Is a Rise in Intracellular Concentration of Free Calcium Necessary or Sufficient for Stimulated Cytoskeletal-associated Actin?

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Abstract. The addition of the calcium ionophore A23187 to rabbit neutrophils increases the amount of actin associated with the cytoskeleton regardless of the presence or absence of calcium in the incubation medium. In the presence of extracellular calcium, the effect of A23187 is biphasic with respect to concentration. The action of the ionophore is rapid, transient, and is inhibited by pertussis toxin, hyperosmolarity, and quinacrine. On the other hand, the addition of pertussis toxin or hyperosmolarity has small, if any, effect on the rise in intracellular calcium produced by A23187. While quinacrine does not affect the fMet-Leu-Phe-induced increase in cytoskeletal actin and the polyphosphoinositide turnover, its addition inhibits completely the stimulated increase in Ca-influx produced by the same stimulus. The results presented here suggest that a rise in the intracellular concentration of free calcium is neither necessary nor sufficient for the stimulated increase in cytoskeletal-associated actin. A possible relationship between the lipid remodeling stimulated by chemoattractants and the increased cytoskeletal actin is discussed.

SOME of the neutrophil responses that are activated by the chemotactic factor formylMethionyl-Leucyl-Phenylalanine (fMet-Leu-Phe)[1] such as cell motility, shape change, and the projection of pseudopodia or ruffles depend on the mechanical displacement of part of the cell, or of the whole cell. The cellular contractile apparatus, of which actin and myosin are the major components, is closely involved in these responses, and an understanding of neutrophil activation requires a detailed knowledge of the organization of these proteins before and after stimulation. Actin filaments in neutrophils are considerably more labile than their counterpart in muscle, and large pools of depolymerized actin are usually found in resting cells (Korn, 1982; Pollard, 1975; Southwick and Stossel, 1983).

Recently, it has been shown that the addition of fMet-Leu-Phe to neutrophils causes a rapid polymerization of actin (Roa and Varani, 1982; White et al., 1983*a*; Fechheimer and Zigmond, 1983; Yassin et al., 1985). The signals that initiate actin polymerization are not known, but are important to determine because of the central role of actin in neutrophil functions (Southwick and Stossel, 1983; Yassin et al., 1985; Fox et al., 1984). Under conditions similar to those under which actin gets polymerized, chemotactic factors also increase the hydrolysis of phosphatidylinositol 4,5-*bis*-phosphate and causes a rise in the level of intracellular concentration of free calcium (White et al., 1983; Roa and Varani, 1982; Volpi et al., 1983; White et al., 1983*b*).

The present studies were undertaken to examine the question of whether a rise in the intracellular concentration of free calcium is necessary or sufficient for the stimulated increase in cytoskeletal-associated actin.

Materials and Methods

Rabbit peritoneal neutrophils (4–12-h exudates) were collected and washed as previously described (Yassin et al., 1985; Showell et al., 1979) and the cells were resuspended in protein and magnesium free modified Hanks' balanced salt solution. The composition of this modified Hanks' solution is (mM): NaCl, 124; KCl 5; Na_2HPO_4, 0.64; KH_2PO_4, 0.66; $CaCl_2$, 0.5 (unless stated otherwise); $NaHCO_3$, 15.2; Hepes, 10.0; and glucose 5.56; pH 7.2. A 10-min incubation period at 37°C preceded all experimental manipulations. When pertussis toxin was used, the cell suspensions were divided into two flasks, one containing pertussis toxin (500 ng/ml) and the second left as control. The cells were incubated with the toxin for 1 h at 37°C.

Isolation of Cytoskeletal Proteins

Cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100 as described by Phillips et al. (1980) except that the concentration of EGTA was increased from 5 to 10 mM. The experimental details are the same as previously described (Yassin et al., 1985). Briefly, aliquots (500 μl) from a cell suspension containing 10^7 cells/ml were distributed into various sets of Eppendorf microcentrifuge tubes (1.5 ml capacity). At a preset time after the addition of the stimulus, the reaction was stopped by the addition of 500 μl of cold Triton stock solution which contained 2% Triton X-100, 160 mM KCl, 40

[1] *Abbreviations used in this paper:* fMet-Leu-Phe, formylMethionyl-Leucyl-Phenylalanine; IP_3, inositol, 1,4,5-trisphosphate; PAF, platelet-activating factor; PIP_2, phosphatidylinositol 4,5-*bis*-phosphate; PMA, phorbol 12-myristate, 13-acetate.

