

Effects of Chemotactic Factors and Other Agents on the Amounts of Actin and a 65,000-mol-wt Protein Associated with the Cytoskeleton of Rabbit and Human Neutrophils

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ABSTRACT Stimulation of rabbit neutrophils by the chemotactic factors fMet-Leu-Phe and leukotriene B₄, by platelet activating factor, or by arachidonic acid produces a rapid and dose-dependent increase in the amounts of actin and of a 65,000-mol-wt protein associated with the cytoskeleton. Phorbol 12-myristate, 13-acetate, the calcium ionophore A23187 in the presence or absence of EGTA, and the fluorescent calcium chelator quin-2 also cause an increase in cytoskeletal actin. The stimulated increases in the cytoskeletal actin are not dependent on a rise in the intracellular concentration of free calcium and are not mediated by an increase in the intracellular pH or activation of protein kinase C. The increases in the cytoskeletal actin produced by fMet-Leu-Phe and leukotriene B₄, but not by phorbol 12-myristate, 13-acetate, are inhibited by high osmolarity. The effect of hyperosmolarity requires a decrease in cell volume, is not mediated by an increase in basal intracellular concentration of free calcium, and is not prevented by pretreating the cells with amiloride. Preincubation of the cells with hyperosmotic solution also inhibits degranulation produced by all the stimuli tested. The inhibitory action of high osmolarity on the fMet-Leu-Phe and leukotriene B₄ induced stimulation of cytoskeletal actin is discussed in terms of the possibility that the addition of high osmolarity, either directly or through activation of protein kinase C, causes receptor uncoupling.

Most of the neutrophil responses that are induced by chemotactic factors, such as cell motility, shape change, projection of pseudopodia or ruffles, phagocytosis, and aggregation depend on the mechanical displacement of part or all of the cell. Accordingly, the cellular contractile apparatus, of which actin and myosin are the major components, must be closely involved in these responses. An understanding of neutrophil activation thus requires a detailed knowledge of the organization of these proteins before and after stimulation. Actin filaments in neutrophils, as in most nonmuscle cells, are considerably more labile than their counterpart in muscle, and large pools of depolymerized actin are usually found in resting cells (10, 16, 22).

It has been found that the chemotactic responses of neutrophils are inhibited when the cells are incubated in hyperosmotic buffered solutions (2, 9). The inhibition of the functions of neutrophils by high osmolarity is particularly pertinent to

areas of the kidney and urinary tract, where solute concentrations such as NaCl, urea, and glucose (in the case of diabetics) are high. It has been suggested that the susceptibility to infection of the medulla region of the renal system is mediated by the inhibitory effect on neutrophil functions of the hyperosmotic medullary environment (1, 7, 9). The molecular basis of this inhibition is not known at the present.

Recently, it has been shown that the addition of the chemotactic factor fMet-Leu-Phe to neutrophils causes actin polymerization (5, 17, 26, 28). This increase in polymerized actin is rapid, reaching a maximum within 20 s, and is dose dependent. Because of the probable central role of this event in neutrophil function, it is important to determine the factors that initiate and regulate this change (5, 6, 22, 28, 29). At present, the nature of the intracellular signal that initiates the observed stimulation of actin polymerization is not known.

We undertook the present studies to investigate the effect

of fMet-Leu-Phe and of other agents on the organization of the neutrophils' cytoskeleton, and in particular on actin and a 65,000-mol-wt protein, and to investigate the effect of medium osmolarity on their association with the cytoskeletal proteins.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils (4–12-h exudates) were obtained and washed as previously described (14, 21), and the cells were resuspended in protein- and magnesium-free modified Hanks' balanced salt solution. The composition of this modified Hanks' solution (pH 7.2) is (millimolar): NaCl, 124; KCl, 5; Na₂HPO₄, 0.64; KH₂PO₄, 0.66; CaCl₂, 1.6 (unless otherwise stated); NaHCO₃, 15.2; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10.0; and glucose, 5.56. A 5–10-min incubation at 37°C preceded all experimental manipulations. The osmolarity of the modified Hanks' solution was increased by the addition of NaCl, glucose, or urea. Human neutrophils were isolated on Ficoll/Hypaque gradients as described by English and Anderson (4).

Isolation of Cytoskeletal Proteins: Cytoskeletal structures were isolated as proteins insoluble in 1% Triton X-100 as described by Phillips et al. (15) and Jennings et al. (8) except that the concentration of EGTA was increased from 5 to 10 mM. The experimental details are the same as previously described (28). In brief, 500- μ l aliquots from a cell suspension containing 10⁷ cells/ml were distributed into various sets of Eppendorf microcentrifuge tubes (1.5-ml capacity). One set was always used as a control. The reaction was stopped by the addition of 500 μ l of cold Triton stock solution which contained 2% Triton X-100, 160 mM KCl, 40 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 centrifuged for 10 min (8,000 g) in an Eppendorf microfuge. In some experiments the tubes were centrifuged for 45 min at 150,000 g. The supernatant was then decanted, and the pellet was dissolved in 50–100 μ l of a solution containing 9% SDS, 13 mM mercaptoethanol, 15% glycerol, and 86 mM Tris HCl (pH 6.7) by incubation in a boiling water bath with vigorous vortexing until dissolved, usually 10–15 min. Actin was identified by molecular weight and two-dimensional electrophoresis (28).

