

Stimulated Cytokineplasts from Human Polymorphonuclear Leukocytes Mobilize Calcium and Polymerize Actin

Cytoplasts Made in Cytochalasin B Retain a Defect in Actin Polymerization

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Abstract

Biologically active fragments from polymorphonuclear leukocytes (PMN) are simplified systems that can be used to elucidate specific pathways by which cell function is altered. In the current study we have found that cytokineplasts, which are motile fragments derived from the leading front (protopod, lamellipodium) of human PMN, rapidly increase their intracellular free calcium concentration when stimulated by chemotactic formyl peptide or by leukotriene B₄, as measured by Quin-2 acetoxymethyl ester fluorescence. As in the parent cell, extracellular EGTA blunts this response only partially. Hence, cytokineplasts retain a mobilizable internal calcium pool, despite a general lack of intracellular organelles. In addition, formyl peptide more than doubles the amount of cytoskeleton-associated (polymerized) actin. In contrast, cytoplasts made by high-speed, discontinuous gradient centrifugation of cytochalasin B-treated leukocytes also increase their intracellular free calcium on stimulation, but cytoskeleton-associated actin increases by only ~14%. Thus, defective motile function in the latter cytoplast is associated with compromised effector function (actin polymerization).

Introduction

Two distinct granule-poor cytoplasts from human blood polymorphonuclear leukocytes (PMN)¹ are currently being used to study functional pathways in the parent cell. Cytokineplasts ("kine," to emphasize their motile capacities), induced by the brief application of heat to adherent PMN, are capable of random locomotion, chemotaxis, capping, and other motile functions, but lack respiratory burst oxidase activity (1-5). Other nucleate fragments (herein called cytochalasin B [CB]-cytoplasts), which are produced by the high-speed centrifugation of CB-treated PMN in discontinuous gradients of Ficoll (6), have defective motile function (4, 6), the basis of which is not known, but

retain a number of other stimulatable functions of the parent cell, including respiratory burst oxidase activity, aggregation, and adaptation of formyl-peptide receptors (6-8).

Both intact PMN and CB-cytoplasts have been reported to exhibit a rise in intracellular free calcium concentration upon stimulation with *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (9, 10). If calcium is removed from the external media this response is partially muted in PMN and virtually abolished in CB-cytoplasts (9), suggesting that the latter lack a mobilizable internal calcium pool. That pool was apparently either left behind or functionally compromised during formation of the cytoplast. In this paper we report that stimulated cytokineplasts can increase their intracellular free calcium concentration both in the presence and absence of extracellular calcium. In addition, on stimulation these motile cytoplasmic fragments polymerize actin while CB-cytoplasts do so only poorly.

Methods

Materials. EGTA, CB, phorbol 12-myristate 13-acetate (PMA), Hepes, fMet-Leu-Phe, and Hanks' balanced salt solution were all purchased from Sigma Chemical Co. (St. Louis, MO); NCTC 135 tissue culture medium (with L-glutamine) from Gibco (Grand Island, NY); Quin-2 acetoxymethyl ester (Quin-2/AM) from Lancaster Synthesis Ltd. (England). Leukotriene B₄ was a gift from Dr. P. Borgeat, Centre Hospitalier de l'Université Laval, Quebec, Canada (11). All other reagents were of analytical grade.

Preparation of cytoplasts. Human PMN were obtained from buffy coats and cytokineplasts were made from them as described previously (4). CB-cytoplasts were prepared as described by Roos et al. (6). In some experiments, tritiated CB, 0.26 $\mu\text{Ci}/\mu\text{g}$, was used to make CB-cytoplasts. After the standard number of washes, residual counts were measured in an LS scintillation counter (Beckman Instruments Inc., Spinco Div., Palo Alto, CA) and compared with those of control PMN suspended (but not centrifuged) and washed in a similar manner.

Both cell fragments were initially suspended in NCTC medium. Before loading with Quin-2/AM, they were washed and made up in Hanks' balanced salt solution.