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mM imidazole HCl, 20 mM EGTA, and 8 mM sodium azide, pH 7.0. The tubes were placed on ice for 10 min, and then were centrifuged for 6 min (8,000 g). After centrifugation, the supernatant was decanted and the pellet dissolved in 50–100 μl of a solution containing 9% SDS, 13 mM mercaptoethanol, 15% glycerol, and 86 mM Tris-HCl (pH 6.7) by incubating in a boiling water bath with vigorous vortexing until dissolved. Actin was identified by molecular weight and two-dimensional electrophoresis (Yassin et al., 1985). The cytoskeletal proteins were electrophoresed through a 5–15% gradient or 10% straight polyacrylamide slab gel (with 5% polyacrylamide in the stacking gel) according to the method of Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R250, and the absorption of gel bands were measured at 590 nm using a Transidyne 2955 scanning densitometer.

Measurement of Quin-2 Fluorescence

Quin-2 loading and fluorescence were carried out as previously described (White et al., 1983b). Quin-2 fluorescence was recorded using an SLM 8000 photon-counting spectrofluorometer with a temperature-controlled (37°C) and a magnetically stirred cuvette holder. Stimuli were added by a microsyringe directly into the cuvette without interrupting recording. Fluorescence excitation and emission wavelengths were 339 and 492 nm, respectively.

Labeling, Isolation, and Separation of Lipids

Phospholipid labeling with ^{32}P and subsequent isolation were carried out as described previously (Volpi et al., 1983). The reaction was initiated by adding a known volume of the fMet-Leu-Phe to cell suspension (10^7 cells/ml), and it was terminated by adding 5 ml of n-hexane/isopropanol and concentrated HCl (final concentration, 0.1 M) (300:200:4). The various components of lipids were separated on silica gel pre-coated thin layer chromatography plates without fluorescent indicator (Brinkmann Instruments Co., Westbury, NY).

Radioactive Calcium Influx

Radioactive calcium fluxes were measured using the rapid sampling silicone oil method previously described in detail (Naccache et al., 1977). Radiolabeled calcium and the stimulus were added together, and the amount of radioactivity associated with the cells was measured.

Chemicals

EGTA, Hepes, fMet-Leu-Phe, and actin were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chemicals were supplied by Bio-Rad Laboratories (Richmond, CA). Platelet-activating factor (PAF), A23187, and quin-2/AM were purchased from Calbiochem-Behring Corp. (San Diego, CA). Phorbol 12-myristate, 13-acetate was obtained from CMC, Cancer Chemical (Brewster, NY). Pertussis toxin was a gift from Dr. J. Munoz (National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT), and ^{45}Ca was purchased from New England Nuclear (Boston, MA).

Results

Effect of the Calcium Ionophore A23187 on Cytoskeletal Actin

The addition of 500 nM of the ionophore A23187 to rabbit neutrophils causes an increase in the amount of actin associated with the cytoskeleton (Fig. 1). The effect of A23187 is rapid (reached maximum value within the first 40 s), transient, and it does not require the presence of calcium in the suspending medium. Under the same conditions, the addition of 500 nM A23187 causes a rapid and transient increase in the intracellular concentration of free calcium as measured by quin-2 fluorescence (data not shown). In the presence of 1 mM EGTA and no added calcium, the rise in the intracellular calcium concentration caused by the addition of A23187 is smaller but still significant. The effect of varying the concentration of calcium in the suspending medium on the basal and the A23187-stimulated cytoskeletal actin was also investigated and the results are summarized in Fig. 2. The ionophore-induced increase in cytoskeletal actin is dose dependent and requires concentrations in excess of 10^{-8} M (Fig. 3). Note that, at high concentrations of A23187, the presence of calcium in the suspending medium reverses the stimulation of the incorporation of actin in the cytoskeleton.

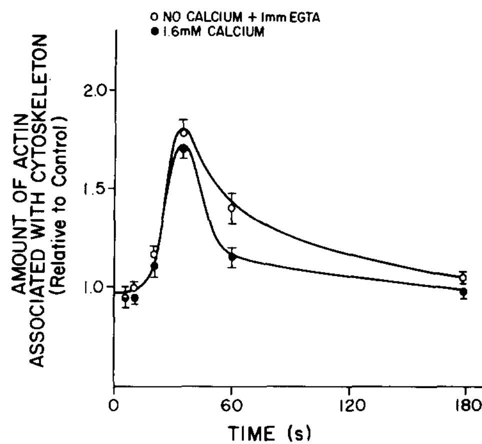

Figure 1. The time course of the A23187-induced increase in the amounts of the actin associated with the cytoskeleton. The concentration of A23187 was 5×10^{-7} M. Each value represents the mean ± SEM of at least three different experiments.

