INTERACTION OF CHEMOTACTIC FACTORS WITH HUMAN MACROPHAGES

Induction of Transmembrane Potential Changes

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ABSTRACT

The electrophysiology of chemotactic factor interaction with cultured human macrophages was investigated with standard intracellular recording techniques. In initial studies, *E. coli* endotoxin-activated serum, added to cell cultures during intracellular recordings, caused membrane hyperpolarizations which were greater than 30 s in duration, 10–50 mV in amplitude, and associated with decreased membrane resistance. Control serum produced smaller hyperpolarizations lasting 10–20 s and 5–30 mV in amplitude. Endotoxin-activated human serum deficient in the third complement component (C3) did not produce hyperpolarizations unless the serum was reconstituted with C3 before activation. Fractionation of normal activated serum by molecular seive chromatography (G-75 Sephadex) indicated that only fractions that eluted with an estimated molecular weight of 12,500 produced membrane potential changes. The active material that was chemotactic for the macrophages was identified as the small molecular weight cleavage product of C5, C5a, by heat stability (30 min at 56°C) and inactivation by goat antisera to human C5 but not C3. 17% of macrophages stimulated with C5a exhibited a biphasic response characterized by a small (2–6 mV), brief (1–10 s) depolarization associated with a decreased membrane resistance preceding the larger and prolonged hyperpolarizations. Magnesium-ethylene glycol bis[β-aminoethyl ether]*N*, N′-tetraacetic acid (Mg [2.5 mM]-EGTA [5.0 mM]) blocked the C5a-evoked potential changes, whereas colchine (10^{-6} M) and cytochalasin B (3.0 μg/ml) did not. Hydrocortisone sodium succinate (0.5 mg/ml) decreased the percentage of cells responding to C5a. In related studies, synthetic *N*-formyl methionyl peptide (f-met-leu-phe), which had chemotactic activity for cultured macrophages, produced similar membrane potential changes. Repeated exposure of macrophages to C5a or f-met-leu-phe resulted in desensitization to the same stimulus. Simultaneous photomicroscope and intracellular recording studies during macrophage stimulation with chemotactic factor demonstrated that the membrane potential changes preceded membrane spreading, ruffling, and pseudopod formation. These observations demonstrate that ion fluxes associated with membrane potential changes are early events in macrophage activation by chemotactic factors.

Activation of leukocytes by chemotactic factors is a complex process in which random locomotion becomes directed locomotion (chemotaxis) when cells are exposed to a gradient of chemotactic factor (13). Chemotactic factors also initiate exocytosis of leukocyte granules and release of intracellular enzymes (1, 2, 14, 20). The precise mechanisms by which chemotactic factors activate these leukocyte functions are poorly understood, although a role for divalent and monovalent cations has been implicated (12, 17) and decreases in membrane surface charge have been demonstrated (11). The studies to be presented assess the initial events of leukocyte activation by chemotactic factors with electrophysiological techniques. Cultivated human macrophages, which respond to chemotactic factors with increased locomotion, were used as the model cell because their size (20–40 μm) makes them better suited for electrophysiological study than neutrophils or peripheral blood monocytes.

Macrophages of guinea pig, mouse, and humans have previously been shown to exhibit spontaneous membrane hyperpolarizations which are associated with increased membrane permeability to potassium (9). Although the functional significance of these hyperpolarizations was not established, the action of both the calcium ionophore A23187 (which produced prolonged hyperpolarizations) and magnesium-ethylene glycol bis[β-aminoethyl ether]*N*,N′-tetraacetic acid (Mg-EGTA; which blocked both the spontaneous and ionophore-induced hyperpolarizations) indicated a role for calcium in these events. The data also suggested that potassium permeability of the macrophage membrane is influenced by the calcium concentration within the cell. Evidence exists that changes in intracellular calcium ions occur during interaction of leukocytes with chemotactic factors (12). If intracellular calcium levels influence the potassium permeability of the leukocyte cytoplasmic membrane as suggested by our previous work, then membrane potential changes reflecting permeability changes to K^+ ions might be expected to occur during chemotaxis.

In the current communication, we correlate transmembrane potential changes in human macrophages with cell activation by chemotactic factors. These observed potential changes precede chemotactic factor-induced pseudopod formation. The membrane potential changes are specific for the chemotactically active materials and reflect ionic events occurring during macrophage activation by these agents.

