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RECEPTOR-MEDIATED ACTIN ASSEMBLY IN ELECTROPERMEABILIZED NEUTROPHILS: ROLE OF INTRACELLULAR pH

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Summary: Neutrophil activation by a variety of stimuli is accompanied by an intracellular acidification, which has been postulated to mediate actin polymerization (Yuli and Oplatka, Science 1987, 235, 340). This hypothesis was tested using 7-nitrobenz-2-oxa-1,3-diazole (NBD)-phallacidin staining and flow cytometry, or right angle light scattering to study actin assembly in intact and electrically permeabilized human neutrophils. Intracellular pH was measured fluorimetrically using a pH sensitive dye. In cells stimulated with N-formyl-methionyl-leucyl-phenylalanine (fMLP) at 21°C, actin assembly clearly preceded the intracellular acidification in response to fMLP. Moreover, actin polymerization persisted in cells where intracellular pH was clamped near the resting (unstimulated) level using nigericin/K^+. Finally, fMLP induced a significant increase in F-actin content in electropermeabilized neutrophils equilibrated with an extracellular medium containing up to 50 mM HEPES. These observations indicate that fMLP-stimulated F-actin assembly is not mediated by a decrease in intracellular pH and suggest that changes in transmembrane potential and ionic gradients are unlikely to mediate actin polymerization.

Despite extensive study, the mechanisms responsible for initiating the conversion of G to F-actin (polymerization, assembly) after receptor-mediated stimulation in neutrophils are

Abbreviations
NBD-phallacidin is 7-nitrobenz-2-oxa-1,3-diazole-phallacidin, fMLP is N-formyl-methionyl-leucyl-phenyalanine, BCECF is 2,7-biscarboxy-ethyl-5(6)-carboxy-fluorescein, pH_i is intracellular pH.

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still incompletely understood (1,2,3,4). Ca^{2+} plays a central role in signal transduction in these cells but recent observations suggest that changes in the intracellular concentration of this cation are neither sufficient nor necessary for actin polymerization to occur (1,2,3,4). The actin polymerization response elicited by the chemotactic tripeptide fMLP is thought to be mediated by a G protein (5), inasmuch as it is inhibited by pertussis toxin. There is also evidence suggesting the involvement of phosphoinositide turnover (6) and protein kinase C (7), but the precise mechanism leading to the conversion of G to F-actin remains unknown (1,2,3,4).

An early decrease in intracellular pH (pH_i) has been recorded after stimulation of neutrophils with a variety of agents (3,8-13). This has led to the suggestion that the acidification might signal the initiation of actin polymerization (3,8-13). Evidence favoring this hypothesis includes (i) the similarity in kinetics and dose response relationships of the acidification and actin polymerization measured at 37°C, (ii) the observation that all the neutrophil agonists that cause actin polymerization also decrease intracellular pH (3), and (iii) the finding that, in the absence of stimuli, cytosolic acidification induced by exposure to acids results in a rapid, pertussis toxin-sensitive polymerization of actin (3,13). In the present report, the causal relationship between pH_i and actin polymerization was investigated further using human neutrophils. The effects of fMLP on actin assembly were monitored under conditions where the acidification was minimized or prevented by means of ionophores or by incubation of permeabilized cells in media with high buffering capacity. In order to preserve receptor-mediated responsiveness, permeabilization was accomplished by electroporation (14).

Materials and Methods

Reagents. Percoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Reagents for Krebs Ringers Phosphate Dextrose Buffer (KRPD) were obtained from Mallinckrodt Inc., Paris, KY. EGTA, fMLP, HEPES, ATP (K^+salt), were obtained form Sigma (ST. Louis, MO). NBD-phallacidin and BCECF-AM were obtained from Molecular Probes (Eugene, OR). Lyso-phosphatidyl choline (lyso PC) was obtained from Avanti Polar Lipids (Pelham, AL) and nigericin was obtained from Calbiochem (San Diego, CA).

Cell Isolation. Human neutrophils (> 98% pure) were isolated from citrated whole blood using dextran sedimentation and discontinuous plasma-Percoll gradients as previously described in detail (15,16). The functional integrity and non-activated state of neutrophils

isolated in this manner has been extensively validated in previous publications (15,16).

pH_i and Light Scattering Determinations. Intracellular pH and right angle light scattering were determined using a Hitachi F4000 fluorescence spectrometer. For the measurement of pH_i, the cells were loaded with BCECF by preincubation with 1 μg/ml of the precursor acetoxymethylester at 37°C for 30 min as described (17). Aliquots of this cell suspension were washed and placed in the indicated medium in a plastic cuvette with magnetic stirring. Fluorescence was measured with excitation at 490 nm and emission at 525 nm, using 5 nm slits. Additions were made to the cuvette through a special port using a Hamilton syringe. pH_i was calibrated using nigericin and K^+ as described earlier (17). Right angle light scatter was monitored at either 340 or 550 nm as described by Sklar (18). Similar results were obtained at both wavelengths. The data are expressed as percent of the initial scattering of untreated cells.