Distribution of Actin between the Cytosol and Cytoskeleton: The percentage of actin associated with the cytoskeleton and the cytosol under control and stimulated conditions was determined as described previously (28). In brief, a cell suspension (5 \times 10⁷ cells/ml) was distributed into two sets of Eppendorf tubes (500 μ l/tube). One set was used to isolate the cytoskeletons, the second set to determine total cell actin. Triton X-100 (500 μ l of stock) was added to the first set and the tubes were transferred to ice. The two sets of tubes were then centrifuged as described previously. 500 μ l of supernatant solution was removed from the first set and added to 150 μ l of stopping solution (9% SDS, 13 mM mercaptoethanol, 15% glycerol, and 86 mM Tris HCl, pH 6.7) and boiled for 5 min. This gave an index of the amount of actin left in the cell cytosol after the cytoskeleton was extracted. The remaining supernatant of all the tubes was decanted and the pellets were dissolved in 1.3 ml of stopping solution and boiled for 10–15 min. Aliquots (130 μ l) of each sample were applied to the electrophoresis gel for analysis. The distribution of actin in the two compartments (cytoskeleton and cytosol) was quantitatively determined as previously described (28).

PAGE: 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 (11). Proteins were stained with Coomassie Brilliant Blue R250 and the absorption of gel bands was measured at 590 nm using a Transidyne 2955 scanning densitometer (Transidyne General Corp., Ann Arbor, MI).

Fluorescence Measurements: The changes in the level of intracellular free calcium ions were measured using the fluorescent probe quin-2. Cells were loaded with quin-2 as described previously (20, 24, 27), and fluorescence was measured with an SLM (model 8000) spectrofluorometer with temperature controlled cuvette and magnetically driven stirrer (SLM Instruments, Inc., American Instrument Co., Urbana, IL). The free calcium concentration in the incubation medium was calculated as described previously (27).

Degranulation: Neutrophil degranulation was measured as previously described (21). In brief, the cells were allowed to equilibrate at 37°C for 10–15 min. The cells were then transferred to tubes containing serial dilutions of various stimuli and of cytochalasin B (5 μ g/ml), if so desired. The reaction was allowed to proceed for 5 min, except when phorbol 12-myristate 13-acetate (PMA)¹ was the stimulus, in which case the incubation lasted for 15 min. The

¹ Abbreviations used in this paper: PAF, platelet-activating factor; PMA, phorbol 12-myristate, 13-acetate.

cells were then centrifuged, and the amount of lactate dehydrogenase, lysozyme, and *N*-acetyl- β -glucosaminidase present in the supernates was determined. In all of the experiments to be reported here, the release of lactate dehydrogenase was <6% of the total cell contents, and no differences were noted between the control and the experimental conditions.

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). Quin-2/AM was purchased from Lancaster Synthesis Ltd. (Lancashire, England). Platelet activating factor (PAF) was purchased from Calbiochem-Behring Corp. (San Diego, CA). Dicaprin was obtained from Nu Check Prep, Inc. (Elysian, MN).

RESULTS

Effect of fMet-Leu-Phe on the Amounts Associated with the Cytoskeleton of Actin and a 65,000-mol-wt Protein

The amounts of actin in human and rabbit neutrophils expressed as micrograms per 10⁶ cells were 0.99 \pm 0.2 (5 experiments) and 5.49 \pm 0.7 (16 experiments), respectively. In resting human neutrophils, actin was distributed between the cytosol (65 \pm 16%) and the cytoskeleton (35 \pm 15%). The corresponding distribution for rabbit neutrophils was 70 \pm 7% in the cytosol and 30 \pm 6% in the cytoskeleton. On the other hand, when the neutrophils were stimulated with the chemotactic factor fMet-Leu-Phe for 20 s, cytoskeletal actin increased to 85 \pm 15 and 71.7 \pm 6% of the total cell actin in human and rabbit cells, respectively (Fig. 1). These distributions were obtained under conditions where the cell suspensions, after Triton X-100 extraction, were centrifuged for 10 min at 8,000 g. A second experiment was carried out under conditions where the suspensions after Triton X-100 extrac-

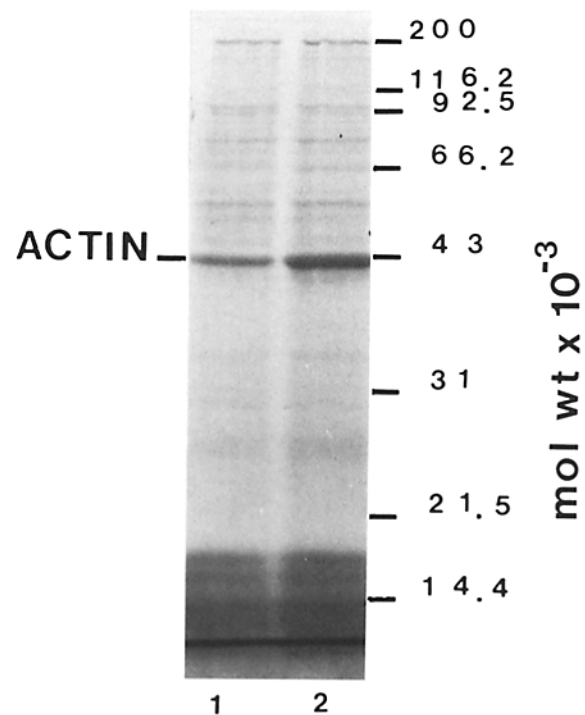


FIGURE 1 SDS PAGE (5–15%) of Triton-insoluble cytoskeletons isolated from control and fMet-Leu-Phe (10⁻⁹ M)-treated rabbit neutrophils. Lanes 1 and 2 show the protein patterns of the cytoskeletons from control and fMet-Leu-Phe-stimulated cells, respectively. The same amount of protein was added to each lane. The numbers on the side of the gel refer to the positions of the molecular weight standards.

tion were centrifuged for 45 min at 150,000 g. The distribution of actin between the cytosol and the cytoskeleton obtained using high speed centrifugation under both control and stimulated conditions was not significantly different from those obtained under low speed centrifugation.