MATERIALS AND METHODS

Media and materials used were: complete culture media consisting of RPMI 1640 Medium (Grand Island Biological Co., Grand Island N. Y.) supplemented with fresh L-glutamine (0.3 mg/ml; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J.), penicillin and streptomycin (Grand Island Biological Co.), and 5% heat-inactivated (56°C for 30 min) human AB serum (Flow Laboratories, Inc., Rockville, Md.); Gey's balanced salt solution with 2% penicillin, 2% streptomycin, and bovine serum albumin (Microbiological Associates, Bethesda, Md.); Hanks' balanced salt solution (National Institutes of Health Media unit); *E. coli* 0127B 8 lipopolysaccharide B (Difco Laboratories, Detroit, Mich.); Sephadex G-75 and Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); hypaque (Winthrop Laboratories, New York); 12-μm Nucleopore polycarbonate filters (Neuro Probe, Inc., Bethesda, Md.); triarylmethane and methyl alcohol fixative, xanthene dye and thiazine dye (Harleco, Gibbstown, N. J.).

Collection and Culture

Peripheral blood mononuclear cells were obtained from normal human subjects by the Hypaque-Ficoll sedimentation method (6). The mononuclear cells were washed twice in Hanks' media and then suspended at 3.0×10^6/ml in complete culture media. The cells were plated on 35-mm plastic Petri dishes. After 23 h of incubation in 5% CO_2-95% air atmosphere at 37°C, nonadherent cells were washed off. Cells were cultured for up to 4 wk, the media being changed every 3 days. For most experiments, cells were used after 2–3 wk of culture when they were 15 to 40 μm in diameter. These cells were viable as assessed by their ability to exclude trypan blue dye.

Macrophage Chemotaxis

3-wk-old macrophages were removed from Petri dishes with a "rubber policeman" and suspended in Gey's tissue culture medium at a density of 2.0×10^6 cells/ml. Macrophage locomotion was assessed by using previously described chemotactic chambers (21) in which a 12-μm pore size polycarbonate filter separated the upper and lower compartments which contained the cells and chemotactic factors, respectively. The chambers were incubated for 90 min at 37°C in 100% humidity, 5% CO_2-95% air mixture; then the filters were fixed in triarylmethane and methyl alcohol and stained with a xanthene and thiazine dye mixture followed by six sequential rinses in distilled water. Cells that had migrated through the filter were counted in 5–10 high-power fields and expressed as cells per high power field (hpf). All samples were done in quadruplicate, and the SE deter-

mined. When two conditions were compared, Student's *t* test was used to calculate the statistical significance except where indicated otherwise.

Preparation of Chemotactic Factors

Fresh frozen human serum was activated with *E. coli* lipopolysaccharide as previously described (10). 4 ml of serum was mixed with 0.17 ml of 0.85% NaCl with 1.2 mg of *E. coli* endotoxin. Control serum had saline without endotoxin. The mixture was incubated at 37°C for 1 h and then brought to 56°C for 30 min. For some experiments, serum was heated at 56°C for 30 min before adding endotoxin. In other experiments, endotoxin was added to 4 ml of saline and carried through the same incubation. Human serum deficient in the third complement component and purified human C3 were the generous gift of Dr. Michael M. Frank.

The cleavage product of the fifth component of complement, C5a, was partially purified from human serum activated with endotoxin as described above, except that instead of heating at 56°C for 30 min, 10 mM EDTA was added to the activated serum. 3 ml of activated serum was applied to a 20.5 × 100-mm column of Sephadex G-75 (Pharmacia Fine Chemicals). 5-ml fractions were eluted from the column with 0.85% saline buffered with 0.04 M phosphate to pH 7.4 at 4°C. The protein elution profile was estimated by measuring the optical density at 280 nm, and the macrophage chemotactic activity in the eluates was measured by using undiluted fractions in chemotactic chambers. Selected fractions were pooled and used for electrophysiological studies.

Pure synthetic peptide chemotactic factor *N*-formyl methionyl (f-met-leu-phe, 19, 20) was the generous gift of Dr. Elliot Schiffman. The pH and osmolarity of the C5a and f-met-leu-phe were the same as those of control media.

Complement Components

Partially purified human C3 (Cordis Laboratories, Miami, Fla.) and goat antibody to human C3 and C5 were commercially obtained (Meloy Laboratories, Inc., Springfield, Va.). The antisera were heated at 56°C for 30 min before use. 0.50-ml of chemotactically active fractions from the Sephadex G-75 column were mixed with 25 μm antisera, incubated at 37°C for 30 min, and used directly. For controls, antisera were incubated similarly with saline.

Electrophysiology

For electrical recordings, tissue culture dishes were fitted into a Lucite chamber mounted on a Zeiss inverted microscope. A 10% CO_2-90% air mixture was passed across the surface of the Petri dish to maintain pH. Experiments were performed at room temperature, 25–28°C. Glass microelectrodes filled with 4 M K acetate with resistances of 60–150 MΩ were used. Ag-AgCl indifferent electrodes were connected to the culture chamber by 2 M KCl agar salt bridges with 10-μm tip diameter. A unity gain active bridge amplifier enabled one electrode to be used for both recording and current injection. Cell input resistance was calculated from the electronic potentials elicited by small current pulses (< 0.5 nA) injected via the microelectrode. The injected current was monitored with a virtual ground-current meter connected to bath ground. The output of both the unity gain amplifier and the current meter were displayed on a Brush chart recorder and a cathode ray oscilloscope.

