The Kinetics of Chemotactic Peptide-induced Change in F-Actin Content, F-Actin Distribution, and the Shape of Neutrophils

T. H. HOWARD and C. O. ORESAJO
Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, Virginia 22908

ABSTRACT Formyl-met-leu-phe (fMLP) induces actin assembly in neutrophils; the resultant increase in F-actin content correlates with an increase in the rate of cellular locomotion at fMLP concentrations $\leq 10^{-8}$ M (Howard, T. H., and W. H. Meyer, 1984, *J. Cell Biol.*, 98:1265–1271). We studied the time course of change in F-actin content, F-actin distribution, and cell shape after fMLP stimulation. F-actin content was quantified by fluorescence activated cell sorter analysis of nitrobenzoxadiazole-phallacidin-stained cells (Howard, T. H., 1982, *J. Cell Biol.*, 95(2, Pt. 2:327a). F-actin distribution and cell shape were determined by analysis of fluorescence photomicrographs of nitrobenzoxadiazole-phallacidin-stained cells. After fMLP stimulation at 25°C, there is a rapid actin polymerization that is maximal (up to 2.0 times the control level) at 45 s; subsequently, the F-actin depolymerizes to an intermediate F-actin content 5–10 min after stimulation. The depolymerization of F-actin reflects a true decrease in F-actin content since the quantity of probe extractable from cells also decreases between 45 s and 10 min. The rate of actin polymerization (3.8 ± 0.3–4.4 ± 0.6% increase in F-actin/s) is the same for 10^{-10}–10^{-6} M fMLP and the polymerization is inhibited by cytochalasin D. The initial rate of F-actin depolymerization (6.0 ± 1.0–30 ± 5% decrease in F-actin/min) is inversely proportional to fMLP dose. The F-actin content of stimulated cells at 45 s and 10 min is greater than control levels and varies directly with fMLP dose. F-actin distribution and cell shape also vary as a function of time after stimulation. 45 s after stimulation the cells are rounded and F-actin is diffusely distributed; 10 min after stimulation the cell is polarized and F-actin is focally distributed. These results indicate that (*a*) actin polymerization and depolymerization follow fMLP stimulation in sequence, (*b*) the rate of depolymerization and the maximum and steady state F-actin content but not the rate of polymerization are fMLP dose dependent, and (c) concurrent with F-actin depolymerization, F-actin is redistributed and the cell changes shape.

Actin is an essential element of the cytoskeleton in nonmuscle cells. Most nonmuscle cells express various motile behaviors, e.g., cytokinesis, locomotion, secretion, and endocytosis, which require change in cell shape and remodeling of the cellular cytoskeleton (1). Restructuring of the cytoskeleton is accomplished in part by modulation of the state of actin polymerization in the cell. Changes in the state of actin polymerization in nonmuscle cells can be induced by external stimuli or by alterations in the functional state of the cell, and these cytoskeletal changes correlate with quantitative changes in the motile behavior of cells (2–6). The correlations suggest that changes in the state of actin polymerization are required for complex motile behaviors such as platelet aggregation (3, 4) and neutrophil chemotaxis (2, 7). In most cases, change in motility is associated with actin polymerization as evidenced by an increase in F-actin or a decrease in G-actin (2–4, 7, 8). To date little is known about the relevance of F-actin depolymerization to changes in the motility of nonmuscle cells.

Although the state of actin polymerization changes in nonmuscle cells and much is known about the ionic and protein factors that regulate the state of polymerization of purified actin in vitro (1, 9), little is known about the molecular mechanisms within cells that regulate actin polymerization and depolymerization. In this paper we report studies on the

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kinetics of actin polymerization and depolymerization in human neutrophils in which we determined the rates of actin polymerization and depolymerization in fMLP-activated neutrophils, and we describe factors that affect cytoskeletal dynamics and F-actin content of this nonmuscle cell, e.g., temperature and cytochalasins. We also introduce a new technique for quantifying F-actin which, unlike the FACS[1] assay (2), is not affected by the shape or F-actin distribution of the cell. Results from these studies show that formyl-met-leu-phe (fMLP) initiates a sequential actin polymerization and depolymerization in neutrophils. The polymerization and depolymerization of actin correlate with changes in the shape and F-actin distribution within neutrophils. The studies suggest that knowledge of the kinetics of actin polymerization and depolymerization in vitro (9) and within nonmuscle cells may help establish testable hypotheses to direct the investigation of the molecular mechanisms that control cytoskeletal organization in nonmuscle cells.

MATERIALS AND METHODS

Preparation of Neutrophils: Leukocytes were prepared from human peripheral blood in EDTA anticoagulant by dextran 60 (Cutter Laboratories, Inc., Berkeley, CA) sedimentation, contaminating erythrocytes were removed by brief (45 s) hypotonic lysis. Neutrophils were purified on Ficoll-Hypaque, yielding 96–97% neutrophils, 2–3% eosinophils, 0–1% mononuclear cells (10). All experiments were done within 5.5 h after the blood sample was drawn (11).

Quantification of F-Actin Content: As previously described, neutrophils were stained with nitrobenzoxadiazole (NBD)-phallacidin (12) (Molecular Probes, Junction City, OR) and analyzed with a FACS (2) with the following modifications. In all cases a two-step stain procedure was used. Neutrophils (0.9 ml of 1.1×10^6 cells/ml) were incubated at the desired temperature in Hanks' balanced salt solution/HEPES buffer (25 mM HEPES, 50 mM phosphate, 150 mM NaCl, 4 mM KCl, 1.0 mM $MgCl_2$, 1.2 mM CaCl, 0.05% delipidated human serum albumin [Sigma Chemical Co., St. Louis, MO]) with fMLP or 0.1% vol/vol dimethylsulfoxide (DMSO, the fMLP solvent); fixed with formalin (3.7% vol/vol) for 15 min at 25°C; and then exposed to a final concentration of 100 μg/ml lysophosphatidyl choline and 1.65×10^{-7} M NBD-phallacidin. Stained cells were filtered (50 μm mesh nylon filter) and analyzed by FACS within 1 h of staining. In all instances the fluorescence histogram of cells yielded a normal distribution, and the fluorescence was recorded as the peak fluorescence channel number. The relative F-actin content is expressed as the ratio of the fMLP peak channel number to the DMSO control peak channel number.