Furthermore, the addition of fMet-Leu-Phe to rabbit neutrophils increased the amount of a 65,000-mol-wt protein associated with the cytoskeleton (Figs. 2 and 3). Note that the effects were rapid and transient, reaching maximum values within 20 s.

Effect of Different Stimuli on the Amounts of Actin and a 65,000-mol-wt Protein Associated with the Cytoskeleton

The effects of various chemotactic factors and other agents on the amounts of actin and the 65,000-mol-wt protein associated with the cytoskeleton of rabbit neutrophils have been investigated, and the results are summarized in Table I. All of the stimuli tested rapidly and dose dependently increased the cytoskeletal amounts of both of these proteins. Note that the addition of A23187 increases actin association with the cytoskeleton both in the presence and absence of calcium in the suspending medium. We have found that

dimethylsulfoxide at concentrations >0.05% produces a dose-dependent increase in cytoskeletal actin. The values reported for the A23187 effect were corrected for the action of dimethylsulfoxide. In the case of leukotriene B₄, correction also should be made for the effect of the solvent, since methanol at a concentration >0.1% causes a significant increase in cytoskeletal actin. Again, the values reported were corrected for methanol action. The effect of PMA is dose dependent (0.01–1.0 μg/ml) and, unlike that of fMet-Leu-Phe, its action is slow and sustained. The time required to obtain maximum effect depends on the concentration used (a lower concentration requires a longer incubation). At the lowest concentration tested, the maximum change is reached by 6 min. The largest increase is obtained by 0.1 μg/ml PMA for 2 min. The effect of A23187 is dose dependent, producing its maximum effect at 10⁻⁶ M and, like that of fMet-Leu-Phe, its effect is transient. Note that we have found that the addition of 5 × 10⁻⁷ M of A23187 causes a significant (>100%) increase in the amount of [³H]arachidonic acid released from prelabeled rabbit neutrophils (data not shown). The increase is rapid (<30 s) and

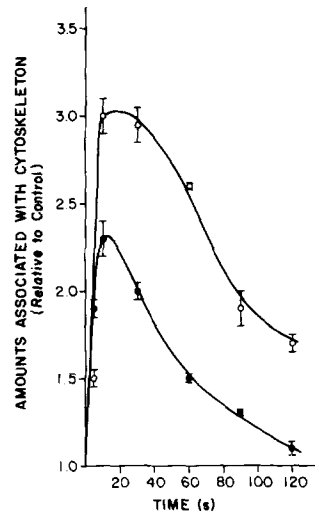


FIGURE 2 The time course of the stimulated increase in the amounts of the 65,000-mol-wt protein and actin associated with the cytoskeleton. The concentration of fMet-Leu-Phe was 10⁻⁹ M. Each value represents the mean ± SEM of three different experiments. ○, the 65,000-mol-wt protein; ●, actin.

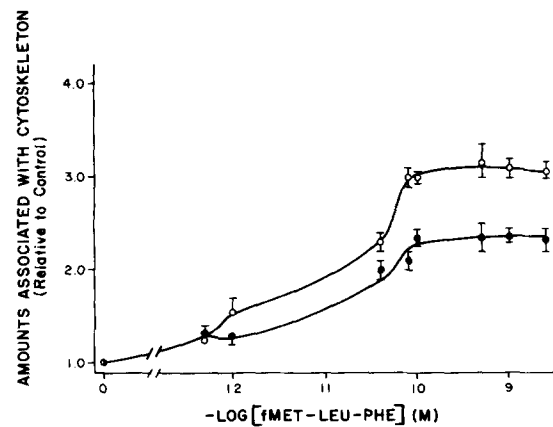


FIGURE 3 The effect of the synthetic peptide fMet-Leu-Phe on the amounts associated with the cytoskeleton of a 65,000-mol-wt protein and of actin. The rabbit neutrophils were allowed to react with the stimulus for 20 s before the reaction was stopped. Each value represents the mean ± SEM of three different experiments. ○, 65,000-mol-wt protein; ●, actin.

TABLE I. Effect of Various Chemotactic Stimuli and Other Agents on the Amounts of Actin and a 65,000-mol-wt Protein Associated with the Neutrophils' Cytoskeleton

Stimulus*	Amounts associated with cytoskeleton relative to control		
	Actin		65,000/Rabbit
	Human	Rabbit	
No addition	1.0	1.0	1.0
fMet-Leu-Phe	2.2 ± 0.15 (9)	2.2 ± 0.1 (40)	2.8 ± 0.2 (8)
Leukotriene B ₄	1.4 ± 0.02 (2)	1.50 ± 0.11 (28)	2.0 ± 0.1 (3)
Arachidonic acid (2 × 10 ⁻⁶ M)	1.5 ± 0.05 (4)	1.6 ± 0.12 (24)	2.1 ± 0.15 (3)
PMA (0.1 μg/ml)	1.3 ± 0.05 (3) [‡]	1.50 ± 0.06 (10)	1.9 ± 0.15 (5)
A23187 (5 × 10 ⁻⁷ M)	—	1.55 ± 0.1 (4)	1.7 ± 0.15 (5)
A23187 + EGTA	—	1.68 ± 0.1 (4)	1.9 ± 0.08 (11)
Quin-2 (0.3 mM) [§]	—	1.20 ± 0.05 (6)	1.8 ± 0.2 (11)
fMet-Leu-Phe + Quin-2	—	2.60 ± 0.10 (6)	2.9 ± 0.3 (11)

* The cells were reacted with fMet-Leu-Phe, leukotriene B₄, or arachidonic acid for 20 s, with A23187 for 40 s, and with PMA for 120 s before the reaction was stopped. The molar concentrations of fMet-Leu-Phe and leukotriene B₄ were as follows: human, 10⁻⁷ and 7 × 10⁻⁸, and rabbit, 10⁻⁹ and 7 × 10⁻⁹. In the case of EGTA, the cells were preincubated in Hanks' buffer containing 0 Ca²⁺ and 1 mM EGTA for 1 min before stimulation with A23187. Each value represents the mean ± SEM, and the number in parentheses is the number of different experiments.

