Relationship of Actin Polymerization and Depolymerization to Light Scattering in Human Neutrophils: Dependence on Receptor Occupancy and Intracellular Ca"^++

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ABSTRACT When exposed to the N-formylated chemoattractant peptides, neutrophils undergo a transient ruffling followed by a polarization that involves a redistribution of F-actin (Fechheimer, M., and S. H. Zigmond, 1983, Cell Motil., 3:349–361). The cells also undergo a biphasic right angle light scatter response whose first phase is maximal 10–15 s after exposure to the stimulus, and whose second phase is longer in duration and maximal only after 1 min or more (Yuli, I., and R. Snyderman, 1984, J. Clin. Invest. 73:1408–1417). We now report that the first phase is accompanied by a transient polymerization of actin (monitored by cytometric analysis of phallacidin staining according to the method of Howard, T. H., and W. H. Meyer, 1984, J. Cell Biol., 98:1265–1271) and the second phase is accompanied by a more sustained polymerization of actin. Based on correlated measurements of ligand binding (Sklar, L. A., D. A. Finney, Z. G. Oades, A. J. Jesaitis, R. G. Painter, and C. G. Cochrane, 1984, J. Biol. Chem., 259:5661–5669) and intracellular Ca"^++ elevation (under conditions where we use the fluorescent Ca"^++ chelator Quin 2 to modulate intracellular Ca"^++ levels), we conclude that this first phase requires less than 100 receptors/cell (out of 50,000) and does not require the release of intracellular stores of Ca"^++. In contrast, the sustained polymerization requires both the occupancy of thousands of receptors (an estimated 10% of the receptors per minute) and may be somewhat sensitive to the availability of intracellular Ca"^++. When ligand binding is interrupted, F-actin rapidly depolymerizes with a half-time of no greater than ~15 s, and the transient light scatter response decays toward its initial value in parallel. Partial disaggregation of the cells follows the recovery of these responses. Based on these observations, we suggest that transient actin polymerization and transient cell ruffling give rise to transient aggregation as long as degranulation is limited.

When exposed to N-formylated chemoattractant peptides, neutrophils respond rapidly with a highly coordinated series of responses. The potential intracellular signals include the turnover of phosphatidylinositol and the transient elevation of both intracellular Ca"^++ and cAMP ultimately leading to the release of inflammatory mediators (O_2^-, proteases, and arachidonate metabolites) and to a cluster of morphological and biochemical events contributing to chemotaxis (for review, see reference 28). Among these latter responses are membrane ruffling (2, 4, 41), actin polymerization and depolymerization (6, 10, 22, 36), cytoskeletal reorganization (2, 6, 37), phosphorylation (6), tyrosination of tubulin (18), cell aggregation, adherence, and polarization (2, 40), all in suspension, and spatial orientation on a surface in a peptide gradient (7, 39, 41). In addition, a biphasic light scatter response of uncertain origin has been described (38). The first phase of this response has been shown to be Ca"^++ independent (31).

While these latter events are under intense investigation, the biochemistry leading to the responses is far from understood. The relationship of the responses to receptor occupancy, to one another, and their dependence on particular signal pathways have not been characterized. Moreover, the conditions that favor the inflammatory components of cell response as compared with the morphological and chemotactic aspects have yet to be defined. A major goal of our research has been to identify the contributions of receptor occupancy to the responses of neutrophils. Cell responses have been examined under conditions where the ligand–receptor inter-

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actions are well-characterized using real-time fluorescent techniques and where cells are exposed to pulse (12, 24, 26, 28–30) and ramp (19, 25) exposure to ligand which regulate independently the number of receptors occupied and the rate at which receptors are occupied.

Our studies have depended primarily upon the application of a fluoresceinated chemotactic hexapeptide, N-formyl-norleucyleucylphenylalaninylorleucyltyrosyllysine-fluorescein (FLPEP) whose binding properties have been characterized elsewhere (23, 29). To place these present studies on a quantitative basis, the fraction of receptors occupied as a function of time and ligand concentration is included here as presented in Table I in the Results.