Data from cell penetrations were used only if the transmembrane potential and input resistance of the cell were stable for at least 2 min and the bridge balance returned to its value before penetration upon withdrawal of the electrode from the cell. Substances tested for their ability to affect the electrophysiological properties of the macrophage were added to the bath either directly through a microliter pipette or put into a broken microelectrode whose tip was 10–20 μm in diameter. The microelectrode containing the test compound was then brought very near to, but not touching, the cell surface with a micromanipulator, and the test compound was allowed to diffuse slowly from the electrode for 30 s or until a response was recorded. In this way, several cells in a single dish were exposed to a gradient of the test compound, although the actual concentration of the compound to which the cells were exposed was not known.

RESULTS

Electrophysiology of Unstimulated Macrophages

Impaled macrophages, 20–40 μm in diameter, were well spread on the surface of the culture dish, often exhibiting a thin veil of peripheral cytoplasm surrounding a mounded inner region. The microelectrode usually penetrated this central region of the cell. During impalement, cells remained attached to the surface of the dish. Cell damage due to microelectrode impalement was immediately observed as swelling accompanied by a decrease in membrane potential and input resistance. Such cells were not used for study. The average transmembrane potential and input resistance of 45 macrophages were -14.5 ± 5 mV (SD; range 6–30 mV) and 84.4 ± 30 MΩ (SD; range 25–120 MΩ), respectively. The current voltage (I/V) relationship was linear in the range of −40 to +40 mV. Occasionally, a membrane hyperpolarization lasting several seconds was seen upon puncturing a cell, but otherwise, spontaneous hyperpolariza-

TABLE I

*Chemotactic Responsiveness of Human Macrophages**

Stimulus	Chemotaxis‡	P§
	cells/hpf	
Buffer	3±1	–
E. coli endotoxin-activated serum	25±3	<0.001
C5a (5 μg/ml)	20±2	<0.001
f-met-leu-phe (10^{-6} M)	17±2	<0.01

* Macrophage (3-wk cultures of monocytes) migration through 12-μm polycarbonate filters after 90 min, using chemotactic chambers. See Materials and Methods.

‡ Mean ± SEM, four determinations.

§ Significance level of difference compared to buffer controls.

tions were infrequent. In this respect these cells, cultured from peripheral blood monocytes, differed from guinea pig peritoneal exudate macrophages which exhibit more frequent spontaneous hyperpolarization (9). Hyperpolarizations could be induced, however, by either mechanical or electrical (large depolarizing pulses) stimulation as we previously reported in macrophages of guinea pig, human, and mouse (9).

Endotoxin-Activated Serum and Control Serum

CELL LOCOMOTION: Addition of 5% endotoxin-activated serum to Petri dishes containing 2- to 3-wk-old macrophage cultures resulted in increased membrane ruffling and pseudopod formation, confirming a previous report (3). As shown in Table I, when cultivated macrophages were removed from the Petri dishes and placed in chemotactic chambers, they migrated across as 12-μm polycarbonate filter in response to endotoxin-activated serum, partially purified C5a, and the synthetic peptide chemotactic factor f-met-leu-phe. In related studies, migration was significantly greater when a gradient of endotoxin-activated serum was present (directed migration of chemotaxis) than when no gradient was present with the stimulus on both sides of the filter (stimulated random migration): 25±3 vs. 13±1 cell/hpf ($P < 0.05$). When cells were exposed to buffer as a stimulus (spontaneous random migration), very few cells migrated across the filter. The locomotory response to control serum was significantly greater than to buffer: 11±2 vs. 3±1.0 cells/hpf ($P < 0.01$), indicating that control serum has weak chemotactic activity.

ELECTROPHYSIOLOGY: Endotoxin-activated serum added directly to the bath through a microliter syringe to give a final concentration of 5.0% initiated a large membrane hyperpolarization within seconds. As shown in Table II, the mean hyperpolarization in 14 out of 15 cells tested was 28.8±4.2 mV (range 10–50 mV). In addition, the endotoxin-activated serum-induced response was more prolonged than the spontaneous or electrically induced hyperpolarizing activations previously reported (9). Cells often took more than 1 min to repolarize. In 10 different cells from the same group, control serum with no endotoxin added to it produced hyperpolarizations in 40% of the cells. As shown in Table II, the average hyperpolarizations produced by control sera were significantly smaller in amplitude (mean amplitude = 12.3±4.3 mV, range 5–30 mV) than those produced by endotoxin-activated serum. Control serum to which endotoxin was added after heating at 56°C for 30 min did not significantly differ from control serum alone. The concentration of control or endotoxin-activated serum used in these studies did not change the pH or osmolality of the cell bath. In addition, *E. coli* endotoxin alone had no effect on these cells in concentrations of 15–600 μg/ml.