For some experiments relative F-actin content was determined by methanol extraction of NBD-phallacidin stained cells. Cells (2×10^6) were fixed and stained in 3.3×10^{-7} M NBD-phallacidin as described above and sedimented at 16,000 *g* for 1 min. The supernatant was removed, and the pellet was overlaid with 350 μl absolute methanol and extracted in the dark with frequent vortexing for 1 h. The cells were again sedimented, the supernatant was removed, and the relative fluorescence intensity (RFI) of the supernatant was determined on an Aminco-Bowman spectrofluorometer (excitation at 465 nm; emission at 535 nm). Results are expressed both as the RFI of the supernatant and relative F-actin content, i.e., the ratio of RFI stimulated to RFI control. This procedure extracts >95% of the bound NBD-phallacidin and >95% of the extractable NBD-phallacidin binding is blocked by nonfluorescent phalloidin in 100–500-fold molar excess.

Quantification of Cell Shape and F-Actin Distribution: Cells were prepared as described above for FACS analysis and centrifuged at low density onto glass slides with a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA) at 1,000 rpm for 5 min. In the text these preparations are referred to as "cytospins." Cytospins were mounted with 90% glycerol. 35-mm fluorescent images of cells were recorded on Kodak Tri-X film using a Leitz microscope equipped with a photographic system. Cells (30–40 per 400× field) and a micrometer were photographed; all fluorescence photographs were manually exposed for an identical period (always two-thirds of the automatic exposure time for the most intense sample, i.e., 10^{-6} M fMLP for 45 s). Prints (5 × 7 in.) were prepared on F4 Kodabromide paper with identical exposure times for each negative. The numerical values that describe ellipticity (Form PE) and F-actin distribution ratio were determined by analysis of prints on a Zeiss Videoplan. Form PE is the ratio of the area to the perimeter of a form such that a value of 1.0 is that for a circle; lower values reflect a progressively elongating ellipse. Form PE was determined by following the edge of cells with the cursor of a Zeiss Videoplan which was interactively scaled and set on the FORM PE function. F-actin distribution ratio is the ratio of area of intense NBD-phallacidin staining to total area of the cell (values of 1.0 indicate diffuse distribution of stain, and values <1.0 indicate progressively more asymmetric distributions of stain). The total area of the cell and area of NBD-phallacidin staining were quantified in square micrometers by tracing the appropriate area with the cursor of the Zeiss Videoplan. The Videoplan was interactively sealed and set on the AREA function. The F-actin distribution ratio is never 1.0 since the nucleus does not stain with NBD-phallacidin.

Preparation of Cytoskeletons and Cytoskeleton-associated Actin Analysis: Cells (2×10^6 in 250 μl Hanks'/HEPES buffer) were mixed with 250 μl of Hanks'/HEPES containing twice concentrated fMLP or DMSO. After the indicated times, 7 mM diisopropylfluorophosphate and 500 μl lysing buffer (20 mM EGTA, 20 mM imidiazole HCl, 80 mM KCl, and 2% Triton-X 100, pH 7.15) were added, and the mixture was vortexed for 10 s. The pellet was then centrifuged at 16,000 *g* for 60 s, overlaid with an equal mixture of Hanks'/HEPES buffer and lysing buffer without Triton X-100, and sedimented at 16,000 *g* for 60 s. The resultant pellet was solubilized in 10% SDS, 4 M urea, 20% glycerol, 2% mercaptoethanol, pH 8.0, at 100°C for 10 min. Proteins were separated by 10–20% gradient SDS PAGE. Coomassie Blue–stained gels were analyzed with an LKB Ultrascan Laser Densitometer (LKB Instruments, Inc., Gaithersburg, MD), and the cytoskeleton-associated actin (CAA) was expressed as the percentage of whole cell actin retained in the Triton-insoluble pellet.

RESULTS

The Shape and Distribution of F-Actin in Neutrophils: Effect of Time after Stimulation

Previous studies indicate that the shape of fMLP-stimulated neutrophils depends upon the concentration of fMLP and the time elapsed after exposure to fMLP (13, 14); furthermore, it is known that actin in polarized neutrophils is localized to the lamellipodium of the cell (15, 16). To study the relationship of F-actin distribution to the shape of fMLP stimulated neutrophils, the degree of cellular polarization and the F-actin distribution in cells exposed to 10^{-9} or 10^{-6} M fMLP were quantified by Zeiss Videoplan analysis of 35-mm fluorescence photomicrographs. Examples of photomicrographs at different fMLP concentrations (10^{-6} and 10^{-9} M) and at increasing time after stimulation (45 s and 10 min) are shown in Fig. 1. The time- and dose-dependent effect of fMLP on the shape and F-actin distribution in neutrophils is striking. As shown in Fig. 1, 45 s after fMLP stimulation the intensity of NBD-phallacidin fluorescence is greater than the intensity in control cells at 45 s or 10 min and greater than the intensity of stimulated cells at 10 min. Also at 45 s the cells are rounded and F-actin is diffusely distributed throughout the cell; 10 min after stimulation the cells are polarized and, as determined from differential interference contrast images (not shown), F-actin is concentrated in lamellipodia and other focal submembranous sites.

Fig. 2 provides quantitative data on changes in the shape and F-actin distribution which are expressed as change in the form factor (Form PE) and the F-actin distribution ratio (ratio of the area of NBD-phallacidin fluorescence to the whole cell area). These quantitative results confirm the impressions derived from viewing the photomicrographs. The form factor indicates that control cells and cells exposed to 10^{-6} or 10^{-9} M fMLP are circular 45 s after stimulation (Fig. 2*A*). The F-

[1] *Abbreviations used in this paper*: CAA, cytoskeleton-associated actin; DMSO, dimethylsulfoxide; FACS, fluorescence activated cell sorter; fMLP, formyl-met-leu-phe; NBD, nitrobenzoxadiazole; RFI, relative fluorescence intensity.

