Chemotactic Peptide-induced Changes in Neutrophil Actin Conformation

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ABSTRACT The effect of the chemotatic peptide, *N*-formylmethionylleucylphenylalanine (FMLP), on actin conformation in human neutrophils (PMN) was studied by flow cytometry using fluorescent 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin to quantitate cellular F-actin content. Uptake of NBD-phallacidin by fixed PMN was saturable and inhibited by fluid phase F-actin but not G-actin. Stimulation of PMN by >1 nM FMLP resulted in a dose-dependent and reversible increase in F-actin in 70–95% of PMN by 30 s. The induced increase in F-actin was blocked by 30 μM cytochalasin B or by a t-BOC peptide that competitively inhibits FMLP binding. Under fluorescence microscopy, NBD-phallacidin stained, unstimulated PMN had faint homogeneous cytoplasmic fluorescence while cells exposed to FMLP for 30 s prior to NBD-phallacidin staining had accentuated subcortical fluorescence. In the continued presence of an initial stimulatory dose of FMLP, PMN could respond with increased F-actin content to the addition of an increased concentration of FMLP. Thus, FMLP binding to PMN induces a rapid transient conversion of unpolymerized actin to subcortical F-actin and repetitive stimulation of F-actin formation can be induced by increasing chemoattractant concentration. The directed movement of PMN in response to chemoattractant gradients may require similar rapid reversible changes in actin conformation.

The force for polymorphonuclear neutrophil (PMN) movement is generated by its cytoplasmic microfilament lattice which has as its most prevalent component polymerized actin (1). As neutrophils initiate movement, actin microfilaments increase in number and distribute themselves toward the leading portion of the moving cell (2). For sustained movement to occur, the microfilament lattice must be continuously remodeled. Consequently, actin polymerization and microfilament production are important determinants of cell motility.

Actin comprises 10% of neutrophil cytoplasmic protein (3) and has two interchangeable physical states with widely different polymerization potentials (4, 5). G-actin exists primarily as a monomer due to the high critical concentration required for its polymerization. F-actin has a lower critical polymerization concentration and at the concentration of actin in cytoplasm, oligomers and polymers form. The presence of high concentrations of potassium and magnesium in the cell favors the F-actin conformation (5), whereas, cytoplasmic proteins such as profilin stabilize G-actin (1, 6). Thus, a pool of G-actin monomers exist in equilibrium with F-actin oligomers and polymers. Actin monomers are added to microfilaments only after a change from the G to the F conformation (7–9).

Our studies examined the effect of the chemoattractant molecule *N*-formylmethionylleucylphenylalanine (FMLP) on neutrophil actin content and distribution. To accomplish this, PMN were exposed to a fluorescent derivative of an acidic phallotoxin, 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin (10), which binds with high affinity to F-actin but not G-actin. Flow cytometry permitted us to quantitate F-actin in large numbers of cells and to observe separate subsets of the cell response to FMLP.

[1] *Abbreviations in this paper:* FMLP, *N*-formylmethionylleucylphenylalanine; NBD, 7-nitrobenz-2-oxa-diazole; PMN, polymorphonuclear neutrophil.

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MATERIALS AND METHODS

Preparation of Neutrophils (PMN): Leukocyte-rich plasma was separated from heparinized human blood by dextran sedimentation of erythrocytes. The plasma was centrifuged on a Ficollpaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient and the lymphocytes, monocytes, and platelets were removed. The remaining granulocyte-erythrocyte fraction was lysed with hypotonic saline. The resulting cell suspension was 95% or greater PMN and 95% of cells were viable by propidium iodide exclusion studies. Cells were suspended at 5×10^6/ml in isotonic PBS pH 7.4 and kept at room temperature. Before and after addition of FMLP and other agents (see below), aliquots of 1×10^6 PMN were fixed by their addition to 1 ml 3.2% paraformaldehyde in PBS at 4°C. Fixed cells were kept at 4°C in paraformaldehyde for 48 to 72 h.

Fluorescence Staining for Flow Cytometry: A stock solution of 3 μg/ml NBD-phallacidin (Molecular Probes, Junction City, OR) in methanol was diluted in PBS for use. Unstimulated paraformaldehyde-fixed PMN were washed twice in PBS/0.1% BSA and then incubated with NBD-phallacidin solution in the dark. This and all subsequent procedures were performed at room temperature unless otherwise noted. NBD-phallacidin uptake by PMN, measured as fluorescence emission by flow cytometry, was saturated following incubation for 30 min at a concentration of 0.3 μg/ml NBD-phallacidin. In subsequent experiments cells were fixed for 48–72 h and incubated with 0.6 μg/ml NBD-phallacidin for 30 min unless otherwise noted. In some experiments, PMN were stained for fluorescent measurement of total cellular immunoreactive actin by successively incubating paraformaldehyde-fixed PMN for 20 min in 95% ethanol, 1 h in rabbit antiactin antisera (Miles Laboratories, Elkhart, IN), and 1 h in FITC-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). NBD-phallacidin and antibody-treated cells were suspended in 0.1% BSA-PBS at a density of 2×10^6 PMN/ml for assay in the flow cytometer.