In this paper, we show that the right angle light scatter response and actin polymerization are tightly coupled events. Because the cell responses are examined under conditions where the ligand–receptor interactions are well-characterized, we are able to determine the receptor occupancy requirements for actin polymerization. Methods are used to modulate intracellular calcium and define the calcium requirements for the responses (5, 15, 31). By taking advantage of methods of simultaneous analysis using fluorometry (27) and cytometry (17), we have begun to unravel the relationship of the right angle scatter response to actin polymerization and the potential roles that these phenomena and Ca" play in cell aggregation.

MATERIALS AND METHODS

Neutrophils: Neutrophils were obtained from fresh human blood by the method of Henson and Oades (9) or Berkow et al. (1) and prepared for these studies as described previously (25). The buffer for all cell experiments contained 5 mM KCl, 147 mM NaCl, 1.9 mM KH2PO4, 1.1 mM Na2HPO4, 5.5 mM glucose, 0.3 mM MgSO4, 1 mM MgCl2, and 1.5 mM CaCl2 (unless indicated otherwise).

Reagents: The N-formyl peptide receptor blocker t-butoxycarbonyl-phe-leu-phe-leu-phe-t-Boc was obtained from Vega Biotechnologies, Inc., Tucson, AZ. 7-Nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin and rhodamine-phalloidin were obtained from Molecular Probes, Inc. (Junction City, OR) and used interchangeably. Fluorescein-labeled N-formyl-norleucyl-leucyl-tyrosine-tyr-lys was prepared and characterized as described previously (28).

Assays of Neutrophil Activation: Assays were performed on cell suspensions containing 4 x 10^6 cells/ml unless otherwise indicated. Analyses of right angle light scattering and transmitted light were performed using the SLM 8000 photon counting spectrotuorometer (SLM Instruments, Inc., Urbana, IL) interfaced to an Apple Computer (Apple Computer Inc., Cupertino, CA). Since excitation and emission were at 340 nm (i.e., in the ultraviolet), it was possible to leave the top off the fluorometer sample compartment and withdraw aliquots for fixation (for actin staining and cytometric analysis, see below) while monitoring the right angle light scatter response. This permitted essentially simultaneous analysis of right angle light scatter, aggregation, and actin polymerization even though the latter two analyses were completed at a later time on the fixed cells.

In all of these spectrometric assays, the cell responses were monitored continuously while cells, held in plastic cuvettes, were stirred in the thermostatted (37°C) sample chamber of the fluorometer.

Cytometric Analysis of F-Actin Content, Right Angle Light Scatter, and Aggregation: The staining procedure was adapted from Howard and Meyer (10). 100 µl of cell suspension were withdrawn at timed intervals after stimulation from the stirred sample compartment during light scattering measurements and mixed with 100 µl of staining cocktail containing 8% formaldehyde, 0.33 mM NBD-phallacidin or rhodamine–phalloidin, and 200 µg/ml lysosomatin. In all experiments, the simultaneous right angle scatter response was recorded on the fluorometer. The fluorescence of these fixed cells was observed to be stable at 4°C for at least several days when no wash step was used. When using the stains at these concentrations, the contribution of unbound stain to the cytometric determination of total cellular staining was insignificant.

The stained cells were analyzed with the Becton-Dickinson FACS IV cytometer (Becton-Dickinson & Co., Paramus, NJ) essentially as described by Howard and Meyer (10) using the 488 and 514 lines of a 2-W argon ion laser for NBD and rhodamine, respectively. The fixed samples were analyzed by counting a total of 10,000 events. These events represented not only single neutrophils but also neutrophil aggregation. For analysis of actin content by cellular fluorescence, the singlet neutrophil population was selected by gating out aggregated cells based on their forward angle and right angle light scattering characteristics, and the mean fluorescence channel was calculated (29). Staining analyses therefore represent the average actin content per cell.