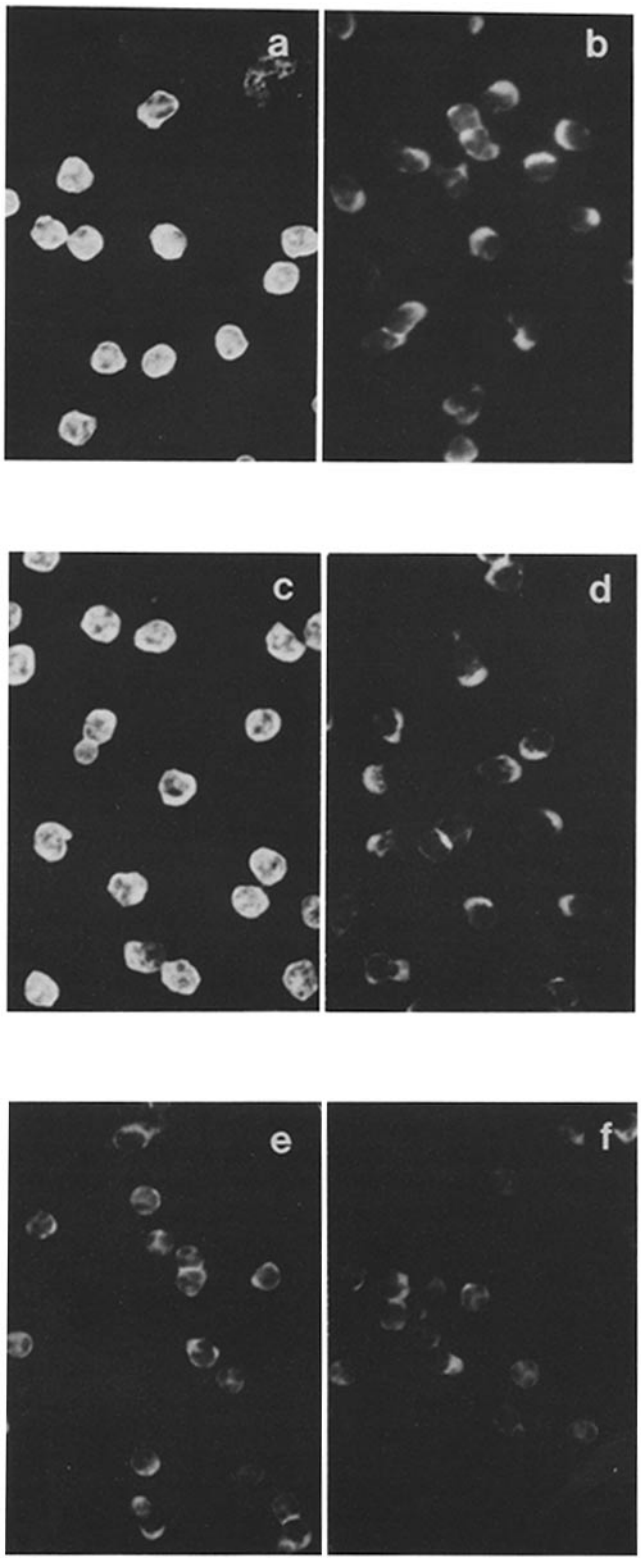

FIGURE 1 Time course of shape and F-actin distribution in fMLP-stimulated neutrophils. 1.0×10^6 neutrophils exposed to 10^{-6} or 10^{-9} M fMLP at 25°C for the indicated times were fixed (3.7% formalin for 15 min) and then permeabilized and stained with lysophosphatidyl choline and NBD-phallacidin (1.65×10^{-7} M) and spun onto coverslips. Presented are 35-mm fluorescence photomicrographs (400×) of cells in the presence of 10^{-6} M fMLP at 45 s (*a*) and 10 min (*b*) or 10^{-9} M fMLP at 45 s (*c*) or 10 min (*d*), or DMSO (0.1% vol/vol) at 45 s (*e*) or 10 min (*f*).

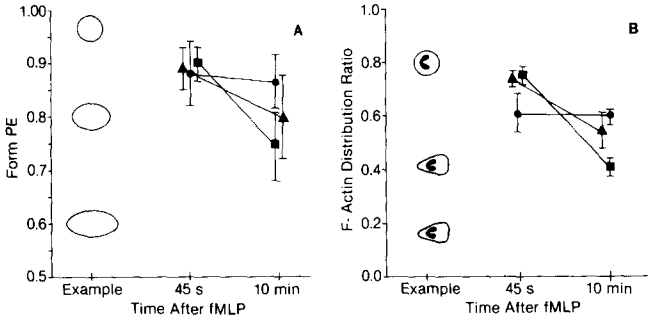

FIGURE 2 Time course of change in cell shape and F-actin distribution. (*A*) Change in ellipticity as measured by Form PE for control (●), 10^{-9} M fMLP (■), and 10^{-6} M fMLP (▲) stimulated cells. Form PE is the ratio of cell area to cell perimeter determined by the Zeiss Videoplan; a ratio of 1.0 indicates a circle, <1.0 an ellipse. Values are mean ± SD for 50–72 cells in each sample. The examples are forms that yield the indicated Form PE values with Videoplan analysis. (*B*) Change in F-actin distribution ratio for control (●), 10^{-9} M fMLP (■), and 10^{-6} M fMLP (▲) stimulated cells. The F-actin distribution ratio is ratio of intense NBD-phallacidin staining to total cell area determined by the Zeiss Videoplan. Note that 0.8 indicates a diffuse distribution because the nucleus does not stain. Values are mean ± SD for 45 cells in each sample. The examples are model forms that yield the indicated F-actin distribution ratios. In the examples, the white areas are areas of F-actin distribution.

actin distribution ratio is high (0.60–0.75) and indicates that the F-actin is diffusely distributed throughout control and stimulated cells (Fig. 2*B*). 10 min after stimulation the F-actin is distributed over a smaller percentage of the area of the fMLP-stimulated cell (F-actin distribution ratio of 0.40, 0.55, and 0.60 for 10^{-9} M, 10^{-6} M, and control cells, respectively; Fig. 2*B*), and the 10^{-9} M stimulated cell is significantly more polarized, or elliptical, than the control or the 10^{-6} M stimulated cell (Fig. 2*A*). At 10 min after stimulus, the distribution of F-actin, as reflected by F-actin distribution ratio is more focal for cells exposed to 10^{-9} M fMLP than for control cells or cells exposed to 10^{-6} M fMLP. These changes in shape are in agreement with those described by Keller (13) and Zigmond (14) who, respectively, noted an fMLP dose-dependent and time-dependent change in neutrophil shape. The redistribution of F-actin from diffuse to focal suggests that redistribution may play an important role in determining the shape and the locomotive behavior of the cell (17, 18).

