THE REGULATORY ROLE OF DIVALENT CATIONS IN HUMAN GRANULOCYTE CHEMOTAXIS

Evidence for an Association between Calcium Exchanges and Microtubule Assembly

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ABSTRACT

Optimal human granulocyte chemotaxis has been shown to require both calcium and magnesium. Exposure of granulocytes to three different chemotactic factors (C5a, kallikrein, and dialyzable transfer factor) yielded a rapid calcium release, depressed calcium uptake, and was associated with a shift of calcium out of the cytoplasm and into a granule fraction. Colchicine, sodium azide, and cytochalasin B, in concentrations that inhibited chemotaxis, also inhibited calcium release while low concentrations of cytochalasin B, which enhanced chemotaxis, also enhanced calcium release. Microtubule assembly was visualized both in cells suspended in C5a without a chemotactic gradient and in cells actively migrating through a Micropore filter. The data suggest microtubule assembly is regulated, at least, in part, by the level of cytoplasmic calcium. It is proposed that asymmetric assembly of microtubules may be instrumental in imparting the net vector of motion during chemotaxis.

INTRODUCTION

The mechanism by which directional granulocyte movement is initiated and modulated is not understood. Of particular interest were the recent observations of Becker and Showell which indicated that the divalent cations calcium and magnesium are critical for optimal rabbit granulocyte chemotaxis (1). Although divalent cation fluxes were not measured, it was hypothesized that if granulocyte chemotaxis were analogous to muscle contractile mechanisms, then calcium movement across the cell membrane would play an essential role in initiation of granulocyte motility.

To further evaluate the role of divalent cations in leukocyte motility we characterized the calcium and magnesium ion requirements for human granulocyte chemotaxis. In addition, the dynamics of calcium exchanges between the granulocyte and the surrounding environment were assessed as a function of time, concentration, and type of chemotactic factor. The effect of known inhibitors of granulocyte chemotaxis on calcium exchanges were also evaluated. This communication provides evidence for an association between cytoplasmic calcium concentration, microtubule assembly, and directional granulocyte movement.

MATERIALS AND METHODS

Leukocytes

Leukocytes were obtained from healthy volunteers. For most studies granulocyte-rich leukocyte preparations were prepared by dextran sedimentation of heparinized

whole blood, a method which yielded about 85% granulocytes and 15% mononuclear cells (2). Residual erythrocytes were eliminated by two to three cycles of hypotonic saline lysis (2). In some experiments granulocytes or mononuclear cells were each separated from whole blood by the Hypaque (Winthrop Laboratories, New York)-Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) technique (3); such separation routinely resulted in granulocyte and mononuclear cell populations each of greater than 98% purity.

Chemotactic Assay

Granulocyte chemotaxis was evaluated using a chromium-51 (^{51}Cr; Amersham/Searle Corp., Arlington Heights, Ill.) radioassay previously described (4). Labeled cells were placed in the upper compartment of a modified Boyden chamber that was separated from a lower chamber by two 5-μm Micropore filters (Millipore Corp., Bedford, Mass.). The ^{51}Cr-labeled cells were suspended at a concentration of 2.3×10^6 granulocytes/ml in calcium- and magnesium-free Gey's balanced salt solution containing 2% bovine serum albumin, penicillin, and streptomycin (Gey's Medium, Microbiological Associates, Inc., Bethesda, Md.) to which were added various divalent cations. The chemotactic stimulus was added to the lower compartment. We have reported previously that under these conditions only granulocytes migrated into the lower of the two filters and that the number of granulocytes entering the lower filter is proportional to the radioactivity incorporated into the lower filter (4). In addition, the trace amount of ^{51}Cr incorporated by the granulocytes has no influence on cell movement (4). After adjusting for variability in specific activity and incorporation of the ^{51}Cr by the granulocytes, chemotaxis is expressed as corrected counts per minute lower filter (cor cpm LF). The chemotactic response for each experimental condition is the average of four chambers and means of different experiments were compared using the Student's *t* test.

Chemotactic Factors

For most experiments the chemotactic stimulus was the cleavage product of the fifth component of complement, C5a, generated by endotoxin (*Escherichia coli* 0127:B8 lipopolysaccharide B; Difco Laboratories, Detroit, Mich.) activation of complement from whole human serum by incubating 4 ml serum with 0.17 ml 0.85% NaCl containing 1.2 mg *E. coli* endotoxin for 1 h at 37°C. The generation of C5a was terminated by the addition of 10 mM ethylenediaminetetraacetic acid (EDTA) (5). The C5a was partially purified from activated whole serum and the precursor products C3 and C5 by gel filtration on a 2.5×100 cm column of Sephadex G-75 (Pharmacia Fine Chemicals, Inc.). The column was equilibrated with 0.04 M phosphate-buffered saline pH 7.4 and run by descending flow at 4°C with 6-ml fractions collected as previously described (6). For molecular weight estimations the column was calibrated with the following proteins of known molecular weight: blue dextran (2,000,000), ovalbumin (45,000), cytochrome *c* (12,384), and insulin A chain (2,550).

To assess the divalent cation requirements for granulocyte chemotaxis it was necessary to have a divalent cation-free chemotactic stimulus. Fig. 1 shows the partial purification of C5a by G-75 Sephadex chromatography of 3.0 ml of EDTA-treated endotoxin-activated human serum. No free calcium was found in any of the column fractions when they were screened for ionized calcium with a calcium electrode (Orion Research, Inc., Cambridge, Mass.). In contrast a large peak of calcium was detected in elution volumes 500–580 ml when the fractions were screened for calcium with an atomic absorption spectrophotometer (Fisher Scientific Co., Pittsburgh, Pa.); this appears to represent calcium complexed to EDTA.[1] Since the amount of EDTA used to terminate the complement reaction was in excess of that necessary to chelate any magnesium present in the reaction mixture, the fractions containing chemotactic activity (300–360 ml) were considered free of both calcium and magnesium. The chemotactic activity of the 300–360-ml fractions was considered to be due to C5a on the basis of its elution volume on gel filtration (7) and because goat antibody to human C5 inhibited its activity whereas anti-C3 did not (8). Chromatography fractions containing chemotactic activity were desalted and concentrated by ultrafiltration (Amicon Corp., Lexington, Mass.) using a UM-2 membrane and 60 lb/in^2 nitrogen pressure. In some experiments highly purified preparations of the chemotactic enzyme kallikrein (provided by Dr. Allen Kaplan) (9) and partially purified (G-25 Sephadex chromatography) dialyzable transfer factor (provided by Dr. Charles Kirkpatrick), which has recently been shown to be a potent chemotactic stimulus (10), were used as chemotactic agents. No detectable calcium was present in the transfer factor or kallikrein preparations.

Divalent Cations

The following divalent cations (chloride salts) were used for chemotaxis studies: calcium (Allied Chemical Corp., New York), magnesium, manganese, nickel, and zinc (Fisher Scientific Co.), and cobalt (MC&B Manufacturing Chemists, Norwood, Ohio).

Protein Determinations

Quantitative protein measurements were made by the method of Lowry et al. (11).

Measurement of Calcium Fluxes

EFFECT OF CHEMOTACTIC FACTORS ON CALCIUM RELEASE (EFFLUX): Leukocyte preparations were washed twice in calcium- and magnesium-free

[1] Based on elution identity with predicted elution of material having the molecular weight of calcium-EDTA.