[‡] The concentration of PMA was 1 μg/ml and the cells were preincubated with PMA for 60 s.

[§] The value refers to the intracellular concentration of quin-2. The results are significantly (P < 0.05) different from control.

dose dependent ($>10^{-8}$ M). These results are similar to those reported by Takenawa et al. (23). The increase in cytoskeletal actin by A23187 may be indirect and is mediated through the generation of arachidonic acid.

The effect of the PAF on the cytoskeletal actin was also investigated. The effect was rapid (maximum at 10 s), transient (returning to basal value by 1 min), and dose dependent (0.1–100 nM). The addition of 100 nM PAF causes a 70% increase in the cytoskeletal actin.

To investigate a possible role of the Na^+/H^+ antiport system in the observed increase in actin polymerization, we examined the effect of amiloride on the basal and fMet-Leu-Phe-stimulated level of cytoskeletal actin. The addition of amiloride (0.01–1 mM) did not affect the basal or the stimulated amount of actin associated with the cytoskeleton (data not shown). Varying the pH of the suspending medium (6.8–7.6) did not affect the fMet-Leu-Phe-stimulated increase in cytoskeletal actin (data not shown). The basal level of cytoskeletal actin remained unchanged when the external pH was increased from 7.0 to 7.6, and it decreased by $\sim 10\%$ when the external pH was decreased to <7.0 .

Effect of Hyperosmolarity on Actin Association with the Cytoskeleton

Since increasing medium osmolarity is known to inhibit the chemotactic and phagocytic activities of neutrophils, the effect of varying medium osmolarity on the degree of actin association with the cytoskeleton was studied. In the first set of experiments, the cells (5×10^6 cells/ml) were incubated for 1 min in Hanks' buffered solution containing different concentrations of NaCl, and then stimulated with fMet-Leu-Phe. The results of these experiments are shown in Fig. 4A. The observed inhibition was not due to the extraction procedure. We ruled this out by performing a second set of experiments in which the cells were first stimulated while being incubated in Hanks' buffered solution containing the normal concentration of NaCl, and then stopping the reaction by the addition

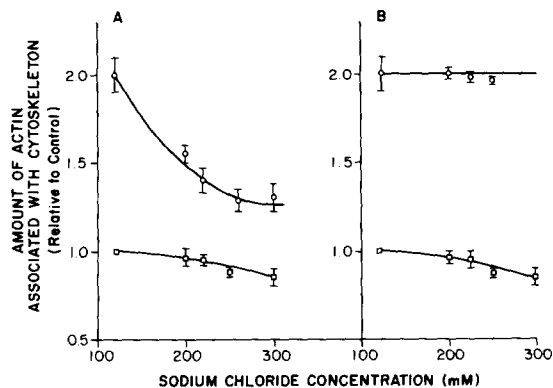


FIGURE 4 The effect of increasing the NaCl concentration in the incubation medium on the amount of cytoskeletal actin under control (\square) and stimulated (\circ) conditions. The fMet-Leu-Phe concentration was 10^{-9} M and the cells were stimulated with fMet-Leu-Phe for 1 min before the reaction was stopped. Each value represents the mean \pm SEM of at least three separate experiments. In A, the cells were incubated for 1 min with Hanks' buffered solution containing the desired concentration of NaCl before being stimulated. In B, the cells were incubated in Hanks' buffered solution containing the normal concentration of NaCl and then stimulated. The reaction was stopped by the addition of Triton X-100 stopping solution that contained the desired concentration of NaCl.

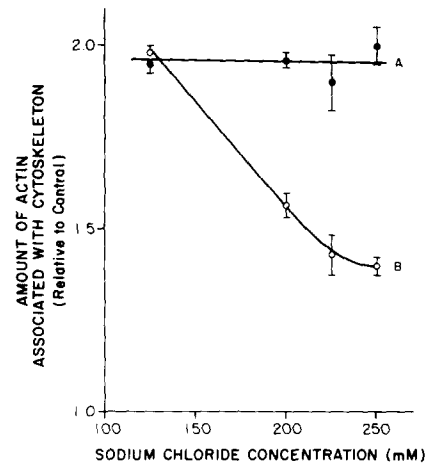


FIGURE 5 Reversibility of the inhibitory action of high concentration of NaCl. Curve A, the cells were incubated for 1 min in Hanks' buffered solution containing the desired concentration of NaCl. The cells were washed extensively with Hanks' buffered solution containing the normal concentration of NaCl, resuspended in the isotonic buffer, and then stimulated with fMet-Leu-Phe (10^{-9} M) for 1 min. Curve B, the cells were treated the same except that they were always suspended in Hanks' solution containing the desired concentration of NaCl. Each value represents the mean \pm SEM of at least three separate experiments.

of Triton X-100 solution that contained different concentrations of NaCl. As shown in Fig. 4B, increasing the NaCl concentrations in the extraction solution did not inhibit the chemotactic factor-induced increase in the cytoskeletal actin. This effect of osmolarity is reversible, as shown in Fig. 5. The order of addition of the fMet-Leu-Phe and the hyperosmotic NaCl solution to the cells was not important. The inhibition is evident at all the concentrations of fMet-Leu-Phe tested and is not prevented by the addition of 1 mM amiloride (Table II). Unlike fMet-Leu-Phe, which causes a rapid decrease followed by an increase in intracellular pH (12, 18), the addition of hyperosmotic solution of either NaCl or glucose causes an amiloride-sensitive increase in intracellular pH (unpublished data).