Time Course of F-Actin Content in fMLP-stimulated Neutrophils

fMLP induces an increase in the F-actin content of human neutrophils as determined by FACS analysis of NBD-phallacidin-stained cells (2). Fig. 3 shows the time course of change in relative F-actin content during 10 min after fMLP stimulation (10^{-10}–10^{-6} M). During the first 60 s after fMLP stimulation there is a dramatic increase in relative F-actin content that is maximal at ~45–60 s and reflects fMLP-induced actin polymerization. This fMLP-induced actin polymerization is inhibited by cytochalasin D in a dose-dependent manner; the F-actin content of neutrophils 45 s after 10^{-6} M fMLP stimulation is reduced by 80% in the presence of 10^{-6} M cytochalasin D (data not shown).

After maximal F-actin content is observed there is a decline in relative F-actin content during the next 10 min. Even 10

min after stimulation the relative F-actin content is elevated above control levels (see Fig. 4) and the elevation persists for as long as 20 min after stimulation (data not shown). The maximal F-actin content at 45 s and the F-actin content at 10 min are both fMLP dose dependent (see Fig. 4); however, maximal F-actin content at 45 s is induced by 10^{-9} M fMLP whereas the maximum F-actin content at 10 min is observed for fMLP concentrations $\geq 10^{-8}$ M.

The decline in fMLP-induced F-actin content between 45 s and 10 min after stimulation probably reflects the depolymerization of F-actin. However, quantification of fMLP by FACS analysis with the fluorescent probe can be affected by the shape of the cell and the distribution of the fluorescent probe, both of which change as noted above (19). To determine whether the decrease in relative F-actin content observed by FACS actually reflects a depolymerization of F-actin, the quantity of NBD-phallacidin associated with neutrophils that are stimulated with 10^{-6} M fMLP was determined by extracting bound NBD-phallacidin from fMLP-stimulated cells into absolute methanol. The quantity of NBD-phallacidin extracted was assayed by spectrofluorometry. This measurement is independent of cell shape and probe distribution. As shown in Table I, the levels of methanol-extractable NBD-phallacidin parallel the results obtained using FACS analysis. At 45 s the methanol-extractable NBD-phallacidin is increased (2.11 ± 0.22-fold) above control levels, and the amount of NBD-phallacidin extracted by methanol decreases to a lower level 10 min after stimulation. This result, paired with the data from FACS analysis, shows that the decrease in fluorescent signal observed between 45 s and 10 min after fMLP stimulation actually reflects a net decrease in F-actin content. These results indicate that after exposure of neutrophils to fMLP there is a rapid initial polymerization of actin which, at 25°C, is maximal at 45 s; the polymerization is followed by depolymerization of actin during the subsequent 9–10 min. Concurrent with these changes in relative F-actin content, rapid changes in cell shape and F-actin distribution also occur.

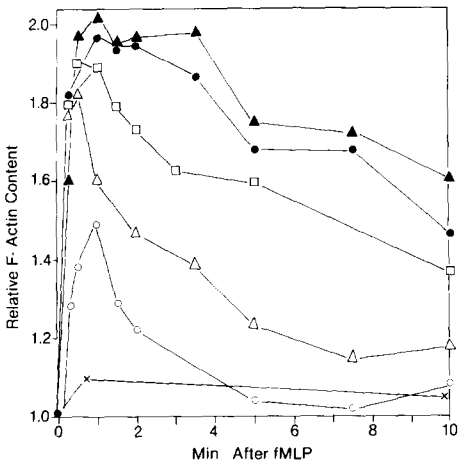

FIGURE 3 Time course of changes in F-actin content of fMLP-stimulated neutrophils. 1.0 × 10^6 cells were exposed to 10^{-10} M (○), 5 × 10^{-10} M (△), 10^{-9} M (□), 10^{-8} M (▲), 10^{-6} M (●) fMLP, or DMSO (0.1% vol/vol) (×) at time = 0 min, at 25°C. At the indicated times (0–10 min) the cells were fixed (3.7% formalin), lysed, stained, and analyzed on FACS for relative F-actin content.

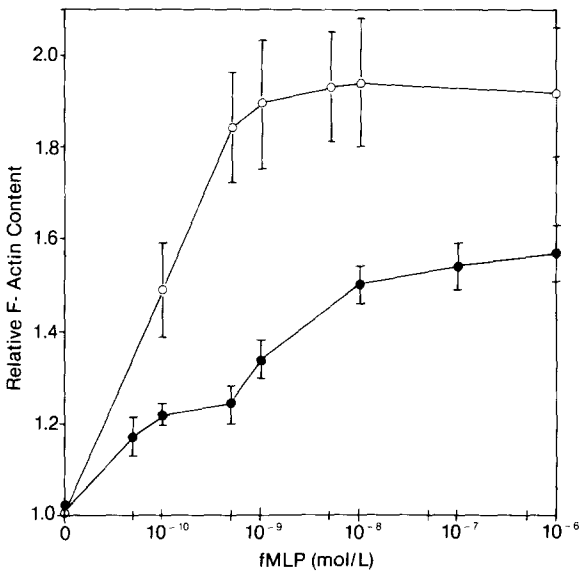

FIGURE 4 Dose response of F-actin content to fMLP concentration. 1.0 × 10^6 cells exposed to increasing concentrations of fMLP were fixed at 45 s (○) or 10 min (●), lysed, and stained with NBD-phallacidin. Fixed stained cells were analyzed by FACS for relative F-actin content. Points plotted are mean ± SD of three trials on three different days.