The response of a macrophage to both control serum and to endotoxin-activated serum is shown in Fig. 1 A. The cell hyperpolarized by 30 mV after 0.1 ml of control serum was added to a bath volume of 2 ml. 3 min after the hyperpolarization (which lasted for 30 s), an additional 0.1 ml of activated serum was added to the bath. The addition of activated serum produced another hyperpolarization which lasted more than twice as long

TABLE II

Comparison of Membrane Hyperpolarizations Induced by Control Sera and E. coli Endotoxin-Activated Sera

Stimulus	Hyperpolarization (mV change)*	P‡
Control sera (5%)	12.3±4.3 (10)	–
E. coli endotoxin-activated sera (5%)	28.8±4.2 (14)	<0.02

* mV change in membrane potential compared to basal conditions. Mean ± SEM. Number of cells tested in parentheses.

‡ Significance of difference, Student's *t* test.

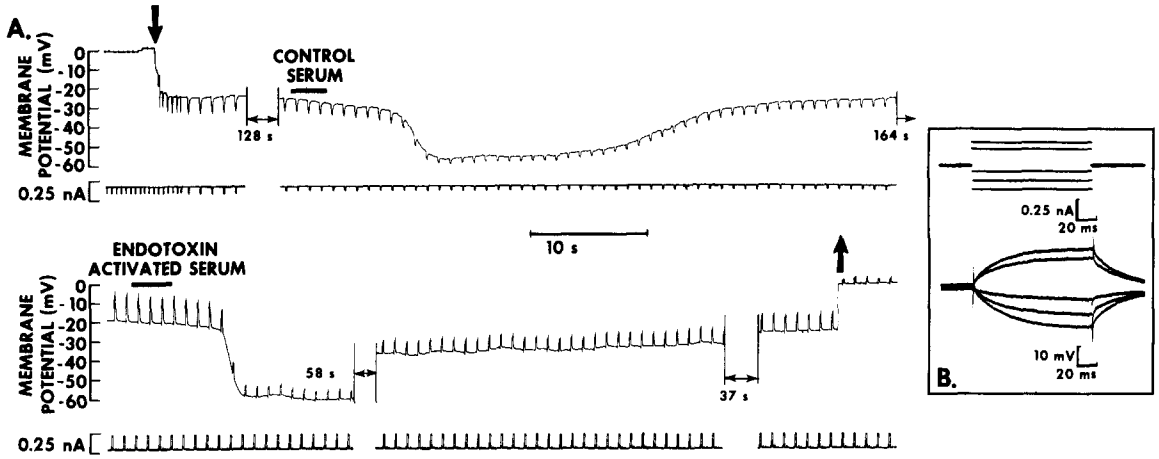

FIGURE 1 (A) Continuous recording from a human macrophage exposed to control serum and endotoxin-activated serum. Vertical arrows indicate penetration (↓) and removal (↑) of the microelectrode from the cell. Small current pulses used to measure membrane resistance are superimposed on the tracing. The cell resting membrane potential = −24 mV. (B) Picture of oscilloscope tracings of current injected through microelectrode (top tracing) and evoked potential changes (bottom tracing). Same cell as A.

as the response to control serum. In fact, the cell was not fully recovered after 140 s when the electrode was removed from the cell.

The repolarization following a hyperpolarizing response was much slower than the recovery seen during the spontaneous hyperpolarizations that we previously reported (9). In addition, the membrane resistance often did not return to prestimulation values. Responses to control serum and endotoxin-activated serum were associated with decreased membrane resistance as indicated by the decreased electrotonic potential produced by the constant current pulses. To accurately determine the input resistance of the cells studied, photographs were taken of oscilloscope traces of the electrotonic potential produced by the constant current pulses. Oscilloscope traces of the current (top trace) pulses used to measure the input resistance of the cell in Fig. 1 A and the membrane potential displacement produced by these pulses (bottom trace) are shown in Fig. 1 B. The input resistance of this cell estimated from the photograph is 65 MΩ. During the hyperpolarizing response to endotoxin-activated serum, the input resistance of this cell decreased from 65 to 20 MΩ.