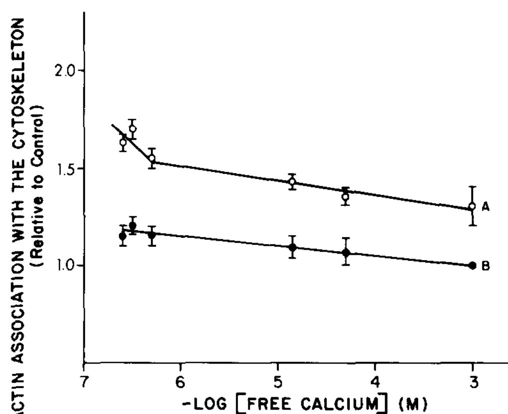

Figure 2. The effect of varying the concentration of calcium in the incubation medium on the cytoskeletal actin in rabbit neutrophils in the presence and absence of the ionophore A23187 (5×10^{-7} M). Each value represents the mean ± SEM of at least three separate experiments. The cells were reacted with the ionophore for 40 s before the reaction was stopped. (*A*) In the presence of the ionophore A23187; (*B*) control cells.

Is a Rise in the Intracellular Concentration of Free Calcium Sufficient to Cause an Increase in Cytoskeletal-associated Actin?

To examine this question, we have carried out three sets of experiments. In the first set, we have tested the effects of pertussis toxin on the increase in cytoskeletal actin and the rise in intracellular concentration of free calcium produced by A23187, PAF, and fMet-Leu-Phe. The results summarized in Table I clearly show that while the addition of pertussis toxin to neutrophils has no effect on the rise in calcium produced by A23187 or PAF as measured by quin-2 fluorescence, it abolishes the increases in cytoskeletal actin produced by these two stimuli.

In the second set, the effect of increasing the medium osmolarity on both the rise in calcium and the increase in cytoskeletal actin produced by A23187 and fMet-Leu-Phe

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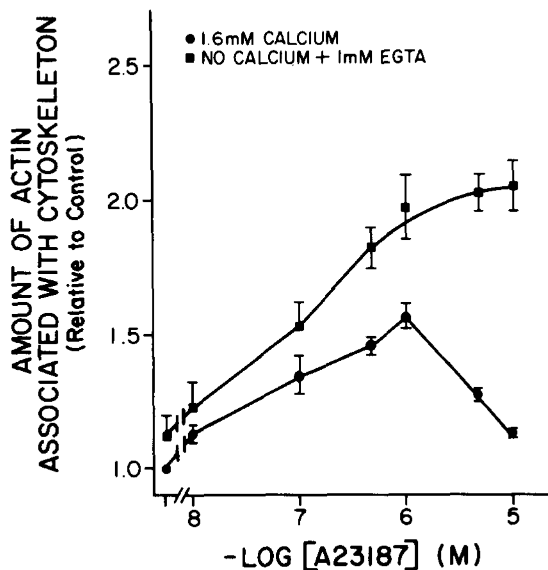

Figure 3. The effects of various concentrations of the ionophore A23187 on the amount of actin associated with the cytoskeleton in rabbit neutrophils. Each value represents the mean ± SEM of at least three different experiments. The cells were reacted with the ionophore for 40 s before the reaction was stopped. EGTA was added 20 s before the ionophore.

Table I. Effect of Pertussis Toxin on the Stimulated Increases in the Intracellular Concentration of Free Calcium and Cytoskeletal Actin

	Control cells		+ Pertussis toxin[(c)*]	
Condition	ΔActin[‡]	Δ[Ca_i][§]	ΔActin	Δ[Ca_i]
No addition	0[‖]	0[‖]	−3 ± 2	−5 ± 3
fMet-Leu-Phe (1 nM)	38 ± 7	390 ± 150	−3 ± 3	10 ± 8
A23187 (500 nM)	20 ± 5	700 ± 200	−2 ± 3	710 ± 150
PAF (10 nM)	30 ± 6	365 ± 70	−5 ± 3	380 ± 80

* The cells were incubated with pertussis toxin (500 ng/ml) for 1 h.

‡ *ΔActin*, cytoskeletal actin under stimulated-control condition expressed as percent of total cell actin.

