-actin content was determined at the indicated times. Results are an example experiment. Values are expressed relative to cells preincubated with DMSO for 10 min.

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