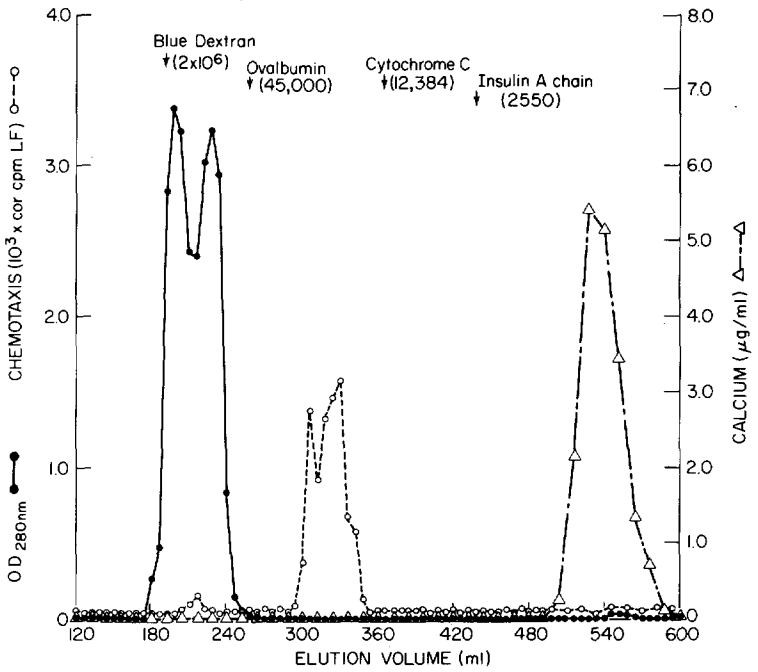

FIGURE 1 G-75 Sephadex chromatography of *E. coli* endotoxin-activated normal human serum. Chemotactic activity was determined using human granulocytes, and calcium measurements were performed with an atomic absorption spectrophotometer.

Hanks' solution (National Institutes of Health Media Unit). Cells were then labeled with calcium-45 (^{45}Ca, New England Nuclear, Boston, Mass.) by incubating each 10^6 cells with 1 μCi ^{45}Ca for 1 h at 37°C in a shaking water bath, washed twice in Ca- and Mg-free Hanks' solution, and then three times in Hanks' solution containing 0.5 mM Mg^{++} and 1.0 mM unlabeled Ca^{++}. The ^{45}Ca-labeled cells were then adjusted to a density of 5×10^6/ml in Hanks' solution containing 1 mM Ca and 0.5 mM Mg. 2 ml of the ^{45}Ca-labeled cells were placed in a 6-ml plastic syringe and 2 ml of the chemotactic stimulus into another 6-ml plastic syringe. The two syringes were connected by a disposable three-way stopcock (Pharmaseal Laboratories, Glendale, Calif.) whose outflow channel was attached to a disposable 0.45-μm Millipore Swinnex filter apparatus (Millipore Corporation). At 0 time, the contents of the two syringes were mixed. At varying intervals thereafter the chemotactic stimulus-leukocyte mixture was filtered through the Swinnex apparatus. The leukocytes remained trapped on the filter while the leukocyte-free media was collected in a test tube. Calcium efflux, as measured by the amount of ^{45}Ca present in the filtrate, was determined by counting 1.0 ml of the filtrate in a liquid scintillation counter using 10 ml of Aquasol (New England Nuclear) counting fluid. Although ^{45}Ca effluxes were highly reproducible within a given experiment, there was considerable variability between different individuals' leukocyte preparations in the amount of ^{45}Ca taken up during the "loading period." In order to compare data among experiments, calcium efflux was therefore expressed as net percent change from control

$$\left(100 \times \left[\frac{\text{cpm experimental}}{\text{cpm buffer control}}\right] - 100\right)$$

except in kinetic studies where it was expressed as percent initial counts per minute per milliliter filtrate.

EFFECT OF CHEMOTACTIC FACTORS ON CALCIUM UPTAKE (INFLUX): Calcium influx experiments were performed in a manner similar to calcium efflux experiments. Leukocytes were washed twice in calcium-free Hanks' solution containing 0.5 mM Mg^{++} and suspended to a concentration of 5×10^6 cells/ml of the 0.5 mM Mg^{++} Hanks' solution without Ca^{++}. 0 mM calcium was used in the stable environment after preliminary experiments demonstrated that the best ^{45}Ca uptake data were obtained when ^{45}Ca was the only calcium supplied. 2 ml of the cell suspension was placed in a 6-ml plastic syringe and 2 ml of the chemotactic stimulus containing 10 μCi of ^{45}Ca in another syringe. Both syringes were inserted into a three-way stopcock-0.45-μm filter apparatus as described above. At 0 time the contents of the two syringes were mixed and the experi-

ment was completed by flushing the reaction mixture through the 0.45-μm Micropore filter. The filter with trapped leukocytes was immediately flushed with 30 ml of Hanks' solution containing 1 mM nonradioactive Ca^{++} and 1 mM Mg^{++}. The washed leukocyte-rich filter was then placed in 10 ml of scintillation counting fluid; ^{45}Ca uptake was the amount of ^{45}Ca taken up from the media by the leukocytes. Again, to minimize variability among subjects the data were expressed as net percent change from control as calculated above except for kinetic studies in which calcium influx was expressed as percent initial counts per minute per filter.

Effect of C5a on the Intracellular Distribution of Calcium

An estimate of the effect of the chemotactic stimulus C5a on the intracellular distribution of calcium was made by prelabeling the cells for 45 min at 37°C with ^{45}Ca in the presence or absence of C5a under the conditions described above for calcium influx studies. After labeling with ^{45}Ca, the cells were washed twice at 4°C in Ca- and Mg-free modified Hanks' solution and then three times in Hanks' solution containing 1 mM Ca^{++} and 1 mM Mg^{++}. 30 million cells were suspended in 2 ml of 0.34 M sucrose containing 5,000 U of heparin and fractured by shearing forces with 25 cycles of aspiration through a 25-gauge spinal needle, and the cellular components were separated by differential centrifugation.[2] The broken cells were then examined under phase-contrast microscopy to confirm breakage of virtually all cells; additional spinal needle aspirations were performed if necessary. 10 ml of 0.34 M sucrose were then added to the 2 ml lysate and the broken cells were fractionated by differential centrifugation. The first centrifugation was done at 400 *g* for 10 min at 4°C. The supernate was saved, the pellet resuspended in 5 ml of 0.34 M sucrose, and recentrifuged at 400 *g* for 10 min at 4°C. This supernate was also saved. The 400 *g* pellet, which contained nuclear debris and unbroken cells, was suspended in 6 ml of 0.34 M sucrose. The supernates were pooled and centrifuged for 30 min at 27,000 *g* at 4°C.

The 27,000 *g* pellet or granular fraction, which contained granules and mitochondria, was suspended in 6 ml of 0.34 M sucrose. The 27,000 *g* supernate was the "cytoplasmic fraction." 1 ml of each fraction was counted for ^{45}Ca in a liquid scintillation counter as described above.