The increase in cell aggregation was quantified on the fixed cells by observing the percentage of total particles that were singlets (based on scatter parameters). The aggregation observed in fixed cells was approximated by transmittance measurements on cell suspensions (unpublished observation). The aggregation of fixed cells was insensitive to shear forces, since attempts to disrupt aggregates by passage through a syringe did not change the distribution of aggregates.

To verify that the fixing of the cells was rapid, the right angle scatter parameters were measured by fluorometry of live cells and cytometry of fixed cells (Fig. 1). The right angle light scatter of fixed cells was determined on the singlet population and quantified as the mean channel number. Thus, the right angle light scatter determined by cytometry represents the average scatter per cell. The rapidity of fixation under these conditions is indicated by a precise correspondence between the right angle scatter observed in the fluorometer and the right angle scatter observed cytometrically in the fixed cells. For the remainder of the experiments performed in this manuscript, right angle light scatter was determined using the fluorometer. While this manuscript was being completed, Wallace et al. (34) demonstrated the application of a similar staining protocol to the kinetics of actin polymerization and depolymerization in human neutrophils. A number of other workers have also found polymerized actin and other evidence of cytoskeletal activation within seconds of the exposure of neutrophils to stimulation (6, 22, 36). Taken together, these observations indicate that cells are rapidly and thoroughly fixed by the protocols used here and that the staining, even at early time points, is indicative of actin polymerization.

FIGURE 1 Pseudosimultaneous analysis of right angle light scatter by (A) cytometry (of fixed cells) and (B) fluorometry (of live cells). 4 x 10^6 cells/ml buffer at 37°C were continuously stirred in suspension. FLPEP was added at time zero, and right angle light scatter was continuously monitored at 340 nm on the SLM fluorometer (B). Simultaneously, aliquots were rapidly removed and mixed with rhodamine–phalloidin cocktail as described in Materials and Methods. Right angle light scatter of the singlet population of these fixed samples was determined on a Becton-Dickinson FACSS IV cytometer (A). Q, 0.01 nM FLPEP; x, 1 nM FLPEP. In B, traces are shown for stimulation by 1 nM (solid curves) and 0.01 nM FLPEP (dashed curves). Error bars in A show the typical range of values determined for duplicate determinations from one donor. B shows typical reproducibility for duplicate determinations from one donor.
Formats of Cell Stimulation: Neutrophils were stimulated with formyl peptide according to three distinct protocols. The standard protocol involved the bolus addition of a known concentration of ligand. The pulse protocol can be achieved by administration of FLPEP as a bolus, but followed after a timed interval by a blocking agent, either antibody to fluorescein (24, 26, 29, 30) or receptor antagonist, t-Boc-phe-leu-phe-leu-phe (12, 24). In a pulse protocol, the number of receptors occupied is a function of the dose of ligand and the length of uninterrupted binding. When binding is interrupted, the occupied receptor dissociates at a rate characterized by the normal dissociation rate, 0.3/min (19). In this study, 2 x 10^{-6} M t-Boc-phe-leu-phe-leu-phe was used. The infusion protocol varies the rate of receptor occupancy. This is accomplished by administering a fixed ligand concentration over different periods of time using a Harvard Infusion pump (Harvard Apparatus Co., Inc., S. Natick, MA) to drive the contents of a syringe into the cell suspension (25). The evaluation of ligand binding during an infusion is discussed elsewhere (19, 20).