Measurement of Fluorescence by Flow Cytometry: Fluorescence histograms of unstained and stained PMN's were determined at 4°C using an EPICS V flow cytometer cell sorter (Coulter Electronics, Hialeah, FL) interfaced to a Coulter MDADS data acquisition system. Fluorescence was excited at 488 nm with 500 mW laser output and detected using a 510 nm interference filter in combination with a 515-nm glass long pass filter. Histograms of cell number versus log fluorescence intensity and forward angle light scatter were recorded for a minimum of 25,000 cells per sample. The mean and mode channel numbers, coefficient of variation, and standard deviation of the log fluorescence intensity distributions were analyzed using ROMP (Rochester multi-parameter data analysis, developed by Drs. James Leary and Roy Robinson, University of Rochester, Rochester, NY) on a Terak 11/23 system. Subsequently, the log fluorescence intensity means were converted to a linear scale using a log-linear calibration method (11) and normalized with respect to the mean fluorescence intensity of unstimulated PMN. Polystyrene spheres (10-μm diam, ⅛ Brights) (Fine Particle Division of Coulter Electronics, Hialeah, FL) were used for calibration and normalization of the flow cytometer from experiment to experiment. All experiments were performed three or more times.

Specificity of NBD-Phallacidin for Actin Conformation: Rabbit skeletal muscle actin (Sigma Chemical Co., St. Louis, MO) was dissolved at a concentration of 0.5–1 mg/ml in buffer with 5 mM Tris pH 7.4 and 1 mM ATP (G-buffer). In G-buffer, actin assumes the G conformation. To remove nonmonomeric actin and other contaminants, this solution was centrifuged at 80,000 *g* for 3 h at 4°C and the supernatant recovered. Actin concentration was measured before and after centrifugation by absorbance at 280 nM using an extinction coefficient of 1.11 for G-actin in G-buffer. Centrifugation decreased the apparent concentration of actin by 20 to 30%. This G-actin solution was used immediately or frozen at −20°C until use. Centrifuge-purified G-actin in G-buffer was converted to F-actin by addition of 100 μmol KCl and 1 μmol $MgCl_2$ per ml. F-buffer was prepared from G-buffer by addition of 100 μmol KCl and 1 μmol $MgCl_2$ per ml.

NBD-phallacidin was preincubated at a concentration of 0.15 μg/ml in G-actin solution, F-actin solution, G-buffer, or F-buffer 30 min before addition to fixed, pelleted PMN for an additional 30 min. PMN were subsequently washed twice, suspended to 2×10^6 cells/ml, and fluorescence measured by flow cytometry. An autofluorescence control of fixed, washed, unstimulated PMN incubated in F-buffer without NBD-phallacidin was also studied.

NBD-phallacidin, at a concentration of 0.15 μg/ml, was incubated with 0.5 mg/ml F-actin in F-buffer and in F-buffer alone for 30 min. Aliquots of these suspensions were either centrifuged at 80,000 *g* for 3 h at 4°C or kept at 4°C for 3 h. These solutions were assayed on a spectrofluorometer for fluorescence emission at wavelengths >515 nm using a peak excitation wavelength of 488 nm.

Stimulation by FMLP: A 1-mM stock solution of FMLP in dimethyl sulfoxide was prepared and diluted further in PBS so that addition of 10 μl of FMLP solution to 1 ml of PMN suspension yielded a final FMLP concentration of 1 nM to 1 μM. The final concentration of dimethyl sulfoxide was 0.1 ml/100 ml or less. Immediately before and at appropriate intervals after FMLP addition, a 200-μl aliquot of the cell suspension was removed and fixed by rapid addition of 1 ml of 3.2% paraformaldehyde in an ice bath.

In some experiments, PMN suspensions were incubated 30 min with either 30 μM cytochalasin B (Sigma) or t-BOC-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe (t-BOC) (Vega Biochemicals) at 1 and 10 μM prior to addition of 10 nM FMLP.

Repetitive Stimulation: FMLP was added to a PMN suspension to a final concentration of 10 nM and aliquots of cells were fixed at 0 min, 15 s, 30 s, 1 min, 3 min, and 5 min after addition. At 5 min after initial stimulation, the concentration of FMLP was abruptly increased to 100 nM and aliquots of PMN so treated were fixed at similar intervals.