Loading with Quin-2/AM and fluorescence measurements. Quin-2/AM loading was carried out as described previously (10, 12) with the following exceptions: concentration of Quin-2/AM, 10 μM ; loading time, 30 min; concentration of fragments, 10⁷/ml. Experimental details are provided in the legends of Figs. 1 and 2. Fluorescence measurements were performed in a fluorescence spectrophotometer (model 8000, SLM Instruments, Inc., Urbana, IL) with temperature-controlled cuvette and magnetically driven stirrer (10).

Isolation of cytoskeletal proteins. Cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100, and electrophoresed in a 5-15% gradient or 10% sodium dodecyl sulfate (SDS)-polyacrylamide slab gel (13-15). Experimental details are provided in the legends of Figs. 3 and 4. Gels stained with Coomassie Blue were examined at 590 nm in a scanning densitometer for determination of relative amounts of actin (15). Total actin was determined by the addition of actin standards

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1. *Abbreviations used in this paper:* CB, cytochalasin B; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; Quin-2/AM, Quin-2 acetoxymethyl ester.

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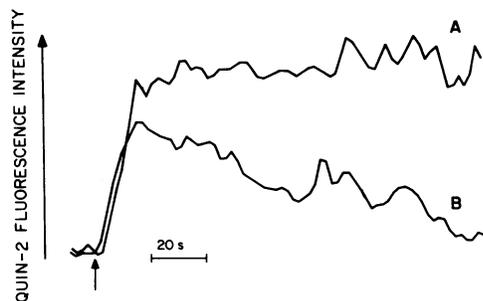


Figure 1. The effect of the chemotactic peptide fMet-Leu-Phe (10^{-8} M) on the fluorescence of Quin-2-loaded cytokineplasts (2×10^6 cytokineplasts/ml) in the presence of (A) 1.6 mM Ca^{2+} in the incubation medium and in the presence of (B) 1.6 mM Ca^{2+} and 2 mM EGTA (final concentration of calcium in the suspending medium was $<10^{-7}$ M). EGTA was added 1 min before fMet-Leu-Phe.

to gels; total protein, by Peterson's modification of the method of Lowry (16).

Results

Intracellular free calcium. The addition of fMet-Leu-Phe (10^{-8} M) to suspensions of cytokineplasts caused a rapid and sustained increase in the intracellular concentration of free calcium (Fig. 1). In the presence of 2 mM EGTA, the rise was diminished, and not sustained. Leukotriene B_4 (8×10^{-8} M) caused a rapid but unsustained increase in the intracellular concentration of free calcium (Fig. 2). Again, EGTA blunted but did not abolish the response.

Actin polymerization. The addition of fMet-Leu-Phe to cytokineplasts resulted in more than doubling of the amount of actin associated with the cytoskeleton (Table I, Fig. 3). In contrast, CB-cytoplasts given fMet-Leu-Phe increased the amount of actin associated with the cytoskeleton by a mean of only 14% (Table I), even though such preparations responded to fMet-Leu-Phe with a brisk rise in Quin-2 fluorescence (not shown). Fig. 4 shows a representative gel in which the increase was 25% (lane C vs. lane B). There was no evidence of polymerization in response to PMA (lane D vs. lane B). Actin in the cytokineplasts and CB-cytoplasts, expressed as percent of total fragment protein,

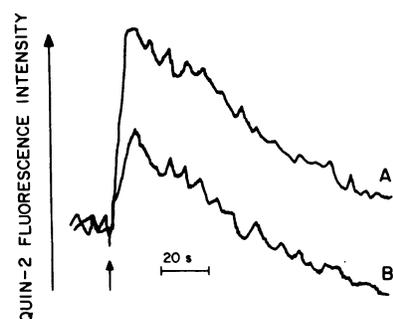


Figure 2. The effect of leukotriene B_4 (8×10^{-8} M) on the fluorescence of Quin-2-loaded cytokineplasts (5×10^6 cytokineplasts/ml) in the presence of (A) 1.6 mM Ca^{2+} in the incubation medium and in the presence of (B) 1.6 mM Ca^{2+} and 2 mM EGTA (final $[\text{Ca}^{2+}]$, $<10^{-7}$ M). EGTA was added 1 min before leukotriene B_4 .