Effect of Different Inhibitors of Chemotaxis on Calcium Fluxes

The following agents, which affect chemotaxis, were evaluated for their effects on calcium fluxes: colchicine and sodium azide (Sigma Chemical Co., St. Louis, Mo.) and cytochalasin B (Imperial Chemical Industries, Ltd., Pharmaceuticals Division, Merside Alderley Park, Macclesfield, Cheshire, England) dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific Co.) at a concentration of 1.5 mg/ml DMSO. An appropriate DMSO control had no effect on chemotaxis or calcium fluxes in the concentrations used. Each agent was made up to 10 times the desired concentration on the day of the experiment and added in a small volume to give the desired final concentration. For chemotaxis and calcium influx studies the granulocytes were preincubated for 30 min with each agent; for calcium efflux studies the last two granulocyte washes before the study were performed with the modified Hanks' solution containing the inhibitor.

Morphologic Studies

ANALYSIS OF LEUKOCYTES SUSPENDED IN C5a: Granulocyte-rich cell suspensions were exposed to buffer or buffer plus the chemotactic factor C5a under conditions identical with those of the lower compartment of the chemotactic chamber. At 30 s, 5 min, and 30 min after exposure to buffer or C5a the cell suspensions were added to pH 7.2 distilled glutaraldehyde (final concentration 1%) and immediately centrifuged at 2,800 *g* in a conical tube. After 60 min of glutaraldehyde fixation, the pellet was washed in phosphate-buffered isotonic sucrose (pH 7.2), postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.2), dehydrated in graded ethanol-propylene oxide, and embedded in Maraglas (Polysciences, Inc., Warrington, Pa.). Plastic "thick" sections were stained with methylene blue and examined in a Zeiss photomicroscope II; thin sections were doubly stained with lead citrate and uranyl acetate and examined in a Philips EM-300 electron microscope.

ANALYSIS OF LEUKOCYTES EXPOSED TO A CONCENTRATION GRADIENT OF C5a: Under conditions identical with those used for the chemotactic assay, granulocyte-rich leukocyte suspensions (non-^{51}Cr labeled) were exposed to a gradient of C5a. After 30 and 60 min of incubation (times before which significant numbers of cells enter the lower filter) the upper filter was removed and fixed in 1% pH 7.2 glutaraldehyde. After postfixation in osmium tetroxide, the whole filter was dehydrated and embedded in plastic as described above. Pieces (1–2 mm^2) of cell-infiltrated areas of the upper filter were stacked and polymerized on edge in Maraglas to permit analysis of cross sections of the filter by either light or electron microscopy.

RESULTS

Divalent Cation Requirements for Granulocyte Chemotaxis

The dose response of the concentration of calcium and magnesium required for granulocyte

[2] H. R. Kimball and G. Ford. Manuscript submitted for publication.

chemotaxis is shown in Fig. 2. It is apparent that each divalent cation has an optimal concentration for supporting chemotaxis (0.5 mM Mg^{++} and 2.0 mM Ca^{++}). The ability of different combinations of calcium and magnesium to support chemotaxis was studied with dose responses of one cation in the presence of different concentrations of the other cation. Addition of 1.0 mM Ca^{++} to the different concentrations of magnesium used to generate the dose-response curve yielded the best results (Fig. 2). Systematic study of various combinations of calcium and magnesium indicated the combination 1.0 mM Ca^{++} and 1.0 mM Mg^{++} gave the optimal chemotactic response. Addition of varying concentrations of either calcium or magnesium during the dose-response study of the other ion never resulted in a lowering of the optimal concentration of that cation required to support chemotaxis (unpublished observation).

When other divalent cations were tested for their ability to support chemotaxis, each cation exhibited an optimal concentration although none supported chemotaxis better than calcium or magnesium alone (Table I). Furthermore, although cobalt and manganese supported chemotaxis as well as calcium ($P > 0.05$) none of the other divalent cations supported chemotaxis as well as calcium or magnesium alone ($P < 0.05$). No correlation was found between the ability of the divalent cations to support chemotaxis and the ionic radius, preferred binding ligand (oxygen, nitrogen, or sulfur), or electronegativity of the cations.

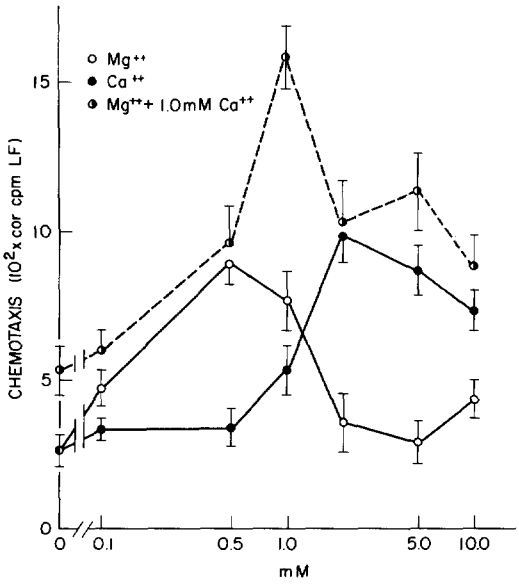

FIGURE 2 Chemotactic activity of human granulocytes in the presence of varying concentrations of calcium or magnesium. The dashed line was obtained by adding 1.0 mM calcium throughout the magnesium dose-response study. The bars denote the standard error of the mean of four separate experiments for each experimental condition as denoted by open, closed, or half-shaded circles.

TABLE I

Ability of Divalent Cations to Support Human Granulocyte Chemotaxis

Divalent cation	Optimal concentration	Chemotaxis (cor cpm LF)*
	mM	
None	—	395 ± 63
Sr^{++}	1.0	795 ± 61
Ni^{++}	1.0	979 ± 130
Ba^{++}	1.0	1,186 ± 238
Zn^{++}	1.0	1,200 ± 136
Mn^{++}	0.1	1,373 ± 314
Co^{++}	0.1	1,831 ± 42
Ca^{++}	2.0	2,098 ± 250
Mg^{++}	0.5	2,529 ± 301

* The chemotactic stimulus was C5a (10 μg protein/ml). Means ± SE of four dose-response experiments.

Calcium Fluxes and Chemotactic Activity of Sephadex Chromatographed Endotoxin-Activated Human Serum

To determine if chemotaxis were associated with calcium movement either into or out of granulocytes, initial calcium flux experiments were performed using fractions from the G-75 Sephadex chromatography column used for isolation of C5a (Fig. 1). As shown in the middle panel of Fig. 3, there was an excellent correlation between fractions which induced calcium efflux and chemotactic activity. A final peak of ^{45}Ca efflux-stimulating activity was detected at 95% bed volume (not shown) in fractions containing the EDTA and presumably was related to EDTA chelation of cell-associated calcium.