In other experiments, the concentration of FMLP was increased at 1 min intervals successively to 1, 3, 10, 30, and 100 nM after which PMN aliquots were removed and fixed at 15 s, 30 s, and 1 min following each dose increment. Additional samples of PMN were fixed at 3 min and 5 min after the final FMLP increment.

Fluorescence Microscopy: PMN fixed at intervals after addition of 100 nM FMLP were washed twice in PBS, stained 30 min with 0.6 μg/ml NBD-phallacidin, rewashed twice in PBS, and suspended in 50% glycerol–PBS at a cell density of 5×10^6/ml. One drop of this suspension on a glass slide was covered with a #1 glass coverslip and observed in a Nikon Fluophot fluorescence microscope. Photographs were taken with Kodak Tri-X film at ASA 1600.

RESULTS

Specificity of NBD-Phallacidin Staining for F-Actin

To confirm that NBD-phallacidin reacted preferentially with F-actin, unstimulated PMN were incubated with NBD-phallacidin that had been previously incubated with G-actin or F-actin or with their respective buffers. Preincubation of NBD-phallacidin with F-actin eliminated subsequent staining and PMN fluorescence was identical to autofluorescence of unstained cells (Fig. 1 *a*). In contrast, PMN stained with NBD-phallacidin preincubated with G-actin had a profound increase in fluorescence intensity (Fig. 1 *c*). PMN stained with NBD-phallacidin preincubated in the buffers used to suspend either G-actin or F-actin, had a similar marked increase in fluorescence intensity (Fig. 1, *b* and *d*). As further confirmation of the binding of NBD-phallacidin to F-actin, centrifugation of a solution of NBD-phallacidin and F-actin at a force adequate to pellet F-actin oligomers and polymers removed 90% of fluorescent emission from the supernatant. The fluorescence emission of NBD-phallacidin in F-buffer, G-buffer, or G-actin suspension was not reduced by similar centrifugation.

Change in F-Actin in Response to FMLP Stimulation

Fluorescence emission of single cells after NBD-phallacidin staining was recorded as a measure of the F-actin content of individual PMN. For a population of unstimulated PMN, a plot of cell number versus log F-actin content formed a single bell-shaped peak with slight negative skewness. In each experiment, the mean F-actin content of unstimulated cells was determined from this distribution and used as a reference value for subsequent observations (Fig. 2 *a*).

At 30 s after addition of 100 nM FMLP, a change in the shape of the log F-actin distribution plot was seen (Fig. 2 *b*). Two components of the plot were present, a narrow peak of cells with higher F-actin content and a broader tail of cells with lower levels of F-actin. This appearance is explained by two overlapping populations of cells, one population with F-actin content similar to unstimulated cells and a second larger population with F-actin present in increased amounts. A symmetric distribution for the narrow peak of cells was extrapolated and its mean value determined as representative of the F-actin content of responding cells. The cells remaining outside the extrapolated responding peak were quantitated to approximate the nonresponding subset of PMN. At 30 s after