The values of the intracellular concentration of free calcium as measured by quin-2 under isotonic (124 mM NaCl) and hypertonic (250 mM NaCl) conditions were as follows (nanomolar): basal, 220 ± 40 and 240 ± 55 , respectively; fMet-Leu-Phe (10^{-9} M), 650 ± 70 and 500 ± 80 , respectively. Consistent with these results, we found that the breakdown of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bis phosphate, and the production of phosphatidic acid produced by fMet-Leu-Phe are slightly inhibited (35%) by high osmolarity (250 mM NaCl; data not shown). However, we should point out that when the concentration of NaCl in the hypertonic buffered solution was increased to 375 mM, the basal value of the intracellular concentration of free calcium was found to be 335 ± 70 nM. Furthermore, the commonly observed fMet-Leu-Phe-induced increases in calcium metabolism as measured by both increased ^{45}Ca -influx and the rise in quin-2 signal were inhibited ($>80\%$) by hyperosmolarity (375 mM NaCl).

Effect on the Cytoskeletal Actin of Increasing Osmolarity by the Addition of Urea or Glucose

To distinguish between a decrease in cell volume and a

TABLE II. Effect of Hyperosmolarity on the Increases in the Cytoskeletal Actin Produced by Various Concentrations of fMet-Leu-Phe in the Presence and Absence of Amiloride

Condition	Cytoskeletal actin (relative to control)	
	[NaCl] = 124	[NaCl] = 250
Control	1.00	0.85 ± 0.10 (3)
+ fMet-Leu-Phe (1 × 10 ⁻¹⁰ M)*	1.48 ± 0.15 (3)	0.97 ± 0.15 (3)
+ fMet-Leu-Phe (5 × 10 ⁻¹⁰ M)	1.69 ± 0.12 (4)	1.03 ± 0.15 (3)
+ fMet-Leu-Phe (1 × 10 ⁻⁹ M)	1.84 ± 0.20 (8)	1.15 ± 0.12 (8)
+ fMet-Leu-Phe (1 × 10 ⁻⁸ M)	1.87 ± 0.10 (6)	1.24 ± 0.10 (6)
No addition	1.0	0.87 ± 0.08 (3)
+ Amiloride (1 mM)*	0.93 ± 0.1 (2)	0.84 ± 0.09 (5)
+ fMet-Leu-Phe (10 ⁻⁹ M for 20 s)	2.10 ± 0.15 (7)	1.30 ± 0.10 (10)
+ Amiloride + fMet-Leu-Phe (20 s)	2.0 ± 0.1 (2)	1.28 ± 0.10 (6)
+ fMet-Leu-Phe (10 ⁻⁹ M for 40 s)	1.85 ± 0.1 (3)	1.21 ± 0.05 (6)
+ Amiloride + fMet-Leu-Phe (40 s)	1.80 ± 0.1 (2)	1.18 ± 0.04 (6)
+ fMet-Leu-Phe (10 ⁻⁹ M for 60 s)	1.80 ± 0.08 (11)	1.10 ± 0.06 (6)
+ Amiloride + fMet-Leu-Phe (60 s)	1.77 ± 0.07 (2)	1.11 ± 0.08 (5)

* The cells were incubated with the stimulus for 1 min. Each value represents the mean ± SEM, and the number in parentheses is the number of different experiments. The values obtained in the presence of fMet-Leu-Phe and NaCl are significantly ($P < 0.001$) different from the values obtained in the presence of fMet-Leu-Phe alone. The cells were incubated with the hypertonic NaCl solution for 1 min before fMet-Leu-Phe was added.

* The cells were reacted with amiloride for 1 min before hypertonic NaCl was added, and the cells remained for an additional min.

direct action of the high osmolarity, the hyperosmolar solution was made by the addition of either urea, a highly permeable substance (19), or glucose. The cells were incubated for 20 s with Hanks' buffered solution containing the normal concentration of NaCl to which 252 mM urea or glucose had been added. Because water crosses the cell membrane of rabbit neutrophils at a very high rate, the cell volume will not change in the presence of urea and will decrease in the presence of glucose. The results, summarized in Table III, show clearly that a decrease in cell volume is necessary for the observed inhibition by hyperosmolarity.

High Salt Concentration and the Increase in the Cytoskeletal Actin Produced by Other Stimuli

The effects of high osmolarity on the stimulated increases in the cytoskeletal actin produced by various stimuli were also investigated. The results, summarized in Table IV, clearly show that, unlike the effects of fMet-Leu-Phe and leukotriene B₄, the increases produced by PAF or PMA are only slightly inhibited by the presence of the high NaCl concentration.

Effect of Hyperosmolarity on Neutrophil Degranulation Produced by fMet-Leu-Phe, Leukotriene B₄, A23187, and PMA

We investigated the effect of increasing medium osmolarity on degranulation produced by various stimuli. The cells were

preincubated in Hanks' buffered solution containing 250 mM NaCl for 1 min before being stimulated. The results summarized in Fig. 6 and Table V clearly show that hyperosmolarity inhibits degranulation regardless of the stimulus used.

DISCUSSION

The data presented clearly show that the amount of actin associated with the cytoskeleton can be greatly increased in neutrophils by the addition of chemotactic factors or other stimuli. We must point out that the stimulated increase in cytoskeletal actin may reflect actin polymerization and/or increased actin cross-linking, and the stimuli used could be affecting these two aspects to different degrees.