§ *Δ[Ca_i]*, the difference in the intracellular concentration of free calcium expressed as nM between stimulated and control conditions.

‖ Values were expressed as mean ± SEM of at least three separate experiments. The cells were stimulated with fMet-Leu-Phe for 20 s, A23187 for 40 s, and PAF for 10 s.

were examined. In these experiments, the cells were preincubated for 1 min in Hanks' balanced salt solution to which NaCl was added to give a final concentration of 250 mM. The results are summarized in Table II. Note that while increasing the osmolarity of the incubation medium reduces the rise in the intracellular concentration of free calcium produced by fMet-Leu-Phe only slightly and does not affect that produced by A23187, it inhibits the increase in the cytoskeletal actin produced by these two stimuli drastically.

In the third set, we have examined the effect of quinacrine on the stimulated increase in the amount of actin associated with the cytoskeleton and the rise in intracellular concentration of free calcium. The transient nature and the inhibition by pertussis toxin and hyperosmolarity of the A23187 effect are characteristics usually associated with receptor-mediated effect. As the addition of A23187 to intact neutrophils is known to release arachidonic acid (Takenawa et al., 1983;

Table II. Effect of Hyperosmolarity on the Stimulated Changes of Cytoskeletal Actin and Intracellular Concentration of Free Calcium

	[NaCl] = 124 mM		[NaCl] = 250 mM[‡]	
Condition*	ΔActin[§]	Δ[Ca_i][‖]	ΔActin	Δ[Ca_i]
No addition	0[¶]	0[¶]	−6 ± 3	30 ± 10
fMet-Leu-Phe (10 nM)	42 ± 5	410 ± 100	13 ± 4	350 ± 80
A23187 (500 nM)	24 ± 7	620 ± 122	2 ± 2	710 ± 150

* The cells were stimulated with fMet-Leu-Phe for 20 s and with A23187 for 40 s.

‡ The cells were incubated for 1 min with Hanks' buffered solution in which the concentration of NaCl was increased to 250 mM.

§ *ΔActin*, cytoskeletal actin under stimulated-control conditions expressed as percent of total cell actin.

‖ *Δ[Ca_i]*, the difference in the intracellular concentration of free calcium expressed as nM between stimulated and control conditions.

Table III. Effect of Quinacrine on the Basal and Stimulated Levels of Cytoskeletal Actin

	Increase in cytoskeletal actin in percent of total cell actin	
Stimulus	Control cells	+ Quinacrine*
No addition	0.0	0.5 ± 0.5
+ fMet-Leu-Phe (5 nM)	44 ± 10	50 ± 12
+ Arachidonic Acid (2 μM)	40 ± 8	37 ± 5
+ A23187 (500 nM)	37 ± 6	3 ± 3

* Cells were preincubated with quinacrine (10^{-4} M) for 5 min before they were stimulated. The cells were stimulated with fMet-Leu-Phe and arachidonic acid for 20 s and with A23187 for 40 s. Values expressed as mean ± SEM of at least three separate experiments.

Volpi et al., 1985), it is possible that the observed increases of cytoskeletal actin caused by the ionophore results indirectly from the generation of one or more lipid mediators. To test this hypothesis, the effect of quinacrine, a compound with antiphospholipase activity (Hirata et al., 1979), on the stimulated amount of actin associated with the cytoskeleton was investigated (Table III). The data presented in Table III demonstrates that quinacrine inhibits the stimulation of the cytoskeletal-associated actin that is induced by A23187 while leaving unaffected the responses of fMet-Leu-Phe and arachidonic acid. The effect of quinacrine (it is a strongly fluorescent compound) on calcium influx as a measure of the rise in intracellular concentration of free calcium produced by A23187 and fMet-Leu-Phe was also investigated. The results summarized in Fig. 4 clearly show that unlike increased actin association with the cytoskeleton, the A23187-induced rise in intracellular calcium (as measured by Ca-influx) is only partially inhibited by quinacrine. These results further support the conclusion that a rise in calcium is not sufficient for actin polymerization. Note that while quinacrine had no effect on the fMet-Leu-Phe–stimulated increase in cytoskeletal actin, it inhibited the rise in the intracellular concentration of free calcium (as measured by Ca-influx) produced by the same stimulus.

Is a Rise in the Intracellular Concentration of Free Calcium Necessary for Stimulated Cytoskeletal-associated Actin?

