Oscillating Actin Polymerization/Depolymerization Responses in Human Polymorphonuclear Leukocytes*

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Leukotriene B₄ and platelet-activating factor induced a rapidly oscillating actin polymerization/depolymerization response in human polymorphonuclear leukocytes. N-Formylpeptides were deficient in the ability to induce these oscillations. Flow cytometric analysis of filamentous actin verified that all cells were synchronously responding in this cyclic manner. The hypothesis was tested that these oscillations were analogous to chemical oscillations, i.e. oscillations of intermediate species in chemical systems that are far from equilibrium (Epstein, I. R., Kustin, K., DeKepper, P., and Orban, M. (1983) Sci. Am. 248, 112). Actin polymerization/depolymerization cycles were terminated by adding receptor antagonist a few seconds after initiation of the response by agonists. Thus the oscillations did not represent chemical oscillations that hypothetically could result from a rapid jump of the intracellular milieu to a state far from equilibrium. Rather, continued occupancy of receptors and/or occupancy of new receptors was required to sustain the oscillations. This suggested that the oscillations resulted from regulated polymerization and depolymerization pathways. In simultaneous measurements of actin-associated right angle light scatter and intracellular calcium, no calcium oscillations were detected. Thus, cycles of actin polymerization/depolymerization were not regulated by calcium oscillations.

Periodicity is a fundamental property of life. Circadian rhythms provide an example of oscillations on a macroscopic scale. On a microscopic scale, oscillations of cellular and subcellular functions have been observed. For example, oscillatory movement of chromosomes occurs during mitosis (1, 2). Recent attention has focused on oscillations in intracellular calcium levels that are thought to be important as intracellular signalling mechanisms (Ref. 3 and references therein). In broken cell systems, glycolytic pathways exhibit oscillatory behavior (4). In addition, microtubules in solution oscillate between oligomeric and microtubular states (5). Indeed, chemical reactions devoid of biological systems can oscillate. In these chemical systems, oscillation is achieved in the coupling of intermediate species of systems that are far from equilibrium (Ref. 6 and references therein). We here describe a new system in which oscillations of actin polymerization and depolymerization are observed in human neutrophils activated with specific chemoattractants.

Polymorphonuclear leukocytes (PMNs) are important migratory cells that contribute to tissue injury and inflammation. Migration is accompanied by dramatic rearrangement of the cytoskeletal structure. We have been studying chemoattractant-induced cytoskeletal activation of human PMNs in suspension. These cells exhibit a transient response to numerous chemoattractants such as leukotriene B₄ (LTB₄), N-formylpeptides, platelet-activating factor (PAF), and the complement component C₅a, in which there is a rapid conversion of monomeric actin (G-actin) to filamentous actin (F-actin) followed by depolymerization of the F-actin to near basal levels (7). This actin polymerization response has been shown to correlate with a right angle light scatter assay (8). This real-time spectroscopic assay permits observation of actin polymerization with time resolution of 1 s or less. Studies utilizing N-formylpeptide as stimulus have indicated that this actin polymerization response correlates with cell locomotion (9, 10). Thus this appears to be related to the force-generating component of chemotaxis.

In our studies of LTB₄- and PAF-induced PMN activation, we frequently have noticed that the right angle light scatter response was oscillatory in nature. This occurred without any effort to synchronize the cells in advance. In this communication we characterize the nature of this oscillatory behavior. We confirm that the right angle light scatter oscillations correlate with actin polymerization/depolymerization. Continued occupancy of receptors is required to maintain the oscillations, thus the underlying mechanism is not analogous to chemical oscillations of intermediate reactants in systems far from equilibrium. There is no correlation of actin oscillations and intracellular calcium oscillation, thus the cytoskeletal oscillations are not regulated by calcium oscillations.