Permeabilization Procedure. Neutrophils were permeabilized by electroporation essentially as described (14). Briefly, 10^7 cells were sedimented and resuspended in 1 ml of ice-cold permeabilization medium (140 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM ATP, 1 mM EGTA, 0.193 mM $CaCL_2$, 10 or 50 mM Hepes as indicated, and pH 7.0). 0.8 ml aliquots of this suspension were transferred to a BioRad Pulser cuvette and subjected to two discharges of 2kV from a 25 μF capacitor using the BioRad Gene Pulser. The cells were sedimented and resuspended in fresh ice-cold medium between pulses. Finally, the cells were equilibrated for 1 min in the indicated medium at 21°C prior to stimulation and measurement of actin polymerization.

Flow Cytometry. Neutrophil content of polymerized actin (F-actin) was determined by NBD-phallacidin staining of fixed and permeabilized cells according to the two step method of Howard and Meyer (19) except that the formalin/lyso PC suspension was sonicated in a bath sonicator for 10 min to facilitate the formation of a micellar suspension of the lyso PC. The cells were analyzed on an Epics 5 fluorescence-activated cell sorter (Coulter, Hialeah, FL). Cells were excited with an argon laser at 488 nm and emission recorded at 520 nm with a band pass and short pass filter. Gating was done on the forward angle and right angle light scatter only to exclude debris and cell clumps. 50,000 cells were measured per condition and all values are expressed as log green fluorescence intensity and as relative fluorescence index (RFI) as indicated in the text. The RFI was calculated according to the formula $RFI=2^{((b-a)/26)}$ where a=mean channel number of the control cell population, b=mean channel number of the cell population in question, and 26=number of channels that represents a doubling of fluorescence intensity.

Statistical Analysis. All data are reported as mean±S.E.M and were analyzed by paired T test with a P value of < 0.05 considered significant.

Results and Discussion

We first compared the kinetics of cytosolic acidification and of the change in right angle light scatter, used here as an index

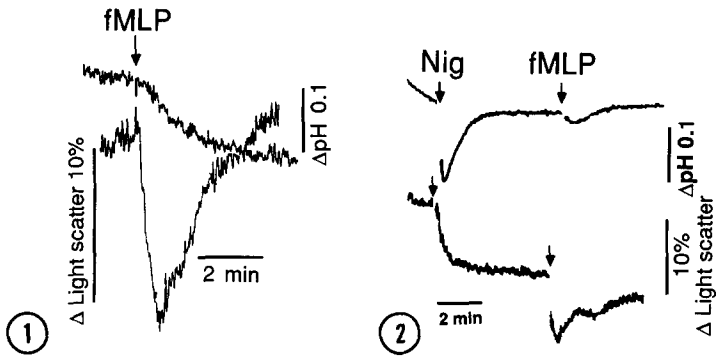

Figure 1. Relative time courses of intracellular pH (top trace) and right angle light scatter (bottom trace) in neutrophils in response to stimulation with 10^{-7}M fMLP. The arrow indicates the time at which fMLP was added. The trace is representative of 4 determinations.

Figure 2. Top trace: Intracellular pH change in response to treatment with 5μM nigericin followed by stimulation with 10^{-8}M fMLP as measured by fluorescence spectroscopy in neutrophils loaded with BCECF. **Bottom trace:** Changes in right angle light scatter in response to treatment with 5 μM nigericin followed by stimulation with 10^{-8}M fMLP.

of actin polymerization (see ref. 18). As illustrated in Fig. 1 and consistent with earlier observations (18), the addition of fMLP to intact cells was followed almost immediately by a decrease in light scattering. At 21°C, the temperature chosen to increase the resolution of the responses, the change in scattering peaked at 15-30 sec, followed by a more sustained, smaller decrease lasting several minutes. In contrast, following the addition of fMLP, there was a discernible lag phase before pH_i decreased measurably, so that at 15 seconds, when the right angle light scatter was near maximal, the acidification was marginal. These observations are not compatible with a causal role of pH_i changes in the initiation of actin polymerization.

We next determined whether actin polymerization in response to the chemoattractant was able to occur in the absence of cytosolic acidification. To preclude the pH_i change induced by fMLP, the cells were incubated with nigericin in a medium containing 140 mM K^+, a concentration approximating that of the cytosol. Under these conditions nigericin, a K^+/H^+ exchanging ionophore, tends to clamp pH_i at a level approximately equal to the extracellular pH (20). As shown in Fig. 2 (top trace), addition of nigericin induced a transient decrease in pH_i, attributable to release of acid equivalents from intracellular compartments into the cytoplasm. Secretory granules are the likely source of acid

equivalents since the nigericin-induced transient fall in pH_i was not observed in degranulated cytoplasts (unpublished observations). The initial acidification eventually subsided, as pH_i equilibrated at a more alkaline level. Under these conditions, addition of fMLP produced only a marginal pH change (< 0.01 units). Parallel measurements of light scattering (bottom trace, Fig. 2) demonstrated that the initial acidification induced by nigericin was accompanied by a change in cell shape (actin polymerization), consistent with results reported using weak acids (13). However, recovery of pH_i was not accompanied by a reversal of the shape change. More importantly, subsequent addition of fMLP produced a further decrease in light scattering, only slightly smaller than that recorded in otherwise untreated cells. As the chemoattractant-induced acidification under these conditions is negligible, the results indicate that fMLP must have induced actin polymerization by a mechanism other than change in pH_i.