The preceding studies with endotoxin-activated normal human serum suggested that the hyperpolarizations were related to products of complement activation stimulating the macrophage membrane. To explore this possibility further, we studied activated serum deficient in C3 in several cells. As shown in Fig. 2, macrophages did not respond to endotoxin-activated human serum deficient in the third component of complement unless the serum was first reconstituted with C3. During the hyperpolarizing response shown in Fig. 2, the membrane resistance decreased from 80 to 40 MΩ. Oscillations in the membrane potential can be seen at the end of each response to C3-deficient serum reconstituted with C3. A number of cells exhibited these oscillations during membrane hyperpolarizations. Control studies in which the following stimuli were used gave no response: C3 in Hanks' media or C3 added to C3-deficient serum after endotoxin activation. Addition of C3 to normal human serum did not modify the normal response.

Macrophage Chemotactic Activity and Membrane Potential Changes Produced by G-75 Sephadex Fractions of Endotoxin-Activated Serum

Assay of the fractions obtained after G-75 gel filtration of unactivated serum revealed minimal chemotactic activity and no change in macrophage membrane potential. However, as shown in Fig. 3, Sephadex G-75 gel filtration of serum-activated *E. coli* endotoxin resulted in a large peak of chemotactic activity with an estimated weight of 12,500 daltons. The chemotactic activity was stable when treated for 30 min at 56°C and was inactivated by

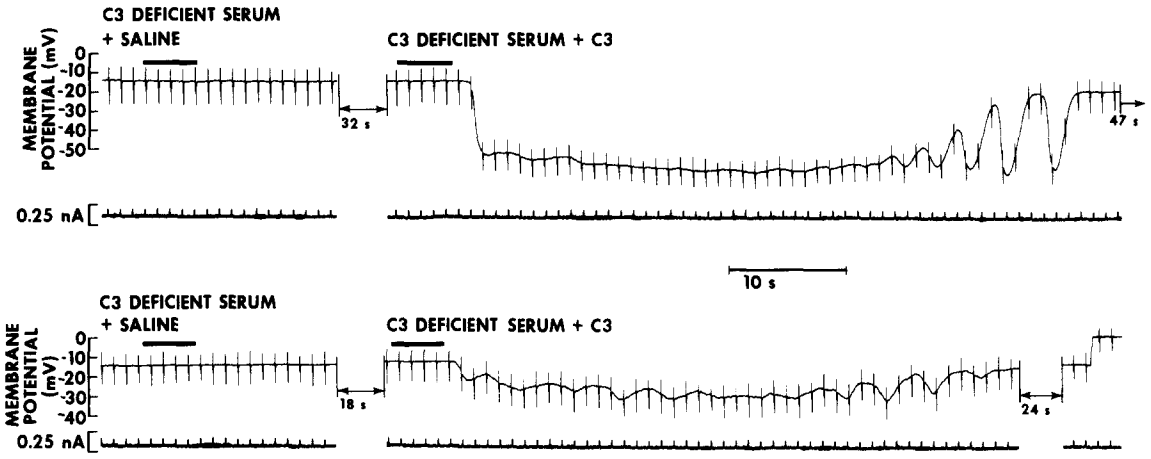

FIGURE 2 Recording from a macrophage exposed to endotoxin-activated C3-deficient serum with and without reconstitution by C3. The upper and lower panels represent a continuous tracing from the same cell. Resting membrane potential = −13 mV.

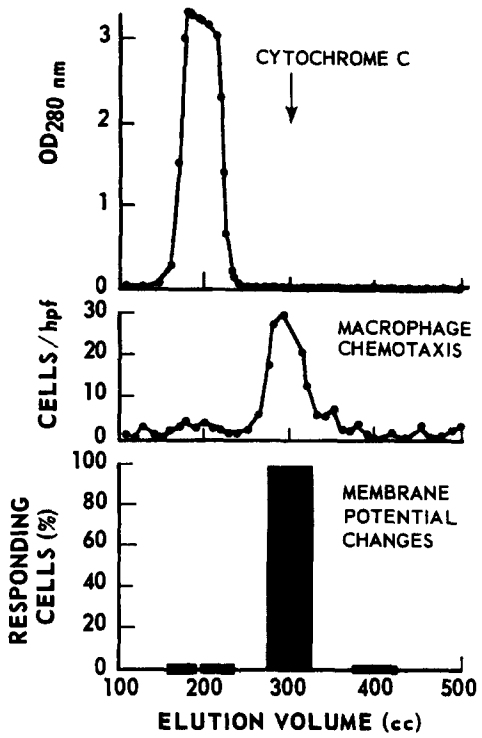

FIGURE 3 G-75 Sephadex chromatography of endotoxin-activated human serum. Upper panel shows absorbance at 280 nm, middle panel shows chemotactic responsiveness of human macrophages (2-wk culture), and lower panel shows the ability of various fraction pools to produce hyperpolarizing potential changes in human macrophages.