Modulation of Intracellular Ca^{2+} Using Quin 2 to Deplete Intracellular Stores and Extracellular Ca^{2+} to Replenish the Stores: The salient features of this procedure are reiterated here (31). Neutrophils (5 x 10^6/ml) suspended in Ca^{2+}-free buffer are exposed to 20 μM 2-(2-bis(carboxylmethyl)-amino-5-methylphenoxy)-methyl-6-aminoquinoline (Quin 2) acetoxytetramethylester (33) for 20 min at 37°C. When washed and resuspended in Ca^{2+}-free buffer, the cells are observed to contain ≤1 mM cytoplasmic Quin 2. When 1.5 mM Ca^{2+} is added to the medium, intracellular Ca^{2+} levels re-equilibrate to resting levels over a time course of 2-3 min. Similar methods have been used by di Virgilio et al. (5) and Lew et al. (15) to modulate intracellular calcium. In our hands, the normal resting levels of Ca^{2+} are ~150-200 nM (i.e., 60–70% Quin 2 saturation). When depleted of Ca^{2+}, the levels are typically <40 nM (<30% Quin 2 saturation). Moreover, the amount of Ca^{2+} released from storage pools from the Ca^{2+}-depleted cells is reduced more than 90%. (Free Ca^{2+} levels in Ca^{2+}-depleted cells hardly rise above resting level after stimulation.)

RESULTS

Kinetics of Actin Polymerization and its Similarity to the Kinetics of the Right Angle Scatter Response

The kinetics of actin polymerization has been examined at doses between 0.01 and 10 nM FLPEP which give rise to receptor occupancy varying between 1 and >90% after 1 min of binding (see Table I).

Typical results for an experiment repeated on four occasions are presented in Fig. 2 B. For ligand concentrations down to at least 0.01 nM, there is a doubling of F-actin detected within 10 s of cell stimulation. For concentrations <0.3 nM, the actin polymerization is transient with considerable recovery within 3 min. At doses above 0.3 nM, a rapid phase of polymerization is followed by a rapid but partial depolymerization and thereafter by sustained polymerization that lasts up to 5 min. Since 0.2% of the receptors (100) are occupied by 0.01 nM after 15 s, it is apparent that only a small number of receptors is required to elicit actin polymerization.

The right angle light scattering of the cells shows a similar time course and dose response (Fig. 2 A). There is a rapid, transient decrease in scatter intensity with a minimum at 8–15 s depending upon the dose (ED_{50} <0.01 nM, see Fig. 4, reference 27). A second phase, whose minimum is near 1 min, is pronounced at doses above 0.3 nM.

Dependence of Actin Polymerization on the Rate of Receptor Occupancy

To examine the impact of the rate of ligand binding, light scattering and actin polymerization were measured during stimulus infusion as shown in Fig. 3. The dose, 0.05 nM, is sufficient only to elicit the first phase of polymerization. For this dose the initial binding rate is 4%/min which is reduced to <1%/min during a 3-min infusion (see, for example, reference 19). As the rate of binding is reduced, both responses elongate and are reduced in magnitude, essentially in parallel.

FIGURE 2 Dose-response curves for pseudosimultaneous right angle scatter and actin polymerization in neutrophils. The data are plotted as the relative right angle scatter (A) or the relative F-actin content (B) versus time. 4 x 10^6 cells/ml at 37°C were continuously stirred in suspension. FLPEP was added at time zero, and right angle scatter was continuously monitored at 340 nm. At various intervals, aliquots of cells were removed, fixed, and stained for F-actin with NBD-phallacidin. The F-actin content was quantified by flow cytometry. Duplicate light scatter determinations are shown in A; duplicate actin data (with error bars) are averaged in B.

FIGURE 3 Actin polymerization during stimulus infusion. The data are collected and plotted as in Fig. 2. The cells were stimulated by 0.05 nM FLPEP injected as a bolus (Q) and during infusions lasting 86 s (○) or 170 s (△). In these experiments, rhodamine-phalloidin was used to stain actin.
Sustained Actin Polymerization Is Nearly Independent of a Stimulated Elevation of Intracellular Ca++

We have shown elsewhere that the first phase of the neutrophil light scatter response occurs even when cells are depleted of intracellular stores of Ca++ (31). In contrast, free radical production, degranulation, and the Quin 2 response are inhibited when the stores are modulated (31). In Fig. 4, we show simultaneous analyses of light scatter responses and actin polymerization in cells where both resting Ca++ levels and the size of the pool released upon stimulation are reduced to 20% or less at their normal level. It is clear that the light scatter response and actin polymerization parallel one another. Even when intracellular Ca++ levels are reduced there is a normal, rapid polymerization that occurs within the first 20 s. This response is sustained at 1 nM FLPEP either in normal Ca++ or Ca+-depleted cells.