MATERIALS AND METHODS

PMN Preparation—Healthy donors were recruited, consent obtained, and blood drawn in accordance with protocols approved by the Human Subjects Review Committee of the Institutional Review Boards of Scripps Clinic and Research Foundation and the University of Michigan. Human neutrophils were purified by counterflow centrifugation as follows (11). Freshly drawn blood was anticoagulated with acid citrate dextrose and red cells sedimented from the white cells in 1.65% gelatin. The PMNs were purified from this white cell preparation by counterflow elutriation yielding granulocytes of >98% purity.

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‡ The abbreviations used are: PMN, polymorphonuclear leukocyte; FLEPP, fluorescein-labeled N-formyl-norleucyl-leucyl-phenylalanine; N-formyl-norleucyl-leucyl-phenylalanine; FMLP, N-formyl-methionyl-leucyl-phenylalanine; LTB₄, leukotriene B₄; PAF, platelet-activating factor; PMA, phorbol myristate acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NBD, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl).
Oscillation of Actin Polymerization/Depolymerization in PMNs

Purity. The purified cells were resuspended in buffer containing 10 mM HEPES, 5 mM KCl, 1.9 mM KH₂PO₄, 1.1 mM Na₂HPO₄, 5.5 mM glucose, 1.5 mM CaCl₂, 0.3 mM MgSO₄, 1 mM MgCl₂ and 147 mM NaCl, at pH 7.4, and kept on ice until assayed. Assays were performed at 37°C after equilibration for 5 min at 37°C in buffer plus 1.5 mM CaCl₂.

Right Angle Light Scatter Assay—Measurements were performed at 37°C on an SLM8000C spectrophotometer with excitation and emission monochromators at 340 nm. The cells were in a stirred suspension at 2 x 10⁶ cells/ml.

Phalloidin Assay for F-actin—The right angle light scatter and F-actin assays were performed simultaneously (8) by spectroscopically monitoring right angle light scatter of a stirred suspension of cells at 4 x 10⁶ cells/ml while aliquots were rapidly removed from the sample and fixed in 3.7% formaldehyde. These fixed samples were then permeabilized and stained for F-actin by addition of a mixture that yielded final concentrations of 3.7% formaldehyde, 0.2 mg/ml lysosome, 165 nM NBD-phallacidin or rhodamine phalloidin. NBD-phallotoxin labeled cells were analyzed with a Becton-Dickinson FACScan, and rhodamine-phalloidin-labeled cells were analyzed with a Becton-Dickinson FACSV. Fluorescence histograms were collected for 5000 cells and the average of bound phallotoxin calculated as the mean fluorescence channel number. F-actin was plotted as the amount of fluorescence relative to unstimulated cells.

Simultaneous Right Angle Light Scatter and Cytosolic Calcium—Intracellular calcium was monitored utilizing Indo-1 (12) as previously described (13). Cells were labeled with Indo-1 and fluorescence at 400 nm (to detect calcium-bound Indo-1) and 340 nm (right angle light scatter) observed simultaneously with excitation at 340 nm.

Reagents—PAF was obtained from Sigma. LT₄ was from Biomol Research laboratories, Inc. Plymouth Meeting, PA. Fluorescently labeled phallotoxins, FLPEP, and Indo-1-acetoxyethyl ester were from Molecular Probes, Inc., Eugene, OR. The PAF antagonist L-695,984 (14) was a gift from J. C. Chabala, Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ. The LT₄ antagonist LY-223882 (15, 16) was a gift from W. W. Jackson, Lilly Research Laboratories, Indianapolis, IN.

RESULTS AND DISCUSSION

Fig. 1 shows an experiment with PAF and LT₄ where 3-4 minima in the right angle light scatter signal were seen. In contrast, N-formylpeptides were deficient in the ability to stimulate this rapidly oscillating pattern. In some donors, the oscillatory behavior was more pronounced than others. Routinely, 2-4 cycles were seen with LT₄ and PAF stimulation. The oscillations were rapid, consistently occurring at intervals of 8-10 s at 37°C, and were observed at doses of stimuli that resulted in maximal stimulation of the response. The frequency of oscillations was temperature-dependent and did not significantly differ for PAF and LT₄. Activation energies (in parentheses are correlation coefficients for combined data from n donors) were 67.4 kJ/mol⁻¹ (0.964, n = 3) for PAF and 69.4 kJ/mol⁻¹ (0.984, n = 4) for LT₄.