goat antisera to the fifth but not the third components of human complement. It was therefore identified as the small molecular weight cleavage product of C5, C5a. Four different pooled column fractions of the endotoxin-activated serum, one of which contained C5a, were tested for their ability to produce membrane potential changes. This was assessed with a blunted microelectrode containing each test fraction. The electrode was brought close to the cell being studied and left in place for 30 s or until a response was elicited. For one preparation of activated serum (shown in the lower panel of Fig. 3), the fractions containing C5a produced hyperpolarizations 100% of the time, whereas the other fractions never produced hyperpolarizations. During the course of our studies, G-75 gel filtration was performed on three separate preparations of endotoxin-activated sera, and the activity of the C5a fractions was evaluated. For each serum preparation, membrane potential changes were restricted to fractions eluting with C5a. The C5a preparations differed in their activity; out of a total of 56 cells tested with all three fractions, 80% responded to C5a with membrane hyperpolarizations.

The effect of goat antisera to the third and fifth components of human complement on C5a-induced hyperpolarizations of the macrophage membrane is shown in Fig. 4. Treatment with anti-C5 completely inhibited the capacity of the pooled C5a to induce hyperpolarizations, whereas antiserum to C3 did not.

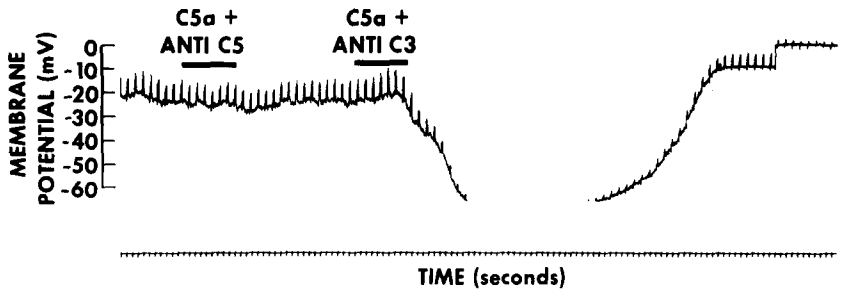

FIGURE 4 Inactivation of C5a response by anti-C5. Recording from a macrophage exposed to C5a which was incubated with goat antiserum to C5 or goat antiserum to C3. Resting membrane potential = −20 mV.

Related studies were performed to see whether there was any association between chemotactic factor-induced potential changes and morphologic events such as pseudopod formation. Because the process of puncturing a cell with a microelectrode occasionally produced both morphologic and electrical changes, we were cautious to perform morphological studies on cells which appeared stable both electrically and morphologically. In every cell exhibiting potential and morphological changes, the electrical events preceded the morphological changes. In nine cells exposed to C5a in which serial photographs were taken, seven cells responded to C5a with membrane potential changes. Four of these cells showed detectable morphological changes, two of these cells were questionable, and one cell showed no detectable changes. The direction of cell movement was usually dependent on the location of the C5a stimulus, although sometimes only a generalized shape change was seen. Control stimuli such as fractions from the G-75 Sephadex column shown in Fig. 3 did not cause electrical changes, did not cause chemotaxis, and produced no obvious morphological changes. Fig. 5 shows both photomicrographs and potential tracings from one of the cells studied. Note that the morphological changes occurring in the impaled cell during the course of the experiment followed the electrical events induced by the chemotactic factor.

Desensitization of the C5a Response by Repetitive Stimulation

Macrophages were repeatedly exposed to C5a by bringing a blunt microelectrode close to the cell until a response was elicited. Then the electrode containing C5a was removed for 10–30 s before repeated stimulation. Repeated exposure of macrophages to C5a resulted in a "desensitization" of the response. An example of this desensitization is seen in Fig. 6, in which a macrophage responded to three successive C5a exposures but did not respond to a fourth exposure. Note that a longer time elapsed with successive stimuli between C5a exposure and the beginning of the evoked response. Each successive response to C5a was decreased in amplitude and duration.

Biphasic response

13 of 56 cells (17%) stimulated with C5a exhibited a biphasic response in which the hyperpolarization was preceded by a small membrane depolarization. The cell in Fig. 6 responded to C5a with a biphasic response. These membrane depolarizations lasted 1–10 s, were 2–6 mV in amplitude, and were associated with decreased membrane resistance. 7% of 56 cells studied depolarized by 2–6 mV without subsequent hyperpolarizations. Thus, 24% of the 56 cells exhibited a depolarizing response. The absence of a detectable depolarizing response in many cells may reflect the fact that the large hyperpolarization obscures the smaller depolarization.

The ionic events contributing to each component of the biphasic response were studied by determining the reversal potential (the membrane potential at which C5a produces no response) of both the depolarizing and hyperpolarizing responses. To do this, the membrane potential was varied by applying a DC current through the microelectrode before stimulation with C5a. The amplitudes of C5a-induced depolarizing and hyperpolarizing responses were then plotted as a function of the adjusted membrane potential. Most cells desensitized to repeated exposure with C5a, rendering such analysis difficult to obtain in a single cell. However, one cell responded to eight repeated C5a exposures before desensitizing.