Although the exact causes of the stimulated increase in cytoskeletal actin cannot be fully deduced from the present data, several possibilities can be clearly ruled out. First, although the addition of fMet-Leu-Phe to rabbit neutrophils produces complex and specific changes in the intracellular pH, with a rapid drop followed by a slower and more sustained rise (12, 18), it is reasonable to conclude that the increase in the cytoskeletal actin produced by these stimuli is not mediated by an increase in the intracellular pH. This conclusion

TABLE III. Cytoskeletal Actin in Control and Stimulated Rabbit Neutrophils Suspended in Hyperosmotic Buffered Solutions*

Experimental conditions	Cytoskeletal actin (relative to control)	
	Control	fMet-Leu-Phe (10 ⁻⁹ M)
<i>mM</i>		
NaCl, 124	1.0	1.82 ± 0.2 (8)
NaCl, 250	0.83 ± 0.06 (7)	1.26 ± 0.15 (7)
NaCl, 124	1.0	1.93 ± 0.09 (3)
NaCl, 124 + 252 urea	0.91 ± 0.05 (3)	1.92 ± 0.07 (3)
NaCl, 124	1.0	1.97 ± 0.10 (4)
NaCl, 124 + 252 glucose	0.96 ± 0.05 (4)	1.36 ± 0.12 (4)

* The cells were reacted with the solute for 20 s and then stimulated with 10⁻⁹ M fMet-Leu-Phe for an additional minute before the reaction was stopped. Each value represents the mean ± SEM, and the number in parentheses is the number of experiments.

TABLE IV. Effect of High NaCl Concentration on the Increase in the Cytoskeletal Actin Produced by Various Stimuli

Stimulus*	Amount of cytoskeletal actin (relative to control)	
	[NaCl] = 124	[NaCl] = 250
Control	1.0	0.81 ± 0.05 (4)
+ fMet-Leu-Phe	1.90 ± 0.15 (4)	1.30 ± 0.10 (4)
Control	1.0	0.84 ± 0.05 (6)
+ A23187 + EGTA (1 mM)	1.60 ± 0.1 (4)	1.05 ± 0.06 (6)
Control	1.0	0.81 ± 0.04 (4)
+ PMA	1.55 ± 0.12 (4)	1.45 ± 0.16 (4)
Control	1.0	0.82 ± 0.03 (3)
+ PAF	1.77 ± 0.05 (3)	1.51 ± 0.05 (3)
Control	1.0	0.87 ± 0.02 (3)
+ Leukotriene B ₄	1.45 ± 0.1 (3)	1.10 ± 0.06 (3)
Control	1.0	0.88 ± 0.15 (2)
Arachidonic acid	1.43 ± 0.1 (2)	0.89 ± 0.08 (2)

* The cells were allowed to react with fMet-Leu-Phe (10⁻⁹ M) for 1 min, with leukotriene B₄ (7 × 10⁻⁹ M) for 30 s, with arachidonic acid (2 × 10⁻⁶ M) for 20 s, with PAF (10⁻⁹ M) for 10 s, with A23187 (5 × 10⁻⁷) for 1 min, and with PMA (0.1 μg/ml) for 2 min before the reaction was stopped. Each value represents the mean ± SEM, and the number in parentheses is the number of different experiments. The cells were incubated with the hyperosmotic solution for <1 min before they were stimulated.

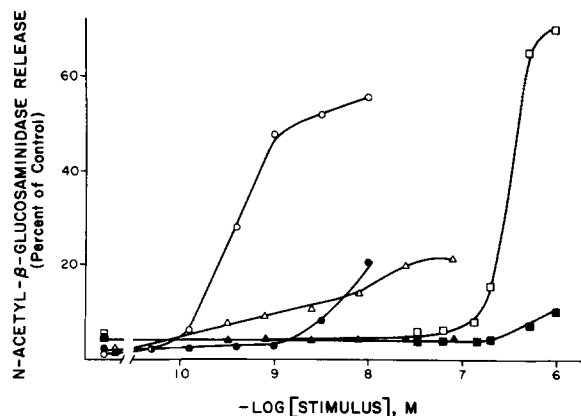


FIGURE 6 Effect of hyperosmolarity on degranulation in the presence of 5 $\mu\text{g/ml}$ of cytochalasin B produced by various concentrations of fMet-Leu-Phe (\circ), leukotriene B_4 (Δ), and A23187 (\square). The cells were incubated with either Hanks' buffered solution containing the normal concentration of NaCl (\circ , Δ , \square) or with Hanks' buffered solution containing 250 mM NaCl (\bullet , \blacktriangle , \blacksquare). No cytochalasin B was present in the case of A23187. The results are taken from a single experiment representative of at least four separate experiments.

TABLE V. PMA-induced Lysosomal Enzyme Release in Cells Incubated in Hanks' Buffered Solution Containing Isoosmotic and Hyperosmotic NaCl Concentration*

PMA concentration $\mu\text{g/ml}$	Lysosome release (% of total)		
	Isotonic	Hyperosmotic	% Inhibition
0.1	50 \pm 5	24 \pm 3	52
1.0	57 \pm 10	32 \pm 5	44

* Cells were treated with the hyperosmotic solution (250 mM NaCl) for 1 min before stimulation with PMA for 15 min. The values represent the mean \pm SEM of at least three separate experiments.

is based on three experimental findings: (a) The time course of the increase in intracellular pH induced by fMet-Leu-Phe is much slower than that of the increase in cytoskeletal actin produced by the same stimulus, and the concentration required is much higher. (b) Amiloride at a concentration (1 mM) that totally abolishes the rise in cellular pH (18) has no effect on the amount of actin associated with the cytoskeleton. (c) The fMet-Leu-Phe-induced rise in the cytoskeletal actin is independent of extracellular pH in the range between 6.8 and 7.6. With respect to the fMet-Leu-Phe-induced drop in intracellular pH, it is possible that the acidification is responsible for part of the increase in cytoskeletal actin. The findings that they have a similar time course are consistent with this view. Further experiments are needed to examine and clarify the relationship between cell acidification and actin polymerization.