Continued Receptor Occupancy Is Required for Sustained Polymerization

To examine the occupancy dependence of the sustained polymerization, neutrophil suspensions were exposed to a pulse of stimulation (Fig. 5). The binding of 1 nM FLPEP was stopped after 30 or 60 s (~35 or 52% occupancy). We observed that the depolymerization of actin began within ~10 s after addition of blocker and approached final levels within ~30 s thereafter. The light scatter signal recovered with identical kinetics. Under these conditions the average rate of ligand dissociation was ~0.3 min⁻¹, i.e., its half-time of 2–3 min was considerably longer than the half-time for decay of cell activities to their basal levels. Thus, under the four different experimental protocols examined, actin polymerization and depolymerization paralleled right angle light scatter in the same cells.

Relationship of Actin Polymerization and Cell Aggregation

Fig. 5 also shows the simultaneous analysis of actin polymerization and cell aggregation. When binding was inhibited, the light scatter recovers with a half-time of no more than ~15 s. In contrast, disaggregation began 30–40 s after binding is inhibited, a time at which light scattering and actin polymerization were essentially recovered.
DISCUSSION

Correlation of Right Angle Light Scattering and Actin Polymerization

Actin polymerization and right angle light scattering are tightly coupled responses. In simultaneous measurements under conditions where the FLPEP dose was varied, the binding interrupted, the rate of binding varied, or the calcium content varied, the kinetics of the right angle light scattering and actin polymerization parallel one another.

It is somewhat surprising, however, that polymerization was accompanied by a decrease in right angle scatter because classical measurement of bulk actin polymerization (or elongation) in solution relies on increases in scattering proportional to the number of G-actins that are incorporated into F-actin (35). Since actin is a major cell constituent, comprising 10–20% of the bulk of the cell protein, a doubling or tripling of the F-actin would be expected to give rise to light scatter changes. Whether the direction (decrease) of the scattering change observed in this study is a consequence of some other higher order of organization of actin (i.e., redistribution, bundling, or reorientation with respect to the cell surface) or a cellular consequence of actin polymerization (i.e., membrane ruffling) has not yet been established. Nonetheless the transient light scatter appears to provide a simple way to monitor actin polymerization and depolymerization or a tightly correlated consequence.

Occupancy Requirement for Actin Polymerization and the Right Angle Response

The binding of FLPEP to its receptor on the neutrophil has been characterized in detail (29). Hence, for any of the formats of cell stimulation described in this paper, the number of receptors occupied at any time during the stimulation of the cells can be estimated. The percentage of receptors occupied, based on typical experimental conditions, as a function of time for FLPEP concentrations relevant to several cellular responses is summarized in Table I. For most cellular responses, the ED₅₀ for FLPEP is in the range of 0.1–0.5 nM (cAMP, depolarization, degranulation, O₂⁻ aggregation, and chemotaxis). Two notable exceptions are Quin 2 (Ca⁺⁺) elevation (ED₅₀ 0.03 nM) and right angle scatter (<0.01 nM) (30).

The first phase of actin polymerization was detected rapidly after exposure of neutrophils to formyl peptide and reached a maximum within 10–15 s to doses as low as 0.01 nM FLPEP. As is the case with the light scattering response, as few as 100 receptors are occupied at the time of the maximal response. In fact, pulse binding experiments performed at 0.01 nM (30) showed that only 2–5 s of binding or 10–20 receptors are required to elicit half-optimal light scattering (and by implication, half-optimal F-actin polymerization). Actin polymerization is sustained for several minutes at least for doses above 0.3 nM, i.e., where the rates of binding after 2 min are 10% of the receptor per minute (see Fig. 4, reference 29). Thus, this sustained polymerization is characterized by a higher requirement for receptor occupancy, which is in the range of thousands of receptors.