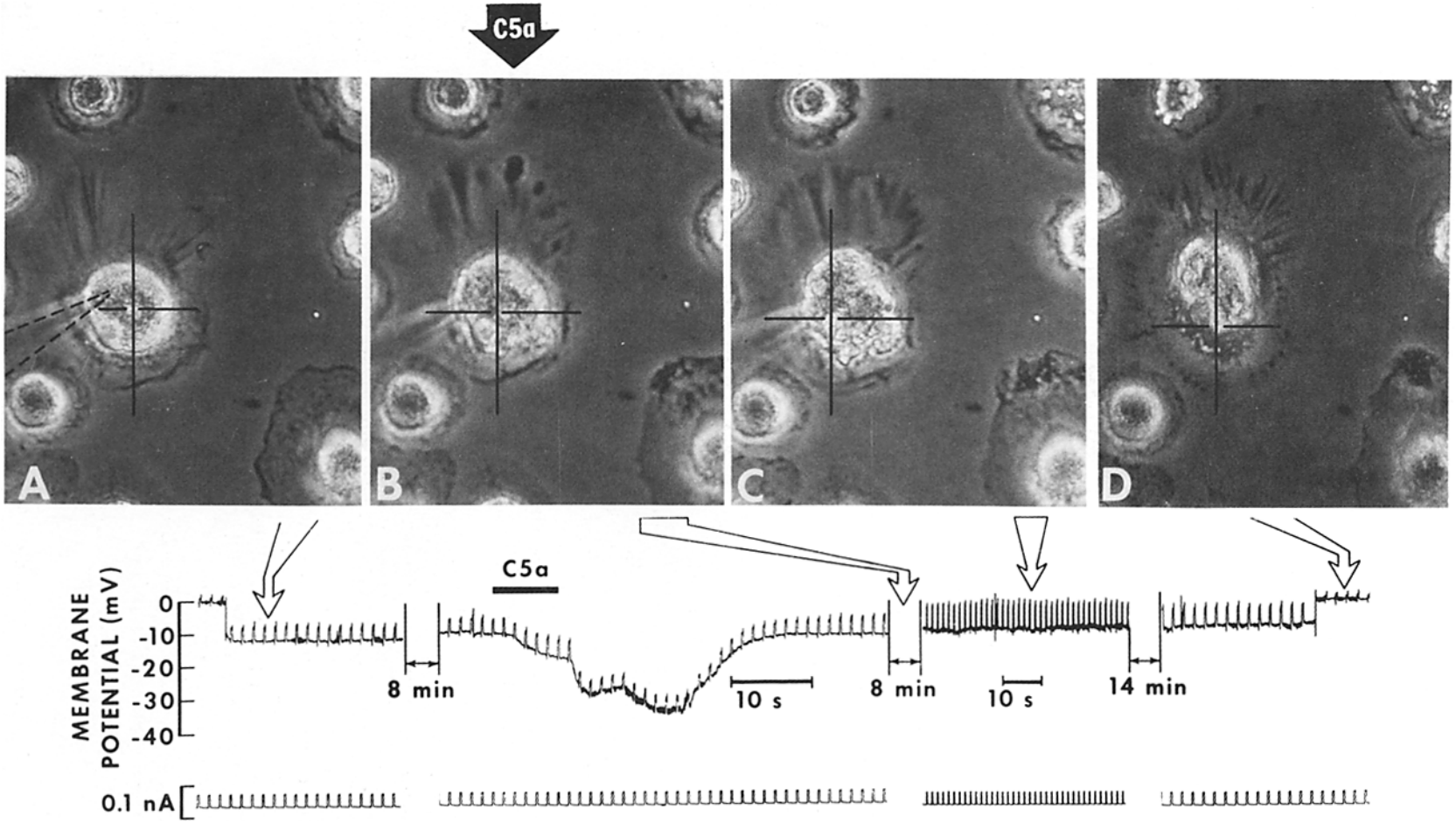

FIGURE 5. Top pictures show morphological changes occurring during course of the membrane potential recording. The arrows connecting the potential tracing (bottom of figure) indicate the time during the recording at which the pictures were taken. The cell was exposed to C5a two times for 30 s each and hyperpolarized each time. The direction of the C5a stimulus is shown by the arrow near picture B. Picture D was taken after the electrode was withdrawn from the cell. Resting membrane potential = −10 mV.