Effect of Temperature on F-Actin Content of Neutrophils

In previous studies we reported that the F-actin content of nonstimulated and fMLP stimulated neutrophils varies with temperature (2). As shown in Fig. 5, a more detailed study of

TABLE I. *Relative F-Actin Content Determined by Methanol Extraction of NBD-Phallacidin-stained Neutrophils*

	RFI*	Relative F-actin content‡
Control	0.042 ± 0.011	1.00
10^{-6} M fMLP, 45 s	0.090 ± 0.029	2.11 ± 0.22
10^{-6} M fMLP, 10 min	0.055 ± 0.007	1.39 ± 0.16

* By spectrofluorometry. Mean ± SD of five trials on different days.
‡ Relative F-actin content = ratio RFI with fMLP/control RFI on a given day; results, mean ± SD of five trials on different days.

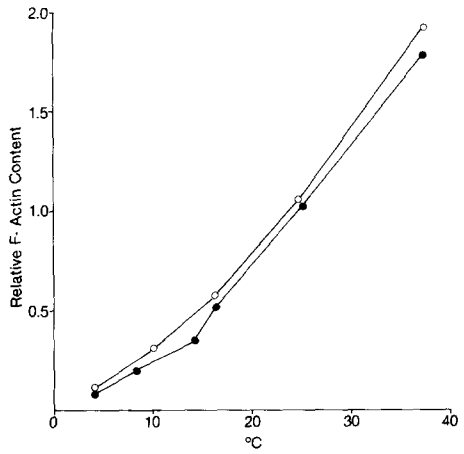

FIGURE 5 Effect of temperature on relative F-actin content as determined by FACS. Neutrophils (1.0 × 10^6 cells) exposed to increasing temperature were fixed, lysed, and stained with NBD-phallacidin and analyzed on FACS for relative F-actin content. Results are expressed relative to F-actin content at 25°C. Plotted are results of two experiments done on two different days.

the effect of temperature (4–37°C) on relative F-actin content of neutrophil shows that the relative F-actin content of non-stimulated neutrophils determined by FACS increases with increasing temperature. The temperature-dependent increase in F-actin content is not due to a temperature dependence of NBD-phallacidin binding to F-actin since cells fixed at 37°C, but stained at 37 or 4°C, contain similar amounts of NBD-phallacidin as determined by FACS analysis. These findings were verified by methanol extraction of NBD-phallacidin-stained cells and are shown in Fig. 6. The effect of temperature on F-actin content as determined by FACS (a 30-fold change from 4 to 37°C) was greater than that determined by methanol extraction (a fourfold change from 4 to 37°C). The difference probably reflects an inaccuracy of determinations of minimal NBD-phallacidin staining by FACS since comparison at higher temperatures are in closer agreement (FACS, 3.3-fold change and methanol extraction, 2.2-fold change from 15 to 37°C). The results indicate that the state of actin polymerization is affected by temperature, and the observation suggests that the rates of fMLP-induced polymerization and depolymerization of actin may be reduced by decreased temperature.

Rates of fMLP-induced Actin Polymerization and Depolymerization in Neutrophils

The rate of actin polymerization at 25 or 37°C was so rapid that not enough samples could be obtained before F-actin content reached the maximal values. However, the rate of depolymerization is slower and can be measured at 25°C. As shown in Table II, the rate of depolymerization of F-actin in fMLP-stimulated neutrophils is inversely proportional to the concentration of fMLP over the range 5×10^{-10}–10^{-6} M fMLP. The depolymerization rate (the percent decrease in relative F-actin content per minute) ranges from 6 ± 1% at 10^{-6} M fMLP to 30 ± 8% at 5×10^{-10} M fMLP. The rate of depolymerization was determined from the first three points after the maximal F-actin content was attained. We determined the initial rate of fMLP-induced actin polymerization with FACS analysis of NBD-phallacidin stained neutrophils by lowering the temperature to 20°C. At 20°C, four data points could be obtained before 30 s had elapsed. Beyond 30 s the rate of change in F-actin content slowed as the F-actin content approached its maximum. Our studies show that the rate of fMLP-induced actin polymerization in neutrophils (expressed as percent increase in relative F-actin content per second) was similar (range 3.75 ± 0.3% to 4.40 ± 0.6% for 10^{-6}–10^{-10} M fMLP) regardless of the concentration of fMLP.

Measurement of bound NBD-phallacidin by methanol extraction of the NBD-phallacidin from neutrophil pellets confirms the observation that the rate of fMLP induced actin polymerization is independent of fMLP concentration (see Fig. 7). By methanol extraction at 17 or 20°C the rate of polymerization is 0.0138 RFI units/10 s and 0.0123 RFI units/10 s for 10^{-6} and 10^{-9} M fMLP, respectively. These values are equivalent to a 3.55 and 3.80%/s increase in relative F-actin content. These observations indicate that the rate of fMLP-induced actin depolymerization is dose dependent,

TABLE II. *Rates of fMLP-induced Actin Polymerization and Depolymerization in Neutrophils Determined by FACS*

fMLP	Polymerization rate	Depolymerization rate
M/L	*%Δ/s**	*%Δ/min‡*
1×10^{-6}	+3.75 ± 0.3§	−6 ± 1§
1×10^{-8}	+4.00 ± 0.3	−10 ± 2
1×10^{-9}	—‖	−20 ± 8
5×10^{-10}	+4.10 ± 0.3	−30 ± 8
1×10^{-10}	+4.40 ± 0.6	−24 ± 5

* Percentage increase in relative F-actin content per second at 20°C.
‡ Percentage decrease in relative F-actin content per minute at 25°C.
§ Mean ± SD determined on three trials.
‖ Not tested.