The number of receptors occupied was readily varied by changing the length of exposure to the stimulus in a pulse protocol. When 1 nM FLPEP was provided for 30 or 60 s (Fig. 5, ~35 or 52% occupancy) and then binding stopped by addition of blocking agent, the actin depolymerization began within 10 s after the inhibition of binding and while occupied receptor remain on the cell surface. Thus it appears that continued occupancy of new receptors is required to sustain actin polymerization.

Ca⁺⁺ Requirement for the Actin Response

The initial rate of actin polymerization was not affected by modulation of intracellular calcium (Fig. 4). However, the sustained polymerization may be somewhat sensitive to intracellular calcium levels. These results raise interesting questions concerning the role of Ca⁺⁺ in the regulation of these processes. Actin polymerization in vitro exhibits a pronounced lag phase that is thought to be associated with the formation of nuclei (for a review, see reference 13). This is followed by a rapid addition of monomer to the preferred barbed end of the growing filament (3, 16, 21). Neither of these processes is strictly Ca⁺⁺-dependent. However, the rapidity of the polymerization process in vivo together with the lack of any appreciable lag phase suggests that the nucleation phase is very rapid in the living cell as compared to that seen with pure actin solutions, or that polymerization is regulated by uncapping of pre-existing filaments.

The rapid depolymerization of actin seen after the first phase of polymerization even in the Ca⁺⁺-depleted cell or after the blockade of binding is noteworthy. At a concentration of filaments present in the cell, protein factors such as gelsolin (37) or villin-like (8) proteins appear to accelerate actin filament disassembly by cutting and blocking further monomer addition (8, 32) in the presence of micromolar levels of free Ca⁺⁺. Our results suggest that the net depolymerization of actin seen in Ca⁺⁺-depleted cells is also significant and could be mediated by other, as yet undescribed, mechanisms. In fact, a recent preliminary report suggests that gelsolin, under certain conditions, can bind to actin filaments in the Ca⁺⁺-independent manner (14). It may be that under in vivo conditions, this calcium-independent activity coupled with other unknown factors could be sufficient for the rapid disassembly of filaments. Given the myriad of proteins that bind to and influence actin assembly and disassembly, it seems unlikely that the action of a single regulatory protein is sufficient to explain our data.

Alternatively, it is conceivable that in the cellular milieu
submicromolar Ca** levels might be sufficient to activate actin depolymerizing proteins even in the presence of millimolar levels of Quin 2. If so, one might predict that enzymes such as the myosin light chain kinase, which are activated by calmodulin-dependent processes, might still be operative in Quin 2–loaded cells. We are currently investigating this possibility.

Relationship between Actin Polymerization and Cell Aggregation

To probe the relationship of actin polymerization and cell aggregation, we examined the evolution of light scatter, actin, and aggregation after pulse stimulation. Once ligand binding to the receptors was inhibited, we observed that both the light scatter and actin responses recovered within 30 s. Zigmond and Sullivan (40) have previously reported that ruffles recede over the same time frame. When these responses recovered, the cells began to disaggregate.

We suggest, as a working hypothesis, that transient cell aggregation may be governed by transient actin polymerization and membrane ruffling. Thus, in the absence of significant degradation (which may involve the release of adhesive proteins), aggregation may depend on the intercellular contact between cell surface projections which are governed by cytoskeletal activation. The extent of these interactions will be a function of the stimulus dose and the time course of receptor occupancy. When stimulation is insufficient to sustain actin polymerization and/or ruffling, such as a result of insufficient binding, the cells disaggregate after the decay of cytoskeletal activation.

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