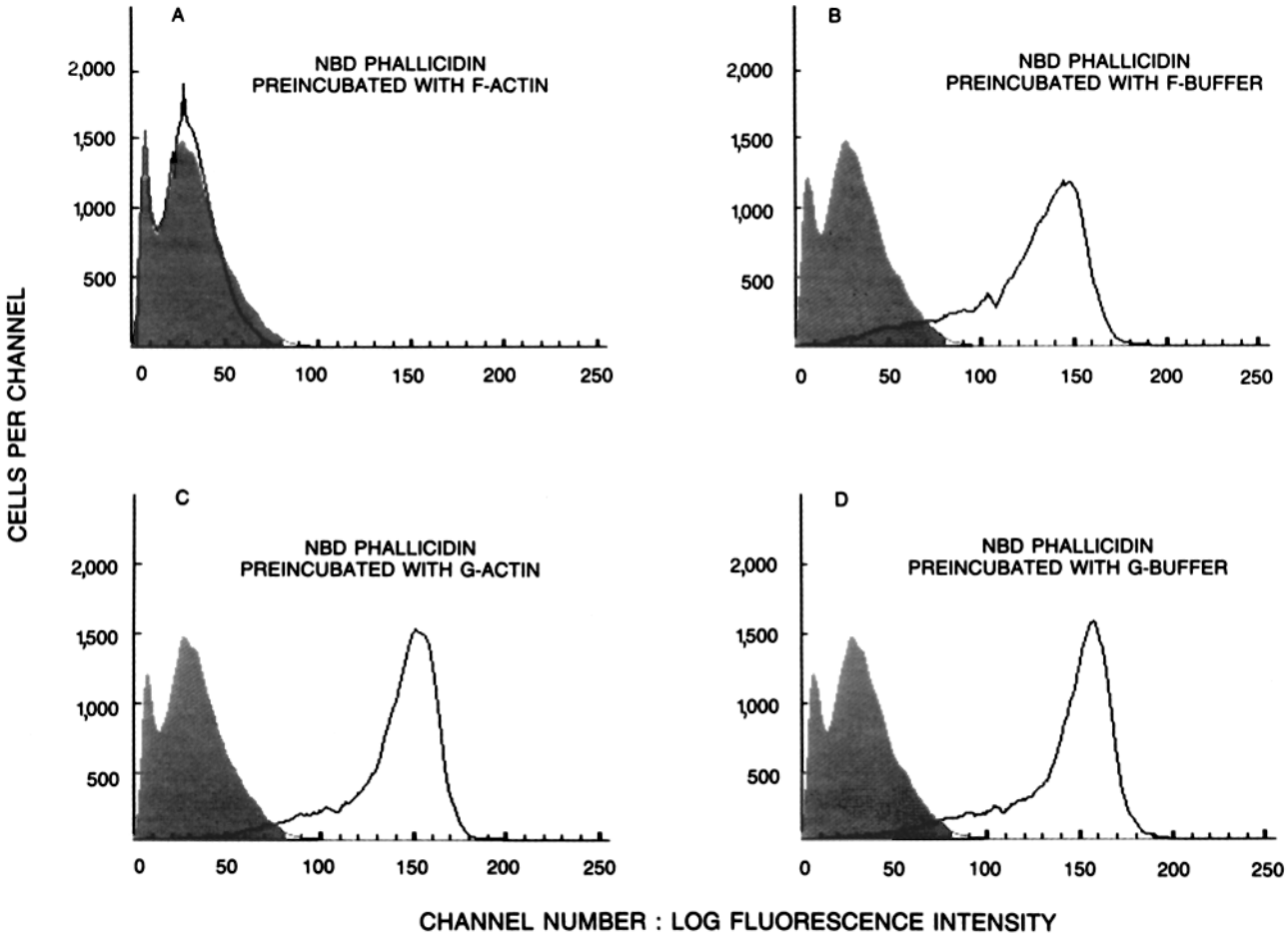

FIGURE 1 Inhibition of NBD-phallacidin staining by fluid phase actin. Unstimulated, paraformaldehyde-fixed PMN were incubated with NBD-phallacidin in the presence of (*A*) fluid phase F-actin, (*B*) F-buffer, (C) fluid phase G-actin, and (*D*) G-buffer. The distribution of log fluorescence intensity of 25,000 cells in each sample, as measured by flow cytometry, is shown. For comparison, PMN autofluorescence is indicated by the crosshatched area. Peak positions were determined to be: autofluorescence-channel 30 (*A–D*), F-actin channel 32 (*A*), F-buffer-channel 146 (*B*), G-actin-channel 150 (C), and G-buffer-channel 156 (*D*). A change in log fluorescence of 25 channel numbers correspond to a twofold change in fluorescence emission while a change in log fluorescence of 83 channel numbers correspond to a tenfold change in fluorescence emission.