In contrast to the calcium efflux results, there was depressed ^{45}Ca influx in the fractions containing chemotactic and ^{45}Ca efflux activity (lower panel, Fig. 3). There were, however, two peaks of material influencing ^{45}Ca influx, one eluting at the same volume as the molecular weight marker ovalbumin and another slightly after the cytochrome *c* marker. The latter peak of ^{45}Ca influx

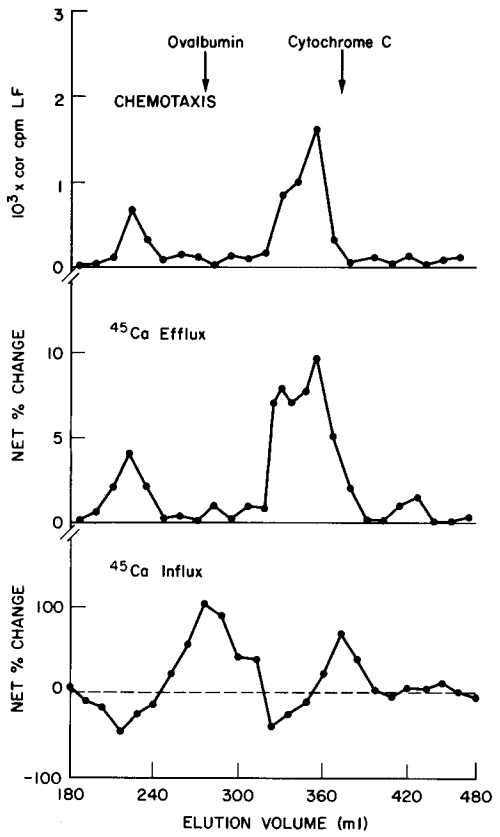

FIGURE 3 Chemotactic, ^{45}Ca efflux, and ^{45}Ca influx activity of G-75 Sephadex chromatography fractions of *E. coli* endotoxin-activated human serum (one representative column screen). Calcium fluxes were values obtained 1 h after exposure of granulocytes to the column fractions and control values were 28,294 cpm/ml filtrate for ^{45}Ca efflux and 7,876 cpm/10^7 washed granulocytes for ^{45}Ca influx.

activity resided in the descending limb of the peak containing chemotactic and Ca^{++} efflux activity and was found fortuitously to be separable from them by UM-2 Amicon filtration (Table II). A final depression of ^{45}Ca influx was detected at 95% bed volume (not shown) in the fractions containing the EDTA and was presumably related to EDTA chelation of the ^{45}Ca.

G-75 sephadex chromatography of nonactivated human serum was also performed under conditions identical with that described above. When the resulting column fractions were screened for chemotactic and ^{45}Ca efflux activity, none was detected in the 300–360-ml fractions, whereas the peak of ^{45}Ca influx activity noted at an elution volume of

TABLE II

Separation of Chemotactic Activity and ^{45}Ca Efflux from ^{45}Ca Influx by Amicon (UM-2) Filtration of Sephadex G-75 Fractions Containing C5a

Amicon filtration	Chemotaxis (cor cpm LF)	^{45}Ca fluxes (net % change)*	
		Influx	Efflux
Nonfractionated C5a	1,513	+29 ± 8	+12 ± 4
Upper fraction	4,025	−48 ± 6	+25 ± 3
Lower fraction	125	+120 ± 9	−15 ± 3

* Net percent change from control cells (those exposed to buffer) 45 min after exposure of granulocytes to C5a (mean ± SE of four determinations).

370 ml persisted. This provides additional evidence that ^{45}Ca efflux and chemotaxis are each a consequence of granulocyte exposure to the chemotactic stimulus C5a.

Evidence that Chemotactically Responsive Leukocytes are the Source of the Ca^{++} Efflux

The maximal increase in calcium efflux observed in granulocytes exposed to C5a was about 15% greater than in cells exposed to buffer alone. Moreover, the percentage of presumably C5a unresponsive cells (lymphocytes) in dextran-sedimented leukocyte preparations was of a similar magnitude. Therefore, experiments were performed to evaluate whether or not the chemotactically responsive granulocyte leukocytes were indeed the source of the ^{45}Ca efflux measured. Calcium-45 efflux studies using Hypaque-Ficoll fractionated granulocyte and mononuclear cell (monocyte and lymphocyte) preparations were compared with studies using dextran-sedimented (mixed) leukocyte preparations (Table III). Calcium-45 efflux using a pure granulocyte population was significantly greater than ^{45}Ca efflux using a mononuclear cell preparation ($P < 0.02$, Student's t test).

Specificity of Chemotactically Active Molecules for Initiating ^{45}Ca Efflux

We next evaluated whether or not a general correlation existed between chemotactically active molecules and ^{45}Ca efflux or whether these events were secondary to nonspecific protein interaction

TABLE III

Specificity of C5a-Induced ^{45}Ca Efflux to Chemotactically Responsive Leukocytes

Leukocyte population	^{45}Ca efflux (net % change)*	P‡
Dextran sedimented leukocytes (85% granulocytes, 15% mononuclear cells)	+12 ± 2	<0.05
Hypaque-Ficoll granulocytes (99% granulocytes, 1% lymphocyte)	+16 ± 1	<0.01
Hypaque-Ficoll mononuclear cells (83% lymphocytes, 17% mononuclear cells)	+4 ± 3	>0.05

* Net percent change from control cells (those exposed to buffer). The data are expressed as mean ± SE of three determinations.
‡ Significance level of difference between cells exposed to buffer or C5a, Student's *t* test.

TABLE IV

Specificity of Chemotactic Factors for Causing ^{45}Ca Efflux from Human Granulocytes

Protein	Chemotactic activity (cor cpm LF)*	Ca^{++} efflux (net % change)‡	P§
Chemotactic factors			
C5a (5 μg/ml)	2,211 ± 125	+16 ± 1	<0.01
Kallikrein (10 μg/ml)	1,136 ± 94	+36 ± 11	<0.02
Transfer factor (15 μg/ml)	1,866 ± 178	+21 ± 1	<0.01
Control proteins			
Ovalbumin (10 μg/ml)	236 ± 36	−7 ± 1	>0.05
Horseradish peroxidase (10 μg/ml)	181 ± 28	+1 ± 3	>0.05
Crystallized human serum albumin (10 μg/ml)	204 ± 12	+6 ± 3	>0.05

* The data are expressed as mean ± SE, four determinations.
‡ Net percent change from control cells (those exposed to buffer). The data are expressed as mean ± SE of four determinations.
§ Significance level of difference of ^{45}Ca efflux between stimulated and control cells, Student's *t* test.

with the ^{45}Ca-labeled leukocytes. As shown in Table IV the chemotactic factors C5a, kallikrein, and partially purified dialyzable transfer factor produced significant ^{45}Ca efflux compared to that of control cells. The differences between C5a- and kallikrein-induced ^{45}Ca release were not significant ($P > 0.05$). However, the trend is for greater ^{45}Ca release with kallikrein than with C5a despite over twice as much chemotactic activity for C5a. The "discrepancy" between chemotaxis and calcium release seen with kallikrein and C5a may reflect different mechanisms of initiation of the chemotactic response by these two molecules. Ovalbumin, electrophoretically purified horseradish peroxidase, and crystallized human serum albumin were not chemotactically active and did not cause calcium efflux. Additional experiments monitoring the binding of calcium by C5a with a calcium electrode and the effect of C5a on ^{45}Ca movement out of a dialysis bag showed that calcium efflux and negative calcium influx were not due to simple chelation of calcium by the chemotactic stimulus.

Effect of Varying Concentrations of C5a, pH, and Temperature on Chemotactic Responses and Calcium Fluxes

As shown in Fig. 4, a log-linear increase in chemotaxis, ^{45}Ca efflux, and a log-linear depression in calcium influx were observed with increasing concentrations of C5a. Concentrations in excess of 100 μg protein/ml were not evaluated.

The pH dependence of calcium efflux paralleled that previously reported for chemotaxis (12) and confirmed in our laboratory, with an optimum at pH 7.25. ^{45}Ca efflux was also temperature dependent as previously reported for chemotaxis (12) with complete inhibition at 4°C, near maximal response at 28°C with maximal levels obtained at 37°C, and a depression at 56°C.