To examine the question of whether or not a rise in the intracellular concentration of free calcium is necessary for

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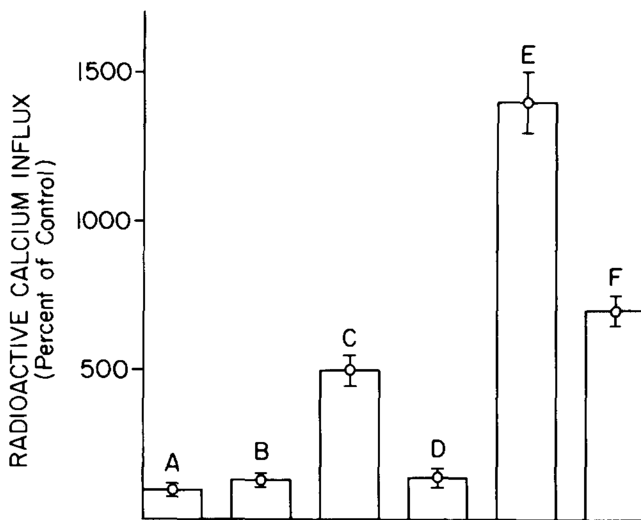

Figure 4. Effect of pre-treating the cells with quinacrine (10^{-4} M) for 5 min on ^{45}Ca-influx in rabbit neutrophils stimulated by fMet-Leu-Phe (5×10^{-9} M) or A23187 (5×10^{-7} M). The cell-associated radioactive calcium was determined 1 min after the addition of the radioactivity and the stimulus. Each value represents the mean ± SEM of at least three separate experiments. (*A*) Control; (*B*) quinacrine; (*C*) fMet-Leu-Phe; (*D*) fMet-Leu-Phe + quinacrine; (*E*) A23187; (*F*) A23187 + quinacrine.

Table IV. Effects of PMA on the Amount of Actin Associated with the Cytoskeleton Isolated from Rabbit Neutrophils under Control, Pertussis Toxin, and Hyperosmotic Conditions

	Cytoskeletal actin relative to control		
Stimulus	Control	+ Pertussis toxin*	+ Hyper-osmostic‡
No addition	1.0	0.90 ± 0.11	0.83 ± 0.15
PMA (0.1 μg/ml)§	1.50 ± 0.13	1.43 ± 0.12	1.34 ± 0.10

* The cells were incubated with pertussis toxin (500 ng/ml) for 1 h.

‡ The cells were incubated with the hyperosmotic Hanks' buffered solution (the solution was made hyperosmotic by increasing the NaCl concentration to 250 mM) for 1 min.

§ The cells were stimulated with PMA for 2 min. The values are the mean ± SEM of at least three separate experiments and the results are significantly different from control ($P > 0.001$).

stimulated cytoskeletal-associated actin, the effect of phorbol 12-myristate, 13-acetate (PMA) on the amount of actin associated with the cytoskeleton was examined. The results summarized in Table IV clearly show that the addition of PMA to rabbit neutrophils causes a significant increase in the cytoskeletal actin that is inhibited neither by pertussis toxin nor by hyperosmolarity. In many systems including the neutrophils, it is generally observed that this concentration of PMA does not cause a rise in the quin-2 signal (Sha'afi et al., 1983; Rink et al., 1983).

Effect of Quinacrine on fMet-Leu-Phe-induced Phosphoinositide Turnover

The addition of the quinacrine inhibits the fMet-Leu-Phe-induced increase in calcium influx almost completely. It is generally accepted that the rise in intracellular concentration of free calcium produced by various soluble stimuli in many cells including the neutrophils is mediated by the generation of inositol 1,4,5-trisphosphate (IP_3) from the hydrolysis of

Table V. Effect of Quinacrine on fMet-Leu-Phe Stimulated Phosphoinositide Turnover

	^{32}P-Radioactivity relative to control			
Lipid*	Control cells		+ Quinacrine-treated cells	
	15 s‡	*40 s*‡	*15 s*	*40 s*
PIP_2	0.84 ± 0.04	0.96 ± 0.03	0.86 ± 0.05	0.94 ± 0.04
PIP	0.88 ± 0.06	0.70 ± 0.10	0.87 ± 0.04	0.68 ± 0.06
PI	0.96 ± 0.08	1.08 ± 0.10	0.99 ± 0.05	0.96 ± 0.08
PA	1.29 ± 0.05	1.96 ± 0.10	1.25 ± 0.08	2.10 ± 0.12

* PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidate.