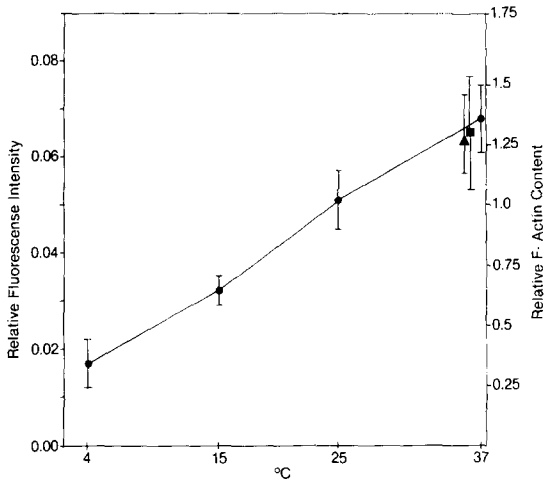

FIGURE 6 Effect of temperature on F-actin content as determined by methanol extraction. Neutrophils (2.0×10^6 cells) were exposed to increasing temperature in the absence of fMLP, fixed, lysed, and stained with NBD-phallacidin. Subsequently, the NBD-phallacidin bound to the cell pellet was extracted into methanol as described in Materials and Methods and quantified by spectrofluorometry. Results (●) are expressed as mean RFI (left ordinate) and mean ± 1 SD relative F-actin content (right ordinate) determined on three trials for each temperature. ■, mean ± SD of F-actin content of cells fixed at 37°C for 10 min, stained at 37°C for 20 min. ▲, mean ± SD of F-actin content of cells fixed at 37°C for 10 min, stained at 4°C for 20 min.

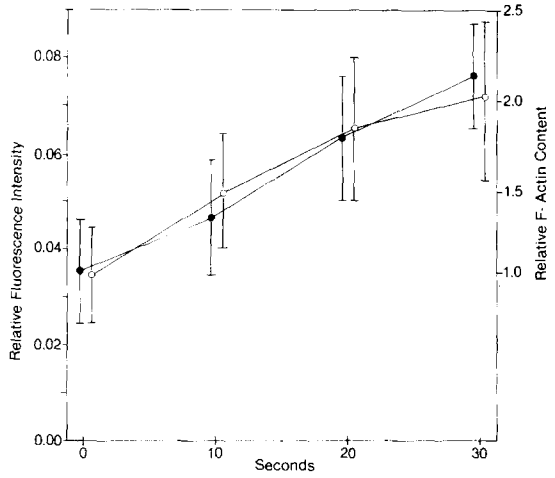

FIGURE 7 Kinetics of 10^{-6} and 10^{-9} M fMLP-induced actin polymerization as determined by methanol extraction. Neutrophils (2.0×10^6 cells) were exposed to 10^{-6} (○) or 10^{-9} M (●) fMLP at 17°C, fixed at the indicated times after stimulus, and stained with NBD-phallacidin. The NBD-phallacidin was extracted from cell pellets into absolute methanol and quantified by spectrofluorometry. Results are expressed as mean RFI (left ordinate) and mean ± 1 SD relative F-actin content (right ordinate) determined on four trials for each fMLP concentration. For the line determined by least squares analysis of mean values, $r = 0.997$ for 10^{-9} M and 0.979 for 10^{-6} M.

whereas the rate of fMLP-induced actin polymerization is independent of fMLP concentration. Since the extent of actin polymerization at 45 s is dose dependent, but the rate of actin polymerization is not fMLP dose dependent, the results suggest that the maximal amount of F-actin present at 45–60 s is determined by the duration of fMLP-induced polymerization and not by the rate of polymerization. However, the results do not exclude the possibility that fMLP dose dependent changes in the size of the pool of polymerizable G-actin could contribute to these differences in maximum F-actin content.

Time Course of Changes in Cytoskeleton-associated Actin in fMLP-stimulated Neutrophils

To confirm our observations on the time course of changes in F-actin content of fMLP-stimulated neutrophils, we determined the amount of CAA by performing SDS PAGE on operationally defined cytoskeletons obtained by Triton X-100 extraction of neutrophils as described in the Materials and Methods. As shown in Fig. 8, the CAA increased rapidly after 10^{-6} M fMLP stimulation, was maximal after 60–90 s (control, 24.4 ± 6.8% and 10^{-6} M fMLP, 44.6 ± 8%), and then returned to control values after 5 min. This result is similar to that observed with FACS analysis of NBD-phallacidin-stained cells. As was the case for F-actin content as determined by FACS analyis, the maximum CAA depends upon fMLP concentration. 60 s after stimulation with 10^{-10}, 10^{-8}, and 10^{-6} M fMLP the CAA was, respectively, 29.5, 33.3, and 38.7% of whole cell actin. These results support the previous observations that fMLP induces a polymerization of actin that is followed by a depolymerization of actin.

DISCUSSION

Cell shape and the expression of cell motility are critically dependent upon the state of the cytoskeleton. In the case of neutrophils, actin is a major contributor to cell shape and motile behavior. Therefore, it is of considerable importance to understand the relationship between the state of actin polymerization and the distribution of F-actin within the cell on the one hand and cell shape and motile behavior on the other hand. In many cell types, external stimuli initiate changes in the state of actin polymerization (2–8) within the cell. Previous work from this laboratory (2) and others (7, 8) demonstrated that chemotactic stimuli induce actin polymerization in neutrophils. The quantity of F-actin in stimulated cells is fMLP dose dependent and correlates, under some conditions ($<10^{-8}$ M fMLP), with the rate of locomotion of the cells (2). The present studies extend these initial observations and define the kinetics of change in F-actin content in response to fMLP stimulation. The results show that immediately after fMLP stimulation there is a rapid polymerization of actin followed by a slower depolymerization of actin. Concurrent with the F-actin depolymerization, there is a marked change in cell shape from rounded to polarized and a redistribution of F-actin from a diffuse organization to a focal concentration in the lamellipodium of the cell. The fMLP-induced polymerization is inhibited by cytochalasin D and is temperature sensitive. The extent of actin polymerization (F-actin content) at 45 s and 10 min after stimulation and the rate of F-actin depolymerization are dose dependent, whereas the rate of polymerization is independent of fMLP dose. These findings have important implications for our understanding of the intracellular mechanisms that control the state of actin polymerization and the shape and locomotive behavior of the neutrophil.