Kinetics of ^{45}Ca Fluxes from Granulocytes Exposed to C5a

In order to assess the contribution of various cell calcium compartments to calcium fluxes, kinetic analysis of calcium exchanges between granulocytes and the surrounding medium in response to the chemotactic stimulus C5a was carried out. Kinetic analysis of C5a induced calcium release from granulocytes is shown in Fig. 5. It is apparent that the release of ^{45}Ca is rapid, occurring within the first minute of exposure of cells to C5a (left panel, Fig. 5). The slope of the line fitted to the ratios of the experimental (C5a treated) to buffer controls over the first minute was 0.129 ± SE 0.037 (lower left, Fig. 5) whereas the slope of the line fitted to the same ratios from 1 to 60 min after exposure to C5a was −0.00016 ± SE 0.00028 (lower right, Fig. 5). Assuming that the (closed) system follows first order kinetics and that the difference between the rates of efflux from treated and untreated granulocytes is not large, then the ratio of the experimental to the control values maybe interpreted as the ratio of the respective rates of net efflux. Accordingly, the ratio of the

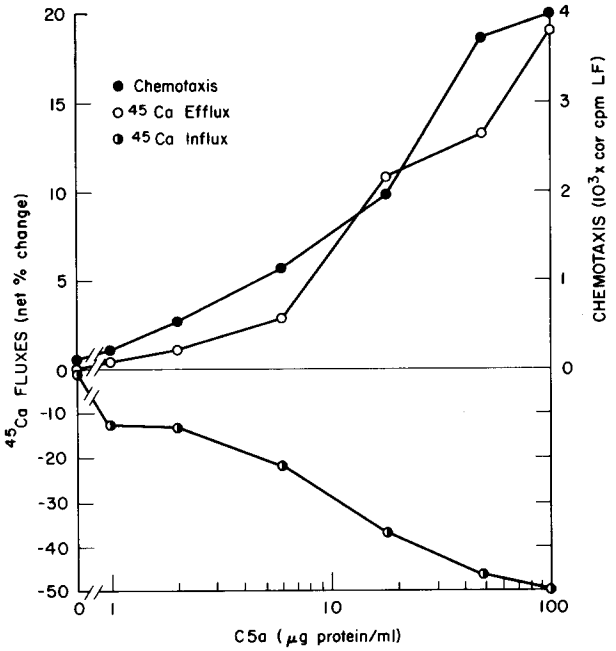

FIGURE 4 Chemotaxis, ^{45}Ca efflux, and ^{45}Ca influx to varying concentrations of C5a. Each point represents the mean of three determinations. Control values for ^{45}Ca efflux were 16,583 ± SE 2,466 cpm/ml filtrate and for ^{45}Ca influx were 47,012 ± 12,320 cpm/10^7 granulocytes.

rate of ^{45}Ca release from C5a- to buffer-treated cells increased over the first minute and this increased ratio was sustained throughout 60 min of study.

Studies of the kinetics of ^{45}Ca influx in C5a-stimulated and unstimulated cells revealed depressed ^{45}Ca uptake throughout the observed time period. As shown in Fig. 6, addition of C5a during ^{45}Ca uptake in unstimulated cells resulted in a decrease in spontaneous ^{45}Ca influx by 30 min; the early changes noted in buffer- and C5a-treated cells are most likely attributable to mixing artifact. The average ratios of buffer to C5a in the four experiments used for Fig. 6 were 1.265 ± 0.099 (t = 2.68, P <0.06), 1.179 ± 0.089 (t = 2.01, P <0.09), and 1.76 ± 0.049 (t = 3.59, P <0.01). The pooled level of significance of the difference between C5a- and buffer-treated cells for these four experiments was P <0.01 (Omnibus test [13]).

Effect of C5a on the Intracellular Distribution of Calcium-45

The effect of C5a on the intracellular distribution of ^{45}Ca in granulocytes was estimated under conditions of limited specificity of separation to minimize the equilibration of calcium in the various fractions with the media in which they were handled. As shown in Table V, there was no difference in the percent radioactivity incorporated in the 400 *g* or nuclear pellet. There was, however, a significant increase in the percent ^{45}Ca present in the 27,000 *g* pellet or granule fraction in C5a-stimulated cells. The increased amount of radioactivity in the 27,000 *g* pellet was associated with a small yet significantly decreased percent of ^{45}Ca in the 27,000 *g* supernate or cytoplasmic fraction.

Effect of Inhibitors of Chemotaxis on Calcium Fluxes

The ability of a variety of agents to alter chemotaxis and calcium-45 influx and efflux was evaluated (Table VI). Each drug, colchicine (14, 15), sodium azide, and cytochalasin B (16), was added at concentrations which either inhibited or enhanced granulocyte chemotaxis. Although each agent inhibited chemotaxis (except 0.1 μg/ml cytochalasin B), they variably affected calcium fluxes. Sodium azide and colchine significantly inhibited calcium influx and efflux (P <0.05). Cytochalasin B (3 μg/ml), at a concentration that inhibited chemotaxis (P <0.01), reduced ^{45}Ca

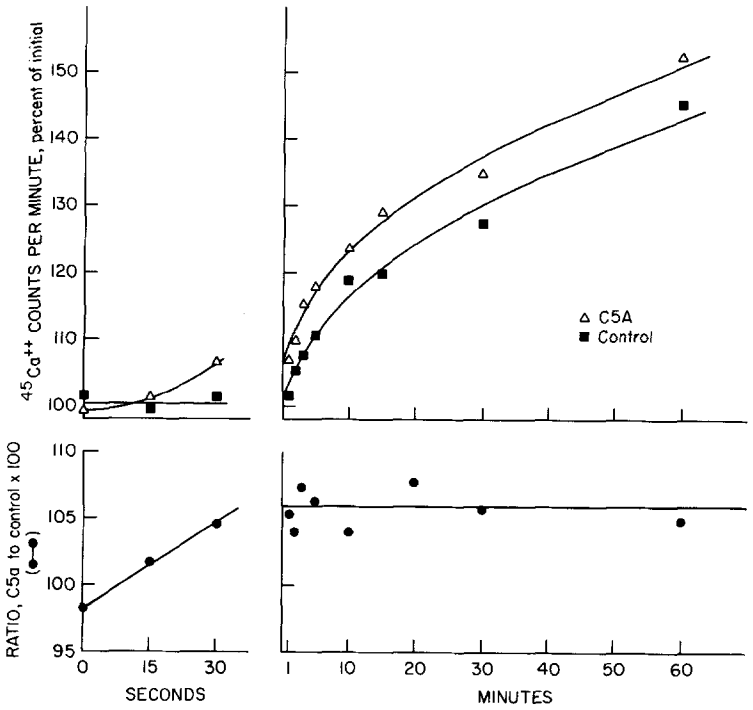

FIGURE 5 Kinetics of ^{45}Ca release from human granulocytes exposed to C5a (10 μg protein/ml). Each point in the upper panels represents the mean of nine different experiments (mean initial counts per minute per milliliter filtrate was 10,832 ± 2,310). Data collected during the first minute are shown on the left panels and data collected from 1 to 60 min are shown on the right panels. The lower panels are plots of the ratios of $^{45}Ca^{++}$ released from C5a-treated cells: ^{45}Ca released from buffer-exposed cells. The curves drawn in the upper panels are fitted by eye. The lines drawn in the lower panels were determined by least square analysis and had a slope of +0.129 ± 0.037 for 0–1 min and −0.00016 ± 0.00028 for 1–60 min (see text).