Second, an overall or local (such as displacement of calcium from membrane sites) decrease in the intracellular concentration of free calcium may mediate some small increase in cytoskeletal actin but cannot be the sole signal. The idea that a local decrease in the intracellular calcium concentration may be involved in actin polymerization was first introduced by Stossel et al. (for a recent review, see reference 22). However, an overall decrease in the intracellular concentration of free calcium cannot be the only event regulating the distribution of actin between the cytosol and the cytoskeleton since

the increase in the cytoskeletal actin produced by quin-2, a calcium chelator, is small.

Third, the stimulus-dependent increase in cytoskeletal actin does not appear to be mediated through a rise in the intracellular concentration of free calcium. This conclusion is supported by several lines of evidence: (a) Stimuli that increase (fMet-Leu-Phe, leukotriene B_4 , etc.), as well as those that decrease (PMA) the intracellular concentration of free calcium enhance actin association with the cytoskeleton. (b) The stimulated increase in cytoskeletal actin is independent of outside calcium concentration. (c) Calmodulin inhibitors such as trifluoroperazine do not inhibit stimulated cytoskeletal actin (28). (d) The increase produced by the addition of A23187 occurs in both the presence and the absence of calcium. (e) PMA at 0.1 $\mu\text{g/ml}$ increases the amount of actin associated with the cytoskeleton without inducing an increase in the intracellular concentration of free calcium (20). (f) The two responses (calcium rise and increased cytoskeletal actin) produced by fMet-Leu-Phe can be dissociated from each other in cells pretreated with Hanks' buffered solution containing 250 mM NaCl.

Fourth, the stimulus-dependent increase in cytoskeletal actin is not mediated by the activation of protein kinase C. Although very attractive, there are several experimental observations that rule out this possibility: (a) The dose-response curve for the fMet-Leu-Phe-induced phospholipid changes (the production of 1,2 diacylglycerol; reference 25) and stimulated cytoskeletal actin are very dissimilar, actin polymerization requiring much lower concentrations of the chemoattractant. (b) Trifluoroperazine is known to inhibit with different median infective doses both the protein kinase C system and the calmodulin-dependent protein kinase. The lack of an inhibitory effect of trifluoroperazine (up to 20 μM) on an fMet-Leu-Phe-induced increase in cytoskeletal actin (28) is inconsistent with a role of protein kinase C in actin polymerization.

The results reported here also show that the increases in cytoskeletal actin produced by fMet-Leu-Phe, leukotriene B_4 , or A23187, but not those induced by PMA, are inhibited by high osmolarity. Furthermore, degranulation produced by all the stimuli tested (fMet-Leu-Phe, leukotriene B_4 , A23187, and PMA) are inhibited by a hyperosmotic NaCl concentration. The inhibitory action of high osmolarity requires a decrease in cell volume and is not mediated by an increase in the basal intracellular concentration of free calcium. The inhibition of the fMet-Leu-Phe-induced increase in cytoskeletal actin by the high salt concentration is probably the basis for the decrease in neutrophil chemotaxis and phagocytosis by hyperosmolarity (2, 9). This inhibitory effect on neutrophil responses is particularly pertinent to certain areas of the kidney and urinary tract, where solute concentrations such as NaCl, and glucose in the case of diabetics, may be sufficiently elevated to inhibit actin polymerization, and thus neutrophil functions, rendering these regions highly susceptible to infections.

The inhibition of the fMet-Leu-Phe-induced increase in cytoskeletal actin by high osmolarity (especially 375 mM NaCl) resembles the effect produced by pretreating cells for 3 min with PMA (13). In addition to its agonist activity, PMA has been shown to antagonize the effects of fMet-Leu-Phe and thrombin (13, 30). The inhibitory effect of hyperosmolarity is best explained by the hypothesis that it is acting as a receptor uncoupler (i.e., the hyperosmolarity dissociates re-

ceptor occupancy from subsequent generations of the second messengers). The uncoupling by hyperosmolarity could be mediated either directly by affecting the physical environment of the receptor, or indirectly by activating protein kinase C. The observations that hyperosmolarity causes a mitogen-independent activation of Na⁺/H⁺ antiport in human epidermoid carcinoma A431 cells (3) and increases intracellular pH in rabbit neutrophils (unpublished data) are consistent with this hypothesis. In addition, hyperosmolarity may cause an increase in the viscosity of the cell interior, and this increase may contribute to the inhibition of degranulation.