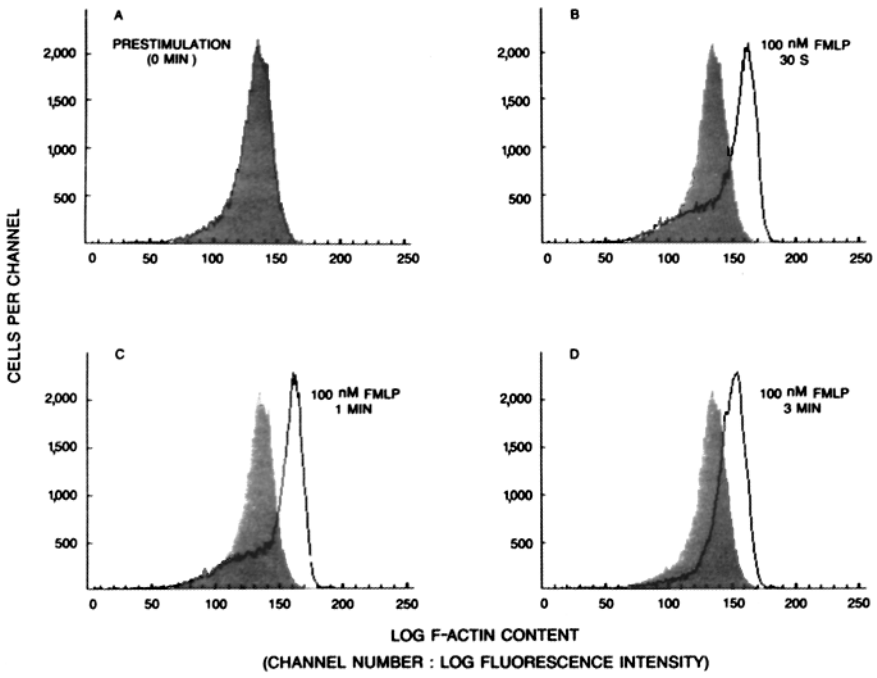

FIGURE 2 Effect of FMLP on log F-actin content. The distribution of the log F-actin content of unstimulated PMN, measured as the log fluorescence emission of 25,000 cells, is shown as the crosshatched area in *A–D*. The distribution of log F-actin content in this representative experiment at (*B*) 30 s, (*C*) 1 min, and (*D*) 3 min after addition of 100 nM FMLP is superimposed. Peak positions were determined, as described in the text, at 0 min-channel 128 (*A*), 15 s-channel 151 (not shown), 30 s-channel 161 (*B*), 1 min-channel 157 (*C*), 3 min-channel 150 (*D*), and 5 min-channel 147 (not shown). A change in position of the log fluorescence peak of 25 channel numbers corresponds to a twofold change in fluorescence emission and a doubling of F-actin content.

exposure to 100 nM FMLP, 70–95% of PMN were in the responding peak.

The mean F-actin content of responding cells reached a maximum value at 30 s and subsequently returned progressively toward baseline level at 1 and 3 min (Fig. 2, *c* and *d*). The number of apparently nonresponsive cells remained constant through the first minute after FMLP addition. However, in some experiments a subsequent small shift of these cells to higher F-actin content was seen at 3 and 5 min after FMLP addition suggesting a delay in onset and smaller magnitude of response in this subset. (Fig. 2*d*).

As shown in Fig. 3, a 33% increase in F-actin content was stimulated by 1 nM FMLP. 10 nm FMLP increased F-actin content to 200% of control while greater concentrations of FMLP resulted in a maximal increase to 220% of control unstimulated level by 30 s. F-actin content began to decrease at 1 min in maximally stimulated samples and fell progressively to 130–160% of unstimulated levels by 5 min. This level was maintained through 10-min poststimulation. The total immunoreactive actin content of PMN, which includes both G and F actin, was unchanged following FMLP stimulation (data not shown).

Inhibition by Cytochalasin B and the t-BOC Peptide

The increase in F-actin was blocked entirely by 30 μM cytochalasin B and was inhibited in a dose-dependent manner by the t-BOC peptide, a competitive inhibitor of the FMLP receptor (Fig. 4). One volume percent dimethyl sulfoxide, the solvent for FMLP and t-BOC did not alter F-actin content. Similarly, 30 μg/ml cytochalasin B and the t-BOC peptide in the absence of FMLP did not alter F-actin content.

Repetitive Stimulation

To observe the PMN response to progressive increments in chemoattractant concentration, cell suspensions were stimulated initially by 10 nM FMLP followed in 5 min by 100 nM FMLP. Following the initial stimulus, a typical maximal increase and subsequent decrease in F-actin occurred. After the second higher stimulus, F-actin content returned to its previous peak level in 15 s and then declined rapidly toward baseline within 3 additional minutes (data not shown).

Similarly, successive increases in FMLP concentration to 1, 3, and 10 nM at 1-min intervals resulted in a dose-related increase in F-actin (Fig. 5). Subsequent increments in FMLP