Further evidence supporting this conclusion was obtained studying actin polymerization directly in intact and electro-permeabilized cells, measuring NBD-phallacidin fluorescence using a flow cytometer. Confirming the results obtained by light scattering, fMLP was found to stimulate the formation of F-actin in cells treated with nigericin and high K^+ (Fig. 3; R.F.I. 1.0 Control vs. 2.31±0.2 fMLP). The extent of the polymerization in response to the chemoattractant was comparable to that of control (nigericin free) cells (RFI=1.0 Control vs. 3.17±0.69 fMLP).

Evidence against a requirement for cytoplasmic acidification for actin polymerization was also obtained using electrically permeabilized neutrophils. Such cells were earlier shown to allow the rapid equilibration of solutes of $M_r \leq 700$, while retaining the ability to respond to fMLP (14). The permeabilization procedure itself caused a slight, variable increase in the content of F-actin, by a mechanism that is presently not clear. Nevertheless, subsequent stimulation with 10^{-8}M fMLP for 1 min resulted in a further large increase in actin polymerization (Fig. 3A and B ; RFI=1.0 Control vs. 2.50±0.50 fMLP). The chemoattractant-stimulated polymerization was evident not only in the regular permeabilization medium, which is buffered with 10 mM Hepes (Fig. 3), but also when the cytosol was equilibrated with a more heavily buffered solution, containing 50 mM Hepes (Fig. 3; RFI=1.0 Control vs. 1.61±0.14 fMLP). The continuity between the external medium and the cytosol, together with the high buffering capacity of the

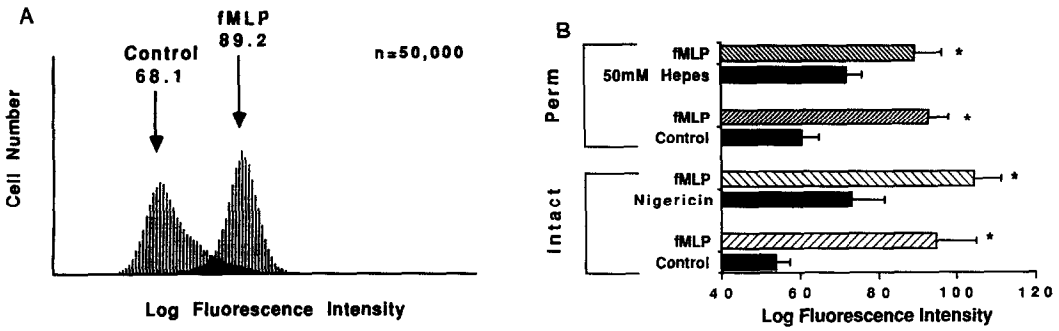

Figure 3 (A). Increase in F-actin content of permeabilized neutrophils as determined by staining with NBD-phallacidin and analysis of fluorescence using flow cytometry. Each histogram represents 50,000 neutrophils and the abscissa has a log scale. The ordinate represents cell number.
(B). Increase in F-actin content of intact and permeabilized neutrophils under the conditions specified by the graph labels. fMLP=10^{-8}M fMLP, nigericin=5μM nigericin. Each bar represents the mean± S.E.M. of 4 experiments conducted with different donors. * $p< 0.05$ as determined by Student's t-test on paired data.

medium used, ensured that changes in pH_i in response to fMLP, if present, were minimal.

In conclusion, several lines of evidence strongly suggest that fMLP-induced actin polymerization can occur in the absence of pH_i changes. First, the decrease in right angle light scatter, a reflection of change in cell shape that results from actin polymerization, clearly preceded the decrease in pH_i (Fig. 1). Secondly, significant actin assembly was noted in cells treated with nigericin and high K^+, under conditions where fMLP had little effect on pH_i (Fig. 2 and 3). Finally, in electropermeabilized cells equilibrated with media containing high concentrations of buffering agents, actin polymerization in response to the chemotactic peptide was still detectable. Though the occurrence of small, localized changes in pH_i can not be rigorously excluded, it appears unlikely that cytosolic acidification initiates the conversion of G to F-actin. Moreover, the data obtained with permeabilized cells also suggest that changes in membrane potential or in the transmembrane distribution of inorganic ions are unlikely to signal actin polymerization. Therefore, the signal responsible for actin assembly remains unknown and is the subject of our current investigations.

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