Table I. Effect of fMet-Leu-Phe on the Amount of Actin Associated with the Cytoskeleton Isolated from Cytokineplasts and CB-Cytoplasts Prepared from Human Polymorphonuclear Leukocytes

Condition	Cytoskeletal actin expressed as percent of total cytoplast actin	
	Cytokineplasts	CB-cytoplasts
No addition	$0.19 \pm 0.05^*$ (n = 8)	0.29 ± 0.06 (n = 10)
fMet-Leu-Phe‡	0.51 ± 0.06 (n = 9)	0.33 ± 0.08 (n = 10)
Percent stimulation	168	14

* The values are means \pm SEM. n, number of experiments, each carried out in duplicate.

‡ The fragments were stimulated with fMet-Leu-Phe (10^{-7} M) for 30 s before the reaction was stopped. 10-, 60-, and 300-s stimulation times were also used with similar results. A doubling of the number of CB-cytoplasts used (which increased the protein per sample to that of the cytokineplasts) did not change the results.

was 24 ± 8 (SEM) and 20 ± 8 , respectively. Stimulation times both shorter (10 s) and longer (60 and 300 s) than the standard 30 s did not result in an increase in actin polymerization by CB-cytoplasts. The failure of normal actin polymerization was not due to its already having been polymerized; the unstimulated actin associated with the cytoskeleton was only a small fraction of the total actin present (Fig. 4, lane B vs. lane A, and Table I).

Nor does it seem to result from significant retention of CB. When we used tritiated CB, counts (cpm/ 2.5×10^5 fragments) fell, after the multiple washes by which fragments are prepared (6), from 12,352 to 69, or 0.6%. As noted earlier (4) and confirmed here, only a small percentage of fragments made in this way and then followed microscopically in warmed sealed preparations, adhered to glass and spread. Some of them exhibited membrane protrusive activity, but translocation did not occur. The 0.6% of initial counts, seen in CB-cytoplasts after washing, is similar to 0.5% (13,475 to 63 cpm/ 2.5×10^5 cells) seen in the

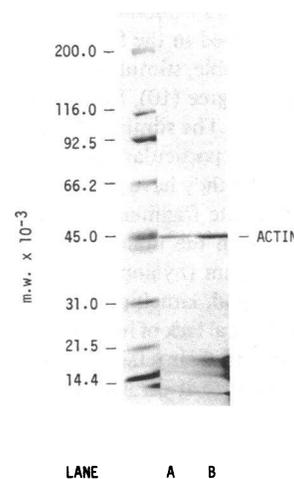


Figure 3. Cytokineplasts: effect of fMet-Leu-Phe on actin association with the cytoskeleton. SDS-polyacrylamide gel electrophoresis (5–15%) of Triton X-100-insoluble cytoskeletons isolated from 5×10^6 control (lane A) and the same number of fMet-Leu-Phe stimulated (lane B) cytokineplasts is seen. The cytokineplasts were given fMet-Leu-Phe (10^{-7} M) for 30 s before the reaction was stopped. Under these conditions, the amount of actin associated with the cytoskeleton, an index of actin polymerization, was doubled.