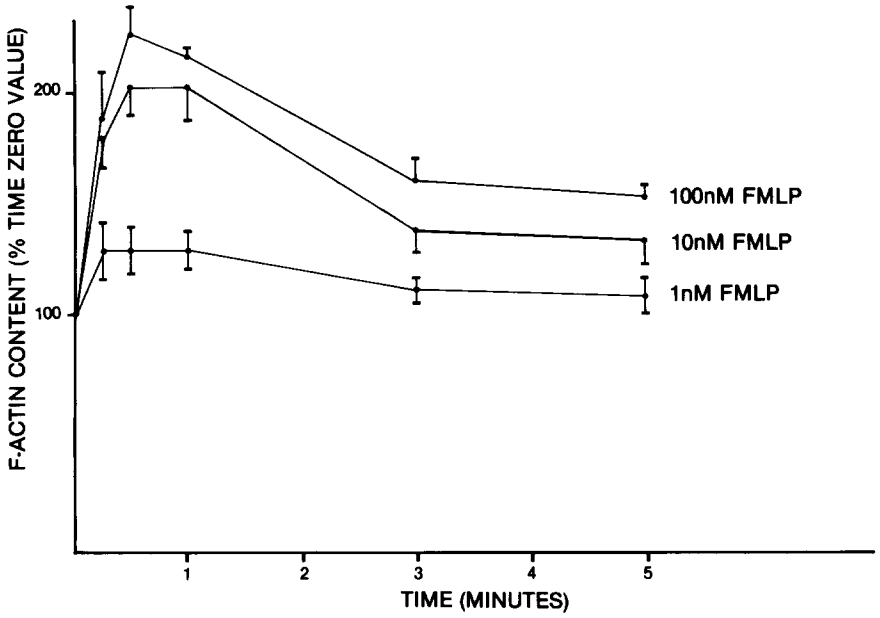

FIGURE 3 Effect of FMLP dose on PMN F-actin content. The F-actin content of PMN is shown at intervals following the addition of 1, 10, and 100 nM FMLP. F-actin content is expressed as percent of time zero value in unstimulated cells. Bars represent the standard error of the mean.

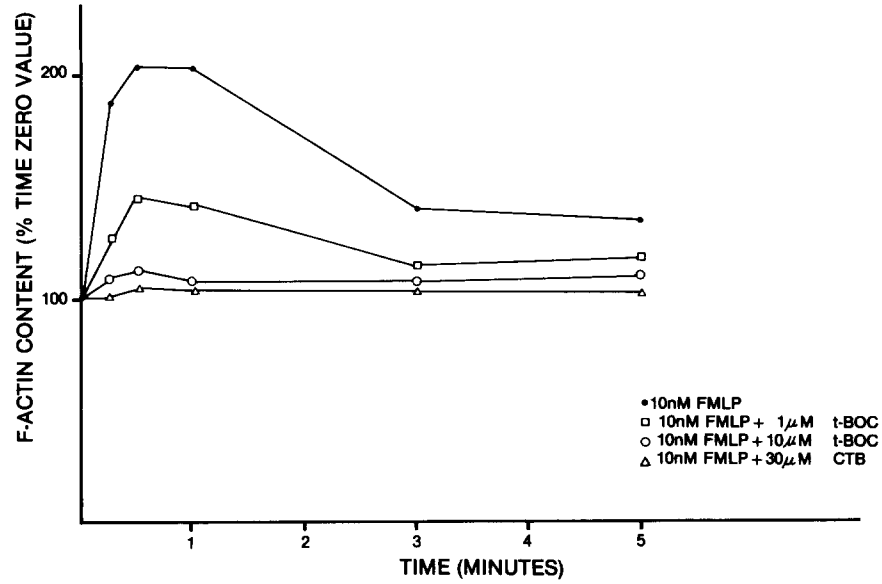

FIGURE 4 Inhibition of the FMLP-induced increase in PMN F-actin by t-BOC and cytochalasin B. The F-actin content of PMN preincubated in PBS (●), 1 μM t-BOC (□), 10 μM t-BOC (○), and 30 μM cytochalasin (△) is shown at intervals following addition of 10 nM FMLP. F-actin content is expressed as percent of time zero value.

concentration between 10 and 100 μM at 1-min intervals resulted repeatedly in a rapid peak in F-actin content 15 s after a dose increase. By 1 min after addition of each increment of FMLP, F-actin had begun to decrease and following the addition of the final stimulatory dose, F-actin content fell approaching prestimulation baseline within 5 min.

Fluorescence Microscopy

Coincident with changes in F-actin content following FMLP stimulation, PMN exhibited changes in the pattern of NBD-phallacidin staining as observed by fluorescence microscopy (Fig. 6). Prior to stimulation (Fig. 6*a*), cells were round with fluorescence homogeneously distributed in cytoplasm. The nucleus appeared as an unstained structure in many cells and around 20–40% of unstimulated cells had a thin, incomplete subcortical rim of more prominent fluorescence. At 15 s after stimulation with 100 nM FMLP, a concentration shown to give a maximal increment in F-actin, the majority of cells remained round and exhibited markedly enhanced staining of the submembraneous cytoplasm in a ring pattern (Fig. 6*b*), which became more pronounced by 30 s (Fig. 6*c*). At 1 min (Fig. 6*d*), subcortical fluorescence remained prominent but irregularity of the cell outline was evident. By 3 min (Fig. 6*e*), cell shape was polarized and fluorescence in many cells was localized in one pole. By 5 min, the polarized shape was less pronounced and distribution of staining more diffuse. A proportion of cells retained prominent areas of fluorescence and polarized shape at 10 min (Fig. 6*f*).