‡ Time (in seconds) after the addition of fMet-Leu-Phe (10 nM); the cells were treated with quinacrine (10^{-4} M) for 5 min; each value represents the mean ± SEM of at least three experiments and each experiment was carried out in duplicate. Addition of quinacrine alone produces no change.

phosphatidylinositol 4,5,*bis*-phosphate (PIP_2) through the action of phospholipase C (Berridge, 1984). Because of this, the effect of quinacrine on the hydrolysis of PIP_2 produced by fMet-Leu-Phe was investigated (Table V). The results clearly show that the addition of quinacrine does not inhibit the breakdown of PIP_2, PIP, and the generation of phosphatidate.

Discussion

The results reported here demonstrate that the addition of A23187 to rabbit neutrophils increases the amount of actin associated with the cytoskeleton. In the presence of extracellular calcium, the effect of A23187 is biphasic with respect to the concentration of the ionophore. The progressive decrease in cytoskeletal actin produced by high concentrations of A23187, in the presence of Ca^{2+}, is probably due to the direct effect of high intracellular calcium ion concentration on actin polymerization. It is known that increasing the concentration of Ca^{2+} reduces the amount of actin associated with the cytoskeleton even after the extraction step with the triton X-100 solution (White et al., 1983*a*).

The action of A23187 on the cytoskeletal actin is probably indirect and mediated by the generation of lipid mediator(s). This conclusion is based on several lines of evidence. First, the effect of A23187 is transient, inhibited by pertussis toxin, hyperosmolality, and the phospholipase A_2 inhibitor, quinacrine. Second, cells pre-treated with A23187 desensitized to the subsequent addition of A23187 (data not shown) with respect to the cytoskeletal response.

The addition of quinacrine to rabbit neutrophils inhibits the increase in calcium influx produced by fMet-Leu-Phe. This effect is not due to an inhibition of PIP_2 hydrolysis.

Based on the data presented here it is reasonable to conclude that a rise in the intracellular concentration of free calcium alone is not sufficient to initiate the stimulated cytoskeletal-associated actin. This conclusion is based on several experimental findings which demonstrate that it is possible to have rises in calcium level in the absence of an increase in cytoskeletal actin. First, incubation of the rabbit neutrophils with pertussis toxin inhibits the increase in cytoskeletal actin but not the rise in intracellular calcium produced by the calcium ionophore A23187 and the PAF. Second, increasing the osmolarity of the Hanks' buffer by increasing the NaCl concentration to 250 mM inhibits drastically the increase in cytoskeletal actin but decreases only slightly the rise in calcium produced by A23187 and fMet-Leu-Phe. Third, incubation

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of the cells with the quinacrine inhibits totally the increase in the amount of actin associated with the cytoskeleton and only partially the rise in calcium produced by A23187.

Conversely, since it is possible to find conditions under which it is possible to elicit an increase in the cytoskeletal actin without a measurable rise in the intracellular concentration of free calcium, it is reasonable to suggest that the latter event is not absolutely required for actin polymerization. This conclusion is supported by two sets of experimental findings. First, PMA increases cytoskeletal actin without elevating intracellular calcium. Second, the increase in calcium influx but not in the amount of actin associated with the cytoskeleton produced by fMet-Leu-Phe is inhibited by quinacrine. It must be clearly pointed out that one cannot completely rule out the possibility that a small, undetected shift in the distribution of intracellular calcium is necessary for stimulated cytoskeletal-associated actin.

As stated in the introduction, the chemotactic factor fMet-Leu-Phe, under conditions similar to those under which it stimulates the amount of actin associated with the cytoskeleton, causes an increase in phosphoinositide turnover in addition to calcium rise. The elevation of intracellular concentration of free calcium is most likely due to the action of IP_3. Since the calcium rise alone does not seem to be necessary or sufficient for stimulated cytoskeletal actin, we would like to propose that stimulated phosphoinositide turnover may be a crucial step in actin polymerization. The finding that quinacrine has no effect on stimulated cytoskeletal actin or phosphoinositide turnover produced by fMet-Leu-Phe supports this hypothesis. Also, consistent with this idea is the observation that the addition of phospholipase C to intact neutrophils or of GTPγS to permeabilized cells increases the cytoskeletal actin (unpublished data). This idea is the subject of further studies.

This work is supported in part by grants AI-13734, GM 17536 and AM 31000 from the National Institutes of Health.

Received for publication 9 August 1985, and in revised form 8 November1985.

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