Most previous studies of actin polymerization in these cells have utilized methods that require lysing and/or fixing the cells to stop the response. The extent of polymerization is then quantified by determining actin associated with Triton X-100-insoluble cytoskeleton (17, 18), measuring DNase I inhibition by G-actin (19), or by using fluorescence methods to measure the amount of fluorescent phallotoxin, a compound that binds specifically to F-actin in the cell (9). These methods in general limit the time resolution to 5-10 s since single aliquots must be removed by hand for fixation. Probably because of this lower inherent time resolution, LT₄- and PAF-induced oscillations of actin polymerization have not been detected in previous studies utilizing such methods (20, 21).

Although it was difficult to detect the PAF- and LT₄-induced oscillations by these conventional methods, we felt it was important to verify that the oscillations were due to actin polymerization/depolymerization reactions since under certain conditions the right angle light scatter may reflect degranulation or other events (22). Thus, we repeatedly removed and fixed aliquots of cells to obtain closely spaced time points after stimulation, permeabilized and stained the cells with fluorescent phallotoxin, and measured the bound fluorescent phallotoxin utilizing flow cytometry (8, 9). There was excellent correlation between right angle light scatter and actin polymerization under these conditions (Fig. 2a). Thus the flow cytometric assay of F-actin content confirmed that the oscillations of the right angle light scatter response represented actin polymerization/depolymerization cycles. With N-formylpeptide stimulation, the actin assay paralleled the right angle light scatter assay that did not display the oscillatory behavior (7, 8, 20; Fig. 2b).

Analysis of the fluorescence histograms obtained by flow cytometry provided further insight into the characteristics of the response. For example, the experiment of Fig. 2b showed that, following stimulation by LT₄, there was a clear shift of all of the cells to higher F-actin content at 5 s, a shift of all cells to near-resting levels at 12 s, and then an increase at 19 s. Thus, the oscillations could not be attributed to subpopulations of cells that were responding with different kinetics. The average fluorescence intensity for all cells in the histogram was represented by the mean channel number, and the variance of the mean of the histogram (CV) gave a measure of the heterogeneity of the cell population. Five seconds after stimulation with LT₄, when the mean channel number was at a maximum, the CV was the same as for unstimulated cells (unstimulated = 15.5%, stimulated = 15.3%). Thereafter, the CVs increased and peaked at ≈30 s (20.7%). This implied that during the first 5 s and the first peak of polymerization, the cells were highly synchronized. At later times the oscillations of individual cells may have lost synchrony, hence the CVs of the histograms increased. This may have contributed in part to the eventual dampening of the oscillations along with recovery of the response in general. This was in contrast to
Oscillation of Actin Polymerization/Depolymerization in PMNs

**FIG. 2.** a, Correlation of right angle light scatter and F-actin in response to 10 nM LTB, and 100 nM PAF. Changes in F-actin were measured as binding of NBD-phallacidin (PAF data) or rhodamine phalloidin (LTB, data) as described under “Materials and Methods.” NBD-phallacidin-labeled cells were analyzed with a Becton-Dickinson FACscan, and rhodamine-phallacidin labeled cells were analyzed with a Becton-Dickinson FACS IV. Fluorescence histograms were collected for 5000 cells and the average of bound phallotoxin calculated as the mean fluorescence channel number. F-actin was plotted as the amount of fluorescence relative to unstimulated cells. The F-actin data are compilations of data points obtained from triplicate (PAF) or duplicate (LTB,) determinations from one donor. For right angle light scatter, triplicate determinations for PAF are averaged, whereas duplicates of the LTB, data are shown to demonstrate reproducibility. The data are representative of at least three different donors. b, fluorescence histograms of rhodamine-phalloidin-stained cells after stimulation by 1 nM FLPEP and 10 nM LTB, Aliquots of cells were fixed and stained with rhodamine-phalloidin and analyzed as described in a. The fluorescence channel number was linearly proportional to the fluorescence intensity per cell.