DISCUSSION

G-actin content in a variety of cells has been analyzed by measuring cell lysates for DNAse I inhibitory activity, a property of G-actin (12). A decrease in cytoplasmic G-actin has been shown to follow platelet activation by thrombin (13) and thymocyte capping induced by antibody binding (14). More recently, PMN stimulation by FMLP and concanavalin A has been shown to rapidly decrease cellular G-actin, presumably due to conversion of G- to F-actin (15, 16). A subsequent return of actin to the G-conformation with con-

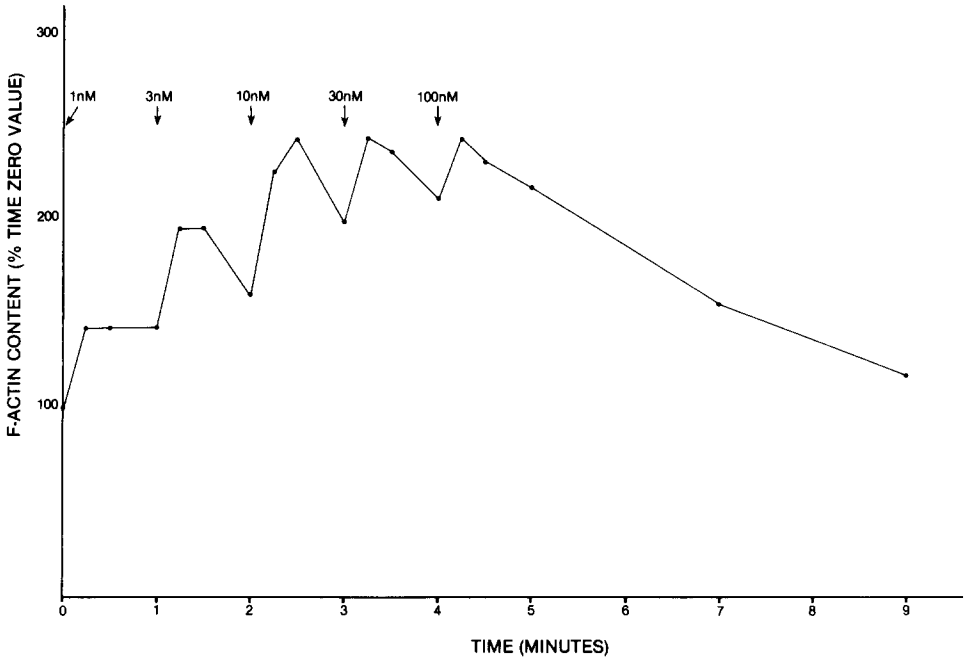

FIGURE 5 Repetitive stimulation: the effect of increasing concentrations of FMLP on PMN F-actin content. PMN were exposed to increments in FMLP concentration at 1-min intervals. Aliquots were removed at intervals following each dose addition for determination of F-actin content (expressed as percent time zero value). The concentration of FMLP present at a given time is shown by arrows (↓).