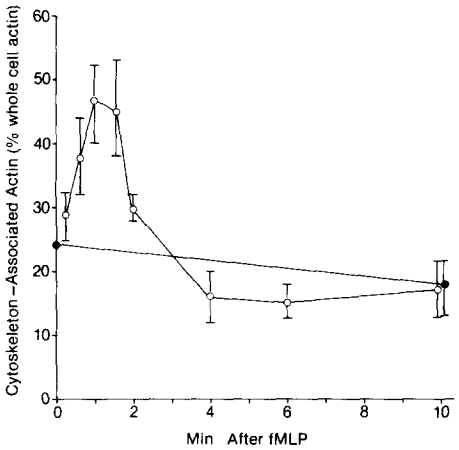

FIGURE 8 Time course of CAA in Tritonized cytoskeletons of fMLP-stimulated neutrophils. Cytoskeletons of fMLP-stimulated neutrophils: 2 × 10^6 cells exposed to 10^{-6} M fMLP (○) or DMSO (●) for the indicated times were treated with diisopropylfluorophosphate and then with 1% Triton X-100, 10 mM EGTA, 40 mM KCl, 10 mM imidazole, pH 7.10, and immediately sedimented for 60 s at 16,000 *g*; Triton-insoluble pellets were solubilized and run on 10–20% SDS-polyacrylamlide gels. Values presented are mean ± 1 SD of percent of whole cell actin associated with the cytoskeleton from four trials.

Our results, which generally agree with a recent report by Wallace et al. (20), indicate that in the fMLP-stimulated neutrophil there is a temporal program of changes in F-actin content as a result of actin polymerization and depolymerization. This sequence of events may be unique to the neutrophil; however, since earlier studies with other cell types used the DNase I inhibition assay to quantify G-actin, early time points could not be assayed and the rapid initial polymerization may have been overlooked (3). The observation of sequentially paired polymerization and depolymerization of actin is confirmed by analysis of CAA in operationally defined cytoskeletons of fMLP-stimulated neutrophils on SDS PAGE. Quantification of the rates of fMLP-induced polymerization and depolymerization reported here mark the first time that the rates of these processes have been determined in vivo.

Parallel changes in the quantity of methanol-extractable NBD-phallacidin in fMLP stimulated cells exclude the possibility that findings with FACS are artifacts generated by change in cell shape or F-actin distribution (19) because the methanol extraction assay is not influenced by cell shape or F-actin distribution. The methanol extraction assay is an inexpensive, rapid method that is applicable to study of cells in suspension or on substratum and is a technique ideally suited for studies of cytoskeletal organization in nonmuscle cells.

Although much is known about polymerization and depolymerization of actin in vitro (9), the intracellular events that modulate the state of actin polymerization in nonmuscle cells are not clearly understood. The observations reported here on the effect of cytochalasin D and the dose dependence of depolymerization suggest directions for future investigation of the molecular mechanisms that control the state of actin polymerization in this nonmuscle cell. Such investigations should consider our observations on the cytochalasin D effect

and the dose dependence of depolymerization as well as two well-established facts: fMLP increases cytosolic Ca^{++} (21, 22), and neutrophils contain actin regulatory proteins— gelsolin, profilin, and acumentin (23–29).

Inhibition of actin polymerization by cytochalasin D indicates that the fMLP-induced polymerization requires monomer addition at the barbed ends of actin filaments, oligomers, or nuclei (30–32). The observation suggests that although fMLP causes a rise in cytosolic Ca^{++} which could activate gelsolin (23, 28, 29) the fMLP-induced polymerization is probably not initiated by Ca^{++} activation of gelsolin because gelsolin-induced polymerization occurs at the pointed end of actin filaments (33). Alternative and testable mechanisms for fMLP-induced actin polymerization in neutrophils include *de novo* creation of nucleating sites, "uncapping" of previously existing nucleating sites, fMLP-induced dissociation of profilin–actin complexes, or "activation" of acumentin. Further studies are necessary to distinguish between these possible mechanisms.

Our observation that the F-actin content at 45 s and the rate of depolymerization are fMLP dose dependent whereas the rate of actin polymerization is not fMLP dose dependent may offer some important insights into the molecular mechanisms that control F-actin content in neutrophils. First, the results suggest that fMLP-induced polymerization and depolymerization may be activated via different intracellular mechanisms. Second, the results suggest that the trigger for fMLP-induced actin polymerization is virtually an all-or-none response to fMLP binding or requires minimal receptor occupancy. Finally, since the extent of polymerization is fMLP dose dependent and is determined by the duration of the polymerization, the results suggest that the molecular events that terminate actin polymerization are fMLP dose dependent. fMLP-dependent termination of actin polymerization could occur because fMLP frees a dose-dependent number of actin monomers from the profilactin pool or because fMLP activates acumentin and/or gelsolin to cap the ends of actin filaments. The possibility that gelsolin might participate in termination of the actin polymerization is particularly attractive since fMLP causes a dose-dependent increase in the cytosolic Ca^{++} concentration in neutrophils (22) and Ca^{++}-activated gelsolin can cap the barbed ends of actin filaments and sever filaments to cause depolymerization of F-actin (28, 33).

Finally, our studies show that concurrent with fMLP-induced changes in F-actin content there are changes in both the distribution of F-actin in the cell and the shape of the cell. Changes in F-actin distribution and cell shape depend upon fMLP dose and the time elapsed after stimulation. 45 s after stimulation, a point at which F-actin content is maximal, F-actin is diffusely distributed throughout the cytoplasm; 10 min after stimulation F-actin is localized to the leading edge of the cell. This result indicates that the focal distribution of F-actin is the result of a diffuse actin polymerization followed by persistence of F-actin in the lamellipodium of the cell and a net decrease in F-actin content. The redistribution of F-actin in the fMLP-stimulated cell could result from translocation of existing F-actin to the lamellipodium, depolymerization of existing F-actin in the uropodial end of the cell, or a combination of these events. Our studies suggest that the redistribution of F-actin is a critical determinant of the shape of the cell. Since Zigmond demonstrated neutrophil immotility at a time when F-actin distribution is diffuse (14), and she and her co-workers (18) and Howard (17), found that polarized cells move more rapidly than rounded cells, it is clear that cell shape is correlated with locomotive behavior of the cell. These changes in the amount and distribution of F-actin within the cell and the observed correlations with the shape and motility of the cell suggest that studies with this system may help elucidate some of the mechanisms that control F-actin content and distribution in nonmuscle cells.