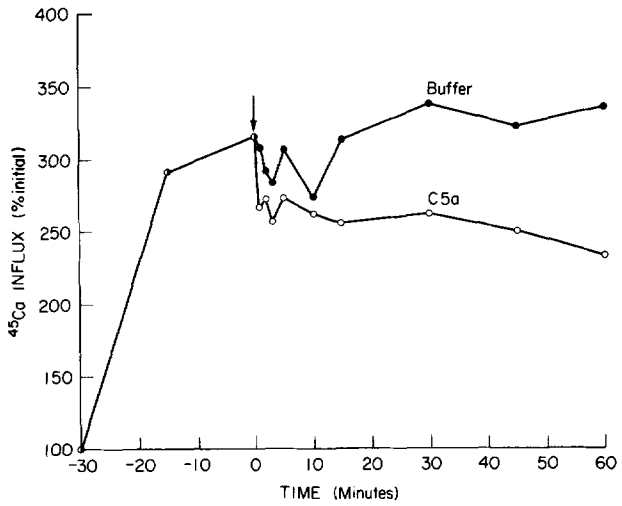

FIGURE 6 Kinetics of ^{45}Ca influx of normal human granulocytes (mean initial counts per minute per 10^6 granulocytes of 24,035 ± 4,389; four different experiments). At 0 time (arrow) either 2 ml C5a (10 μg protein/ml) or 2 ml buffer were added as indicated by the open or closed circles, respectively (see text for explanation).

efflux by 18% ($P < 0.001$), and although it increased ^{45}Ca influx by 23% this increase was not significant ($P > 0.05$), reflecting the greater variability of the influx technique. In contrast a lower concentration of cytochalasin B (0.1 μg/ml), which enhanced chemotaxis ($P < 0.05$), in confirmation of recent observations by Becker et al. (16), increased calcium efflux ($P < 0.01$) but had no effect on calcium influx ($P > 0.05$).

Correlation of C5a-Mediated Chemotaxis, Microtubule Assembly, and Granulocyte Morphology

An association between calcium concentration and isolated microtubule polymerization has been demonstrated (17) and microtubule assembly has been visualized in cytochalasin B-treated granulocytes exposed to C5a (18). Moreover, the microtubular lysing agents such as colchicine, vinblastine, and vincristine inhibit chemotaxis (15, 16). Morphologic studies were therefore performed in the absence of cytochalasin B to determine if microtubule assembly occurred during directed and spontaneous granulocyte motion.

Morphologic analysis of granulocytes migrating through a Micropore filter revealed increased pseudopod formation towards the gradient of C5a concentration (Gallin and Rosenthal, unpublished observation). The simplest interpretation of such configurational changes in granulocyte morphology is that they result as a consequence of movement of the granulocyte through the tortuous and size-limiting pores of the filter. Alternatively, these changes in granulocyte profile might in part be due to the fact that C5a induces a local increase in membrane motility (i.e., pseudopod formation) in a concentration-dependent fashion. Two experiments were performed to evaluate these possibilities. First, granulocytes were placed in a chemotactic chamber and C5a was added to both the upper and lower compartments so that there was no gradient of chemotactic factor. It was reasoned that if C5a were presented to a cell in a nongradient mode, then an increase in membrane motility would result in an increase in random-spontaneous movement, as measured by an increase in the number of granulocytes entering the lower filter. This increase, however, should not exceed that of directed movement resulting from a chemotactic factor gradient. As shown in Table VII, spontaneous migration of granulocytes into the lower filter was significantly ($P < 0.01$) increased over controls when C5a was present in both compartments of the chemotactic chamber. However, this increase was significantly less ($P < 0.01$) than that observed when there was a gradient of C5a. In the second series of experiments, the presence of changes in surface morphology of granulocytes exposed in suspension to the C5a was compared to that of controls. A striking increase in surface

TABLE V

Effect of C5a on the Distribution of ^{45}Ca in Human Granulocytes

Fraction	Cell-associated ^{45}Ca*		
	Buffer	C5a	P‡
400 *g* pellet	4	3	>0.05
27,000 *g* pellet	16	26	<0.01
27,000 *g* supernate	79	70	<0.05

* Mean percent total counts per minute; three experiments.

‡ Paired *t* test.

TABLE VI

Effect of Chemotactic Inhibitors on ^{45}Ca Fluxes in Human Granulocytes Exposed to C5a

Inhibitor	Concentration	Chemotaxis (% control)*	^{45}Ca fluxes (% control)‡	
			Influx	Efflux
Sodium azide	1×10^{-2} M	9	76 ± 8	79 ± 2
Colchicine	4.0×10^{-4} M	45	76 ± 7	88 ± 2
Cytochalasin B	3.0 μg/ml	2	123 ± 10	82 ± 1
	0.1 μg/ml	130	102 ± 1	115 ± 1

* Control cells were exposed to C5a in absence of inhibitor; four determinations.

‡ Mean ± SE percent fluxes of four determinations measured in C5a cells without inhibitor

$$\left(\frac{\text{flux [cpm] in C5a-exposed cells + inhibitor}}{\text{flux [cpm] in C5a-exposed cells}} \times 100\right)$$

TABLE VII

Effect of C5a on the Random Migration of Human Granulocytes

Chemotactic chamber contents		Chemotactic responses* (cor cpm LF)
Upper compartment	Lower compartment	
Granulocytes + buffer	Buffer	62 ± 4
Granulocytes + C5a‡	C5a‡	626 ± 62
Granulocytes + buffer	C5a‡	2,260 ± 210

* Mean ± SE, five determinations.

‡ Final C5a concentration was 5 μg protein/ml chamber fluid.

irregularity (Fig. 7 *a*) was noted as compared to control granulocytes (Fig. 7 *b*). These data suggest that the morphologic changes in surface profile observed in granulocytes moving through a Micropore filter are in part the result of a direct effect of C5a on the cellular processes of movement itself and not necessarily the result of the interaction of a motile cell with a substratum.

Recent studies by Goldstein et al. (18) have demonstrated that addition of C5a to granulocytes in the presence of cytochalasin B results in transient assembly of microtubules detectable in the centriole region. As shown in Table VIII, electron microscope analysis of the above granulocyte suspensions exposed to C5a in the absence of cytochalasin B revealed a significant ($P < 0.001$) increase in the number of microtubules visualized in the centriole region through 30 min of incubation as compared to appropriate controls. In a related series of experiments granulocytes migrating through a Micropore filter towards a concentration gradient of C5a were examined. Few cytoplasmic microtubules were noted in profiles of nonmigrating granulocytes (cells remaining on the upper surface of the filter, Fig. 8 *a*) while granulocytes which had migrated deeply into the filter not only possessed a convoluted surface profile but had easily demonstrable cytoplasmic microtubules (Fig. 8 *b*).

DISCUSSION

Knowledge of the cellular basis of passive (random-spontaneous) migration as well as directional cell movement (chemotaxis) is essential to understanding the normal and pathologic function of granulocytes and of other motile cells. Most investigators agree that divalent cations, a source of energy such as ATP or GTP and some cellular contractile elements (microtubules and microfilaments) are responsible for the translation of a chemoelectrical signal into mechanical work. With regard to the granulocyte in particular, considerable controversy exists as to whether or not random-spontaneous and directional movement each occur as the result of separate physiologic events. For the purpose of this discussion, we have assumed that both types of cell movement require some of the same cellular processes and that directional movement represents a perturbation of the random process by a chemotactic factor.