FLPEP stimulation. FLPEP did not induce rapid oscillations; while the CVs did not change dramatically over the first 30 s, they increased at later times (19.7% after 2 min of stimulation).

Since stimulation of PMNs results in rapid changes in the intracellular milieu (for example, intracellular calcium levels rise from ~100 nM to >1 μM within 2–3 s), we hypothesized that the mechanism underlying this oscillatory behavior might reflect oscillations of intermediate biochemical species (i.e. cytoskeletal components) in a system that has been shifted far from the equilibrium of the resting state. If this were true, we reasoned that stopping the input of activation signal 2–5 s after stimulation would not alter the oscillations. Thus, the PAF receptor antagonist L-659,989 (14) (Fig. 3) and the LTB, receptor antagonist LY-223982 (15, 16) were utilized to stop binding of their respective agonists. Addition of L-659,989 (1 μM) 2 s before PAF inhibited the response verifying that antagonist binding was essentially complete within less than 2 s (Fig. 3A). When L-659,989 was added 2 s after PAF (Fig. 3C), the first phase of actin polymerization/depolymerization occurred, but subsequent oscillations were inhibited. The same result was obtained with LTB, and its antagonist LY-223982 (data not shown). Thus, we conclude that the mechanism driving these oscillations was not analogous to chemical oscillations of intermediate reactants in a
closed system far from equilibrium. In contrast, occupancy of receptors was required to sustain the oscillations, suggesting that the underlying biochemistry involves regulated polymerization and depolymerization pathways.

Recent research in the field of calcium mobilization and signal transduction has revealed the occurrence of calcium oscillations in individual cells (3, 23). Thus an obvious question was whether these cytoskeletal oscillations were regulated by calcium oscillations. Simultaneous measurement of right angle light scatter and intracellular calcium using the calcium probe Indo-1 revealed an absence of calcium oscillations although right angle light scatter oscillations still occurred (Fig. 4). Thus the cytoskeletal oscillations were not correlated with calcium oscillations. Since previous studies show that sequestration of intracellular calcium does not inhibit the N-formylpeptide-induced actin response (8, 24), calcium elevation does not appear to be a signal required to initiate actin polymerization.

A recent report has indicated rapid oscillations in turbidimetric assays of PMNs in response to LTB4, PAF, C5a, and FMLP only after PMN pretreatment with phorbol myristate acetate (PMA) or degranulation inhibitors (25, 26). In our hands, pretreatment of PMNs with 1 ng/ml PMA did not enhance the LTB4- and PAF-induced oscillations and, in fact, diminished the total right angle light scatter changes. PMA enhanced the LTB4- and PAF-induced oscillations and, in fact, oscillations induced by PAF and LTB4 without such prior treatment. In contrast, rapid oscillations were deficient with FMLP only after PMN pretreatment with phorbol myristate acetate (PMA) or degranulation inhibitors (25, 26). In our system herein described, we find rapid oscillations in individual cells (3, 23). Thus an obvious question was whether these cytoskeletal oscillations were regulated by calcium oscillations. Since previous studies show that sequestration of intracellular calcium does not inhibit the N-formylpeptide-induced actin response (8, 24), calcium elevation does not appear to be a signal required to initiate actin polymerization.

The mechanisms that regulate depolymerization are unknown. An understanding of the polymerization/depolymerization of actin within these cells is vital to understanding the dynamics of the structural changes that result in migration of the cells.

REFERENCES