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REFERENCES

1. Stossel, T. P. 1984. Contribution of actin to the structure of the cytoplasmic matrix. *J. Cell Biol.* 99(1, Pt. 2):15s–21s.
2. Howard, T. H., and W. Meyer. 1984. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. *J. Cell Biol.* 98:1265–1271.
3. Carlsson, L., F. Markey, I. Bliksted, T. Persson, and U. Lindberg. 1979. Reorganization of actin in platelets stimulated by thrombin as measured by DNase I inhibition. *Proc. Natl. Acad. Sci. USA.* 76:6376–6380.
4. Cassella, J., M. Flanagan, and S. Lin. 1981. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments during platelet shape change. *Nature (Lond.).* 293:302–305.
5. Lin, S., D. Cribbs, J. Wilkins, W. Magargol, and D. Lin. 1982. Use of cytochalasin-like binding proteins to study actin assembly. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:263–273.
6. Giffard, R. G., J. Spudich, and A. Spudich. Ca^{++}-sensitive isolation of cortical actin matrix from *Dictyostelium* amoebae. *J. Muscle Res. Cell Motil.* 4:115–131.
7. Fechheimer, M., and S. Zigmond. 1983. Changes in cytoskeletal proteins of polymorphonuclear leukocytes induced by chemotactic peptides. *Cell Motil.* 3:349–361.
8. Rao, K. M., and J. Varani. 1982. Actin polymerization induced by chemotactic peptides and conconavalin A in rat neutrophils. *J. Immunol.* 129:1605–1608.
9. Korn, E. D. 1983. Actin polymerization and its regulation by proteins from non-muscle cells. *Physiol. Rev.* 62:672–737.
10. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 97(Suppl. 21):77–85.
11. Seligmann, B., T. Chused, and T. Gallin. 1981. Human neutrophil heterogeneity identified using flow microfluorometry to monitor membrane potential. *J. Clin. Invest.* 68:1125–1131.
12. Barak, L. S., and R. Yocum. 1981. Nitrobenzooxadiazole phallacidin: synthesis of a fluorescent actin probe. *Anal. Biochem.* 110:31–38.
13. Keller, H. U. 1983. Motility, cell shape and locomotion of neutrophil granulocytes. *Cell Motil.* 3:47–60.
14. Zigmond, S., and S. Sullivan. 1979. Sensory adaptation of leukocytes to chemotactic peptides. *J. Cell Biol.* 82:517–527.
15. Senda, N. 1976. The movement of leukocytes. *In* Contractile Systems in Non-muscle Tissues. S. V. Perry, A. Margreth, and R. Adelstein, editors. North-Holland Biomedical Press, Amsterdam. 309–318.
16. Oliver, J., R. Lalchandani, and E. Becker. 1977. Actin redistribution during conconavalin A cap formation in rabbit neutrophils. *J. Reticuloendothel. Soc.* 21:359–364.
17. Howard, T. H. 1982. Quantification of the locomotive behavior of polymorphonuclear leukocytes in clot preparations. *Blood* 59:946–951.
18. Zigmond, S., H. Levitsky, and B. Kreel. 1981. Cell polarity: an examination of its behavioral expression and its consequences for polymorphonuclear chemotaxis. *J. Cell Biol.* 89:585–592.
19. Wheeless, L. L. 1979. Slit scanning and pulse width analysis. *In* Flow Cytometry and Sorting. M. Melamed, P. Mullaney, and M. Mendelsohn, editor. J. Wiley & Sons, New York. 125–135.
20. Wallace, P., R. Wersto, C. Packman, and M. Lichtman. 1984. F-met-leu-phe induces reversible changes in F-actin content of human blood neutrophils. *J. Cell Biol.* 99:1060–1065.
21. Pozzan, T., P. Lew, C. Wollheim, and T. Tsien. 1983. Is cytosolic ionized calcium regulating neutrophil activation? *Science (Wash. DC).* 221:1413–1415.
22. Korchak, H., K. Vienne, and G. Weissmann. 1984. Stimulus response coupling in the human neutrophil. II. Temporal analysis of changes in cytosolic calcium and calcium efflux. *J. Biol. Chem.* 259:4076–4082.
23. Lees, A., J. Haddad, and S. Lin. 1984. Brevin and vitamin D binding protein: comparison of the effects of two serum proteins in actin assembly and disassembly. *Biochemistry* 23:3088–3097.
24. Southwick, F., and J. Hartwig. 1982. Acumentin, a protein in macrophages which caps the "pointed" end of actin filaments. *Nature (Lond.).* 297:303–307.
25. Southwick, F., and T. Stossel. 1981. Isolation of an inhibitor of actin polymerization from human PMN leukocytes. *J. Biol. Chem.* 256:3030–3036.
26. Southwick, F., and T. Stossel. 1983. Contractile proteins in leukocyte function. *Semin. Hematol.* 20:305–321.
27. Yin, H., and T. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature (Lond.).* 281:583–586.
28. Yin, H. L., J. Albrecht, and T. Stossel. 1981. Identification of gelsolin, a Ca^{++} dependent regulating protein of actin gel-sol transformation and its intracellular location in a variety of cells and tissues. *J. Cell Biol.* 91:901–906.
29. Yin, H., K. Zaner, and T. Stossel. 1980. Ca^{++} control of actin gelation. Interactions of gelsolin with actin filaments and regulation of actin gelation. *J. Biol. Chem.* 255:9494–9500.

30. Casella, J., M. Flanagan, and S. Lin. 1983. "Cytochalasins: their use as tools in the investigation of actin polymerization *in vivo* and *in vitro*." *In* Actin: Structure and Function in Muscle and Non-Muscle Cells. Academic Press, Australia.
31. Lin, D., and S. Lin. 1979. Actin polymerization induced by a motility-related high affinity cytochalasin binding complex from human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 76:2345–2349.
32. Flanagan, M., and S. Lin. 1980. Cytochalasins block actin filament elongation by binding to high affinity sites associated with F-actin. *J. Biol. Chem.* 255:835–840.
33. Wang, L., and J. Bryan. 1981. Isolation of calcium-dependent platelet proteins that interact with actin. *Cell.* 25:637–649.