The data presented in this communication provide evidence for a relationship between divalent cation requirements, Ca^{++} exchanges between the granulocyte and the surrounding environment, microtubule assembly, and chemotaxis. In addition we have correlated the effects of a variety of agents which either inhibited or enhanced chemotaxis with calcium fluxes.

The demonstration that both Ca^{++} and Mg^{++} are required for optimal chemotaxis is consistent with the hypothesis that Ca^{++} and Mg^{++} play linked as well as independent roles in human granulocyte movement as recently suggested in studies on rabbit leukocytes (1). Human granulocyte adhesiveness is a Mg^{++}-dependent process (19, 20) while the proposed mechanical work elements (microfilaments and microtubules) are Ca^{++} dependent (21–23). The importance of granulocyte adhesiveness to the vascular endothelium for initiation of in vivo leukocyte emigration from venules has recently been demonstrated in hamsters by Atherton and Born (24). The Ca^{++} and Mg^{++} requirements for optimal chemotaxis, therefore, probably reflect the necessity for optimal function of each independent cell process for directed locomotion.

The data in the present studies show that exposure of granulocytes in suspension to chemoattractants, albeit without a gradient of chemotactic factor, is directly associated with a rapid release of cell-associated calcium (see Fig. 5) similar to that reported in the slime mold amoeba (25). It is tempting to conclude that the observed release of calcium reflects increased calcium efflux. However, it is emphasized that our flux experiments were not directly measuring calcium ion flow across membranes but rather exchange of calcium between granulocytes and the surrounding medium; this may explain the small magnitude of the changes in calcium efflux. Moreover, it is apparent that the possible mechanisms by which a

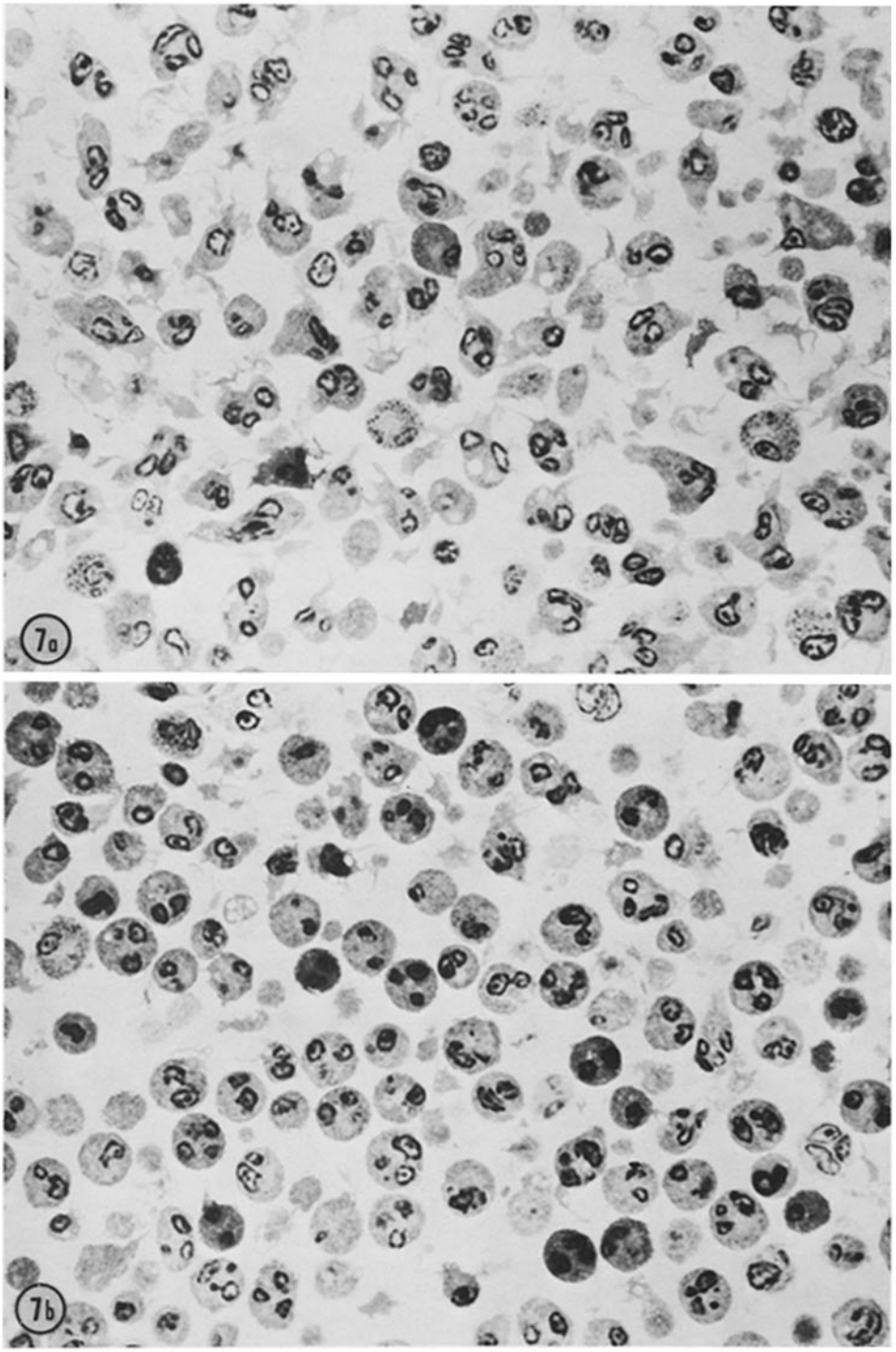

FIGURE 7 Plastic-embedded "thick" sections of granulocyte-rich leukocyte preparations fixed after exposure in suspension for 20 min to the chemotactic factor C5a (Fig. 7 *a*) or to medium alone (Fig. 7 *b*). Note the increase in surface irregularity observed in C5a-exposed cells even though there is no granulocyte contact with a substratum. × 2,400.

TABLE VIII

*C5a Induced Assembly of Microtubules in Human Granulocytes**

Experiment	Number of fields‡ Total	Microtubules present	%Containing microtubules	Chi-squared§	*P*
1. Control	70	6	9	27.4	<0.001
C5a	79	39	49		
2. Control	38	4	11	22.4	<0.001
C5a	25	18	72		
3. Control	67	15	22	17.4	<0.001
C5a	11	10	91		

* Cells in suspension fixed for electron microscope analysis 30 min after exposure to C5a or buffer control.
‡ Fields were examined under × 50,000 magnification. Microtubules were identified as structures that had straight, parallel sides, 240–280 Å apart, were at least 550 Å long and were more electron dense than the ground cytoplasm. Fields were scored as either containing or not containing microtubules.
§ 1 degree of freedom.

net release of cell-associated calcium occurs must include (*a*) transmembrane flow of calcium out of the cell, (*b*) release of membrane surface calcium as suggested by the rapid outflow noted in Fig. 5, or (*c*) decreases in calcium uptake from the surrounding medium as suggested in Fig. 6.