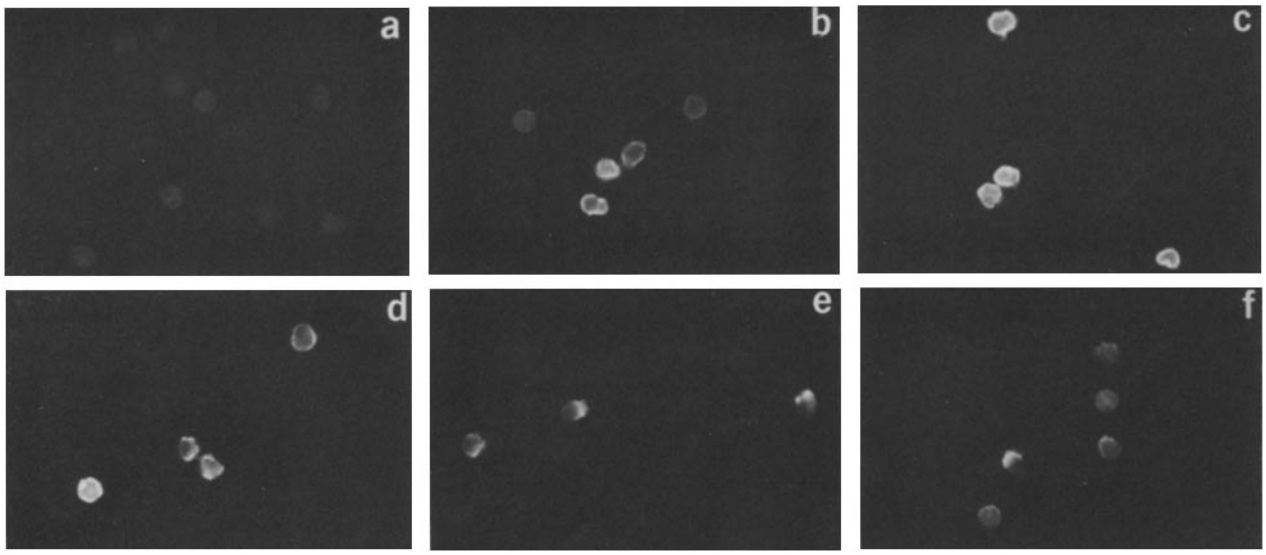

FIGURE 6 Fluorescent photomicrographs of FMLP-stimulated PMN in suspension stained with NBD-phallacidin. Cells are shown prior to stimulation (*A*), and at 15 s (*B*), 30 s (C), 1 min (*D*), 3 min (*E*), and 10 min (*F*) after addition of 100 nM FMLP.

tinued chemoattractant exposure was not seen in these experiments. Since the DNAse I inhibition assay requires preparation of lysates, observation of component subpopulations of cells was not possible.

Our experiments show rapid, reversible increases in subcortical F-actin in human neutrophils following stimulation by FMLP. The timing of these changes in F-actin correlates with the time course of rapid PMN shape change following FMLP stimulation, as observed by phase and electron microscopy. The abrupt addition of FMLP to PMN randomly migrating on a glass surface causes rapid cessation of motion, cell rounding and diffuse membrane ruffling followed within minutes by resumption of random migration despite the continued presence of chemoattractant (17). Similarly, suspended PMN respond by changing shape when abruptly exposed to FMLP (18).

When PMN were exposed to >10 nM FMLP, a maximal increase in F-actin to 220% of the content of unstimulated cells, or a 120% increase above resting level, was seen. This maximal response may represent essentially complete, temporary conversion of the cytoplasmic pool of G-actin to the F conformation. This is a consistent with DNAse I inhibition studies of unstimulated PMN in which the pool of actin in the G state and potentially available for conversion was 150% of the non-G, and presumably F component of cytoplasmic actin (15).

The decline in NBD-phallacidin staining from peak levels poststimulation appears to represent a reversal in actin conformation from F to G state and not an alteration of phallacidin binding sites due to effects of cell activation such as protein oxidation. As evidence for this interpretation restimulation of cells by an increased FMLP dose consistently returned NBD-phallacidin uptake to the same or higher level as seen with preceding stimuli.

The observed response of cytoplasmic actin is mediated through the formyl peptide receptor. The FMLP dose response of F-actin polymerization and inhibition of response by the t-BOC peptide is consistent with the known affinities of this receptor (19, 20). Also, the reversibility of response and capacity for restimulation by higher doses of FMLP agree with the postulated potential for receptor adaptation or the existence of receptor populations of varying affinity (21, 22).

The functional capabilities of circulating PMN are heterogeneous. A subset of cells, in contrast to the majority of circulating PMN, only weakly express the receptor for the Fc portion of IgG, adhere poorly to surfaces, and have diminished chemoattractant responsiveness (23). Further, while most cells undergo membrane depolarization rapidly after FMLP binding, 10–30% of PMN fail to depolarize or hyperpolarize when similarly exposed (24). In our experiments, up to 30% of PMN failed to respond to FMLP with a rapid increase in F-actin content. In several experiments, a small proportion of cells had a transient decrease in F-actin following FMLP stimulation. This bimodal response parallels the other heterogeneous responses of PMN. It is not clear if these subpopulations represent (*a*) cells damaged in the course of separation and study, (*b*) different stages in PMN functional maturation, or (*c*) mature but functionally disparate cohorts of cells.

Cytochalasin B, which completely abolishes PMN random cell motion, surface ruffling activity, chemotaxis, and phagocytosis (25) has been shown in vitro to block polymerization of actin solutions by inhibiting addition of monomers to established polymerization nuclei (26, 27). The mechanism of cytochalasin B induced inhibition of cellular motility is less well defined. In our experiments, cytochalasin B abolished the increase in F-actin following FMLP stimulation implicating either direct or indirect inhibition of actin conformational changes as an end result of its presence.

For neutrophils in a chemoattractant gradient, the rate of concentration change is less abrupt than in our studies. As little as a 1% concentration difference across the cell surface has been proposed as adequate to stimulate cell migration (28). Consequently, the capability of the PMN to modulate microfilament formation appears highly sensitive and finely tuned. Rapid changes in actin molecular conformation allowing microfilament assembly and disassembly may be an important component of many forms of cell movement.

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