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REFERENCES

- Andriole, V. J., and F. H. Epstein. 1965. Prevention of pyelonephritis by water diuresis. Evidence for the role of medullary hypertonicity in promoting renal infection. *J. Clin. Invest.* 44:73-79.
- Bryant, R. E., M. C. Sutcliffe, and Z. A. McGee. 1972. Effect of osmolalities comparable to those of the renal medulla on function of human polymorphonuclear leukocytes. *J. Infect. Dis.* 126:1-10.
- Cassel, D., B. Whiteley, Y. X. Zhuang, and L. Glaser. 1985. Mitogen-independent activation of Na⁺/H⁺ exchange in human epidermoid carcinoma A431 cells: regulation by medium osmolarity. *J. Cell. Physiol.* 122:178-186.
- English, D., and B. R. Anderson. 1974. Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Methods.* 5:249-252.
- Fechheimer, M., and S. H. Zigmond. 1983. Changes in cytoskeletal proteins of polymorphonuclear leukocytes induced by chemotactic peptides. *Cell Motil.* 3:349-362.
- Fox, J. E. B., J. K. Boyles, C. C. Reynolds, and D. C. Phillips. 1984. Actin filament content and organization in unstimulated platelets. *J. Cell Biol.* 98:1985-1991.
- Freedman, L. R., and P. B. Besson. 1958. Experimental pyelonephritis. IV. Observations on infections resulting from direct inoculation of bacteria in different zones of the kidney. *Yale J. Biol. Med.* 30:406-414.
- Jennings, L. K., J. E. B. Fox, H. H. Edward, and D. R. Phillips. 1981. Changes in the cytoskeletal structure of human platelets following thrombin activation. *J. Biol. Chem.* 256:6927-6932.
- Klebanoff, S. J., and R. A. Clark. 1978. *In The Neutrophil: Function and Clinical Disorder.* Elsevier/North-Holland Publishing Co., Amsterdam. 198.
- Korn, E. D. 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. *Physiol. Rev.* 61:672-737.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T₄. *Nature (Lond.)* 227:680-685.
- Molski, T. F. P., P. H. Naccache, M. Volpi, L. M. Wolpert, and R. I. Sha'afi. 1980. Specific modulation of the intracellular pH of rabbit neutrophils by chemotactic factor. *Biochem. Biophys. Res. Commun.* 94:508-514.
- Naccache, P. H., T. F. P. Molski, P. Borgeat, J. R. White, and R. I. Sha'afi. 1985. Phorbol esters inhibit the fMet-Leu-Phe and leukotriene B₄ stimulated calcium mobilization and enzyme secretion in rabbit neutrophils. *J. Biol. Chem.* 260:2125-2131.
- Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Sodium, potassium and calcium transport across rabbit polymorphonuclear leukocyte membranes: effect of chemotactic factor. *J. Cell Biol.* 73:428-444.
- Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* 86:77-86.
- Pollard, T. D. 1975. Functional implications of the biochemical and structural properties of cytoplasmic contractile proteins. *In Molecules and Cell Movement.* S. Inoue and R. E. Stephens, editors. Raven Press, New York. 259-286.
- Roa, K. M. K., and J. Varani. 1982. Actin polymerization induced by chemotactic peptide and Concanavalin A in rat neutrophils. *J. Immunol.* 129:1605-1607.
- Sha'afi, R. I., P. H. Naccache, T. F. P. Molski, and M. Volpi. 1982. Chemotactic factor stimulated changes in pH of rabbit neutrophils. *In Intracellular pH: Its Measurements, Regulation, and Utilization in Cellular Functions.* R. Nuccitelli and D. W. Deamer, editors. Alan R. Liss, Inc. 513-525.
- Sha'afi, R. I., and M. Volpi. 1976. Permeability of rabbit polymorphonuclear leukocyte membranes to urea and other nonelectrolytes. *Biochem. Biophys. Acta.* 436:242-246.
- Sha'afi, R. I., J. R. White, T. F. P. Molski, J. Shefeyk, M. Volpi, P. H. Naccache, and M. B. Feinstein. 1983. Phorbol 12-myristate, 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biochem. Biophys. Res. Commun.* 114:638-654.
- Showell, H. J., D. Williams, E. L. Becker, P. H. Naccache, and R. I. Sha'afi. 1979. Desensitization and deactivation of rabbit neutrophils secretory unresponsiveness induced by incubation with formyl-methionyl-leucyl-phenylalanine prior to the addition of cytochalasin B. *J. Reticuloendothel. Soc.* 25: 1139-1150.
- Southwick, F. S., and T. P. Stossel. 1983. Contractile proteins in leukocyte function. *Semin. Hematol.* 20:305-321.
- Takenawa, T., Y. Homma, and Y. Nagai. 1983. Role of Ca²⁺ in phosphatidylinositol response and arachidonic acid release in formylated tripeptide on Ca²⁺ ionophore A23187-stimulated guinea pig neutrophils. *J. Immunol.* 139:2849-2855.
- Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellular trapped fluorescent indicator. *J. Cell Biol.* 94:325-334.
- Volpi, M., R. Yassin, W. Tao, T. F. P. Molski, P. H. Naccache, and R. I. Sha'afi. 1984. Leukotriene B₄ mobilizes calcium without the breakdown of polyphosphoinositides and the production of phosphatidic acid in rabbit neutrophils. *Proc. Natl. Acad. Sci. USA.* 81:5966-5969.
- Wallace, P. J., R. P. Wersto, C. H. Packman, and M. A. Lichtman. 1984. Chemotactic peptide-induced changes in neutrophil actin conformation. *J. Cell Biol.* 99:1060-1065.
- White, J. R., P. H. Naccache, T. F. P. Molski, P. Borgeat, and R. I. Sha'afi. 1983. Direct demonstration of increased intracellular concentration of free calcium in rabbit and human neutrophils following stimulation by chemotactic factors. *Biochem. Biophys. Res. Commun.* 113:44-50.
- White, J. R., P. H. Naccache, and R. I. Sha'afi. 1983. Stimulation by chemotactic factor of actin association with the cytoskeleton in rabbit neutrophils. Effects of calcium and cytochalasin B. *J. Biol. Chem.* 258:14041-14047.
- Yin, H. L., and T. P. Stossel. 1982. Calcium control of actin network structure by gelsolin. *In Calcium and Cell Function.* Vol. II. W. Y. Cheung, editor. Academic Press, Inc., New York. 325-337.
- Zavoico, G. B., S. P. Halenda, R. I. Sha'afi, and M. B. Feinstein. 1985. Phorbol myristate acetate inhibits thrombin-stimulated Ca²⁺ mobilization and phosphatidylinositol 4,5-bis phosphate hydrolysis in human platelets. *Proc. Natl. Acad. Sci. USA.* In press.