In other studies of ion fluxes calcium exchanges have been closely related to sodium and potassium fluxes. For example, in nerve cells, calcium efflux is coupled to sodium influx and the influx of sodium is influenced by an associated sodium-potassium pump (26). In the barnacle muscle fiber, increased internal sodium results in an increased calcium influx (26). It is possible that chemotactic factors induce changes in the permeability of the cytoplasmic membrane to different ions such as sodium or potassium which could then modify calcium fluxes. In support of the latter hypothesis is the report that ouabain, an inhibitor of sodium-potassium-sensitive ATPases (27) and thus the sodium pump of many different kinds of cells, inhibits chemotaxis (28). Whether sodium and/or potassium fluxes are coupled to calcium fluxes in human leukocytes stimulated by chemotactic factors remains to be determined.

Data obtained from studies of microtubulin proteins isolated from brain indicate that the degree of polymerization of the tubulin protein is regulated by the level of Ca^{++} and the nucleotide triphosphates ATP or GTP with optimal polymerization occurring in a low calcium environment (17, 29, 30). Our demonstration that exposure of granulocytes to chemotactic stimuli results in a Ca^{++} release, decreased Ca^{++} uptake, and an associated shift of cellular calcium from cytoplasmic to granular fractions (perhaps mitochondria) as well as the demonstration that the chemotactic factor C5a induces intracellular assembly of granulocyte microtubules during chemotaxis (Table VIII, Fig. 8) provides cellular correlates with the observation that isolated microtubule polymerization depends in part on a low calcium concentration. The transient nature of microtubule assembly recently noted in cytochalasin B-treated human granulocytes exposed to C5a without a chemotactic factor gradient (18) may be related to the cytochalasin B treatment. In our experiments, in the absence of cytochalasin B microtubule polymerization was not transient, persisted for the duration of the study (1 h), and was noted in cells migrating through a Micropore filter as well as in cells in suspension.

The demonstration of microtubule polymerization on exposure of granulocytes to C5a in association with the redistribution of cell calcium provides the basis for speculations on the role of microtubule function in the randomly and directionally migrating cell. In randomly moving cells microtubules could be envisaged as in a state of polymerization-depolymerization resonance causing the cytoplasmic streaming visualized by phase-contrast microcinematography (31). The increased random motility noted on addition of C5a to cell suspensions (no chemotactic factor gradient, Table

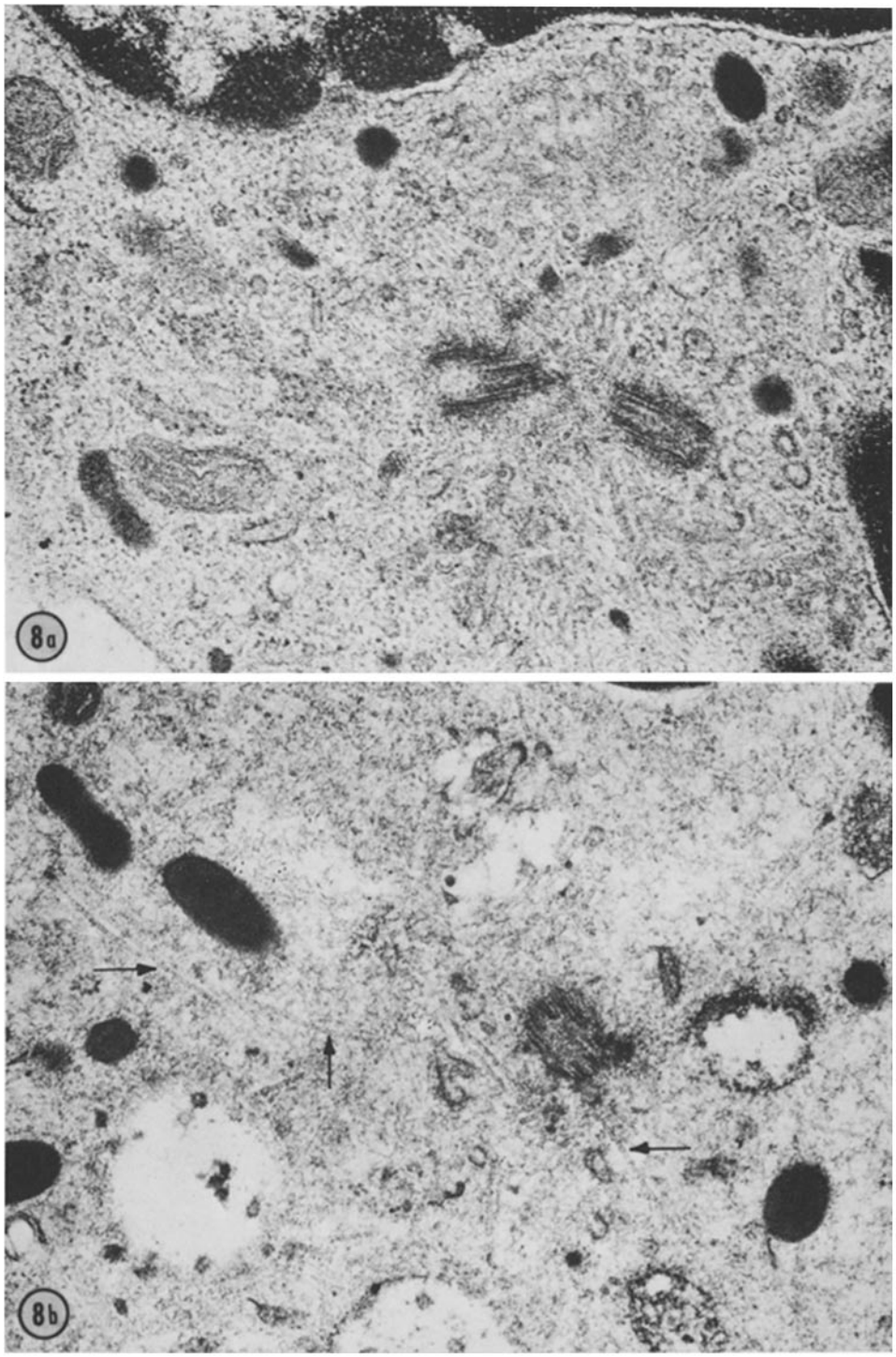

FIGURE 8 Representative electron micrographs of the centriole regions of granulocytes migrating through a Micropore filter under the influences of a concentration gradient of C5a. A nonmigrating cell from the upper surface of the filter, i.e., a region of low C5a concentration, exhibits few microtubules (Fig. 8 *a*), while a migrating cell deep within the filter nearest the source of C5a possesses easily demonstrable centriole-associated microtubules (arrows, Fig. 8 *b*). × 50,000.

VII) would be due to random assembly and disassembly of microtubules in conjunction with microfilament function. In the presence of a chemotactic gradient, a net polar immobilization of the cell membrane and/or cytoplasmic contractile elements would result from a localized decrease in cytoplasmic calcium nearest the chemotactic stimulus with subsequent polarized tubulin polymerization. Thus, the microtubule system would provide the net vector of motion (i.e., polarized pseudopod formation) while the microfilament system, which may require local increases in cytoplasmic membrane Ca^{++} (23), would provide the mechanical work for motion as previously suggested for macrophage chemotaxis (32). The recent demonstration of polarized pseudopod formation on exposure of granulocytes to a gradient of chemotactic factors (33) supports such a concept.

The regulatory role that the divalent cations calcium and magnesium play in human granulocyte chemotaxis suggests that analysis of local membrane and cellular events consequent to the interaction of chemotactic factors with the granulocyte surface is indicated.

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