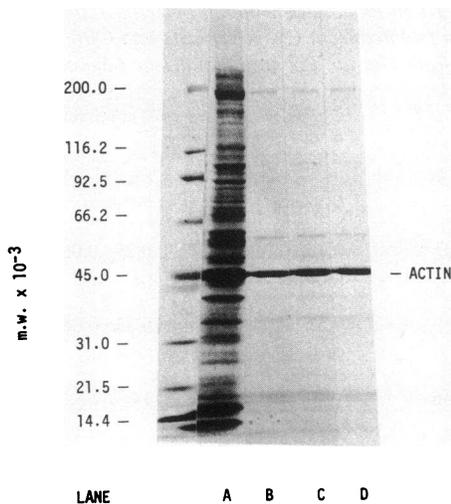


Figure 4. CB-cytoplasts: effect of fMet-Leu-Phe on actin association with the cytoskeleton. SDS-polyacrylamide gel electrophoresis (5–15%) of 5×10^6 unextracted CB-cytoplasts and the same number of Triton X-100-insoluble cytoskeletons is seen. The conditions are as follows: lane A, the whole fragments (no Triton X-100 extraction); lanes B, C, and D, cytoskeletons (Triton X-100-insoluble fraction) isolated from control, fMet-Leu-Phe (10^{-7} M)-stimulated, and PMA ($0.1 \mu\text{g/ml}$)-stimulated CB-cytoplasts, respectively. The CB-cytoplasts were given fMet-Leu-Phe for 30 s and PMA for 2 min before the reaction was stopped. The amount of actin associated with the cytoskeleton increased only slightly (25%) upon stimulation with fMet-Leu-Phe, and not at all with PMA. Note that the unstimulated amount of actin associated with the cytoskeleton (lane B) is only a small fraction of the total actin present (lane A).

parent PMN, whose locomotory behavior, followed microscopically, was intact after washing.

Discussion

Intracellular free calcium. The effects of two commonly used stimuli of intact PMN, the synthetic formyl peptide, fMet-Leu-Phe, and the dihydroxy metabolite of arachidonic acid, leukotriene B₄, on levels of intracellular free calcium in cytokineplasts were measured by the fluorescence of cell-associated Quin-2. As one sees in the parent PMN (10), there was a brisk response to each agonist (Figs. 1 and 2); hence, this transductional signal, and these two sensory triggers, are preserved in the fragments.

When extracellular calcium is unavailable, stimulated intact PMN mobilize free calcium to a lesser degree (10), from intracellular stores whose locations are unclear. The similar response of cytokineplasts (Figs. 1 B and 2 B) is of particular interest in this regard, when one considers how little they have retained of the parent cell's structure. These anucleate fragments contain few or no granules; they are derived from the microfilament-rich, organelle-excluding cortical cytoplasm (hyaloplasm) that is prominent in the leading front (protopod, lamellipodium) of intact, migrating PMN (1–3, 17). This general lack of intracellular organelles in cytokineplasts, combined with their retained ability to store calcium internally and to mobilize it on appropriate stimulation, points to the plasmalemma as a likely calcium depot.

Actin polymerization. Actin is of course essential to cell movement, and one of its critical properties is the ability to

polymerize into filaments capable of bearing tension (15, 18, 19). The increase in cytoskeleton-associated actin in cytokineplasts given fMet-Leu-Phe (Table I, Fig. 3) is of the order seen in intact PMN (15). In contrast, CB-cytoplasts in the same situation increased the amount of actin associated with the cytoskeleton by relatively little (Table I, Fig. 4). This defect was not stimulus-specific; there was no measurable response to PMA in CB-cytoplasts vs. ~30% in intact human PMN (15).

These data may contain an explanation for why cytokineplasts retain the capacity for random locomotion, chemotactic responsiveness, and capping of ligand-receptor complexes, while CB-cytoplasts are defective in each of these motile activities (4, 6), even though the latter fragments retain receptors for fMet-Leu-Phe (i.e., a sensory apparatus) (4) that can respond to stimulation in a number of other ways (6–8). We suggest that in CB-cytoplasts, high-speed centrifugation while microfilaments are in (CB-induced) disarray has resulted in the irreversible loss of normal functioning of the motile machinery of the cell. Although there might also be a transducer defect in CB-cytoplasts, perhaps indicated by the reported lack of mobilizable intracellular calcium (9), a proximal cause of their motile disability may be attributable to compromised effector function (actin polymerization).

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