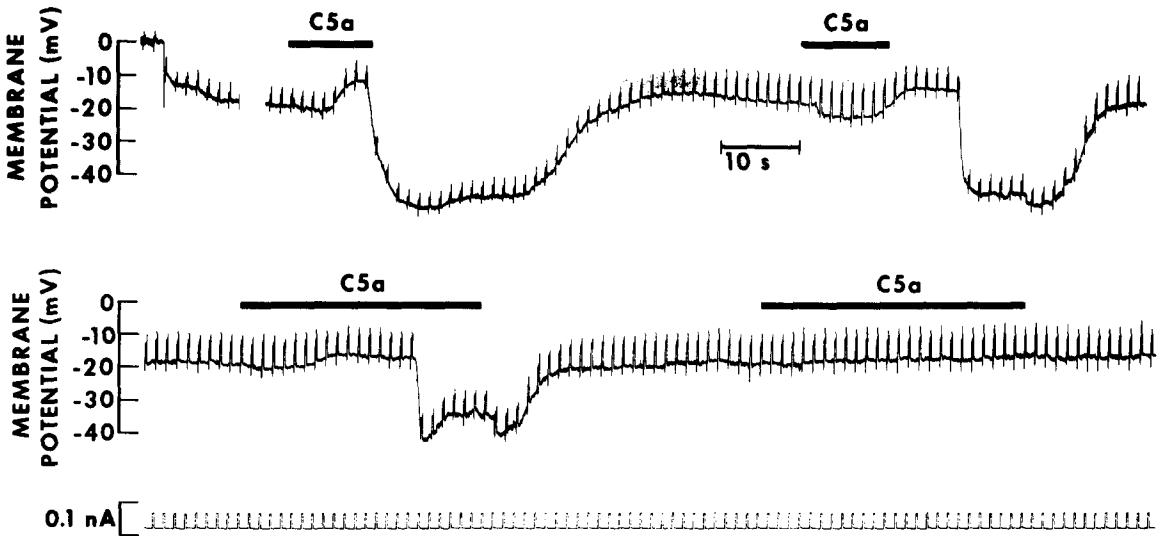

FIGURE 6 Effect of repetitive exposure of a macrophage to C5a contained in a blunt microelectrode. Resting membrane potential = −20 mV.

Tracings of five consecutive responses of this cell to C5a at different membrane potentials (Vm) are shown in Fig. 7 A. A comparison of the amplitudes of the first response at the resting membrane potential (Vm) = −8 mV and the last response at the membrane potential (Vm) = −20 mV indicates that minimal desensitization occurred. In Fig. 7 B the amplitude of the peak depolarizing response and hyperpolarizing response is plotted as a function of the membrane potential for each tracing in Fig. 7 A. At Vm = +30 mV, the depolarizing response is reversed and the peak potential was difficult to determine. The amplitude of the peak "depolarizing" response at this point was arbitrarily taken as the difference between the resting membrane potential and the membrane potential at which the fast hyperpolarization began.

The reversal potential for the depolarizing response (0 mV) would be compatible with an increased permeability to calcium or sodium (which have positive equilibrium potentials) and potassium (which has a negative equilibrium potential). The reversal potential for the hyperpolarization (−70 mV) is consistent with an increased permeability to potassium. Additional experiments involving changing the internal and external concentration of ions are required to further define the ionic events during these potential changes.

Effect of EGTA, Colchicine, Corticosteroids, and Cytochalasin B

Addition of Mg (2.5 mM)-EGTA (5.0 mM) to the bath before stimulation with C5a prevented the potential changes. When Mg-EGTA was added during a hyperpolarizing response, the hyperpolarization promptly terminated. In related studies, Mg-EGTA treatment inhibited chemotaxis by 70% ($P < 0.01$).

The effect of colchicine, cytochalasin B, and hydrocortisone sodium succinate on the ability of C5a to induce potential changes was studied (Table III). Each agent was incubated with the cells for 30 min before electrophysiological study. Such treatment blocked the chemotactic responsiveness of the macrophages. Neither colchicine nor cytochalasin B changed the percentage of cells responding to C5a. In contrast, hydrocortisone decreased the percentage of cells responding to C5a, with 86.4±8.8% of cells responding before treatment vs. 56.6±13.8% responding after incubation with the hydrocortisone ($P < 0.05$, Cochran's weighted mean difference test [7]). Although hydrocortisone decreased the percentage of cells responding to C5a, it had no effect on the amplitude of the C5a-induced potential changes (23.4±2.24 mV in 20 responding control cells vs. 21.59±3.17 mV in hydrocortisone-treated cells, $P > 0.05$, Student's t test).

Response to Synthetic f-met-leu-phe Chemotactic Factor

The small molecular weight f-met-leu-phe attracted 3-wk-old macrophages in the chemotactic chambers, with 17±2 cells/hpf migrating in response to f-met-leu-phe (10^{-6} M) vs. 3±1.0 cells/

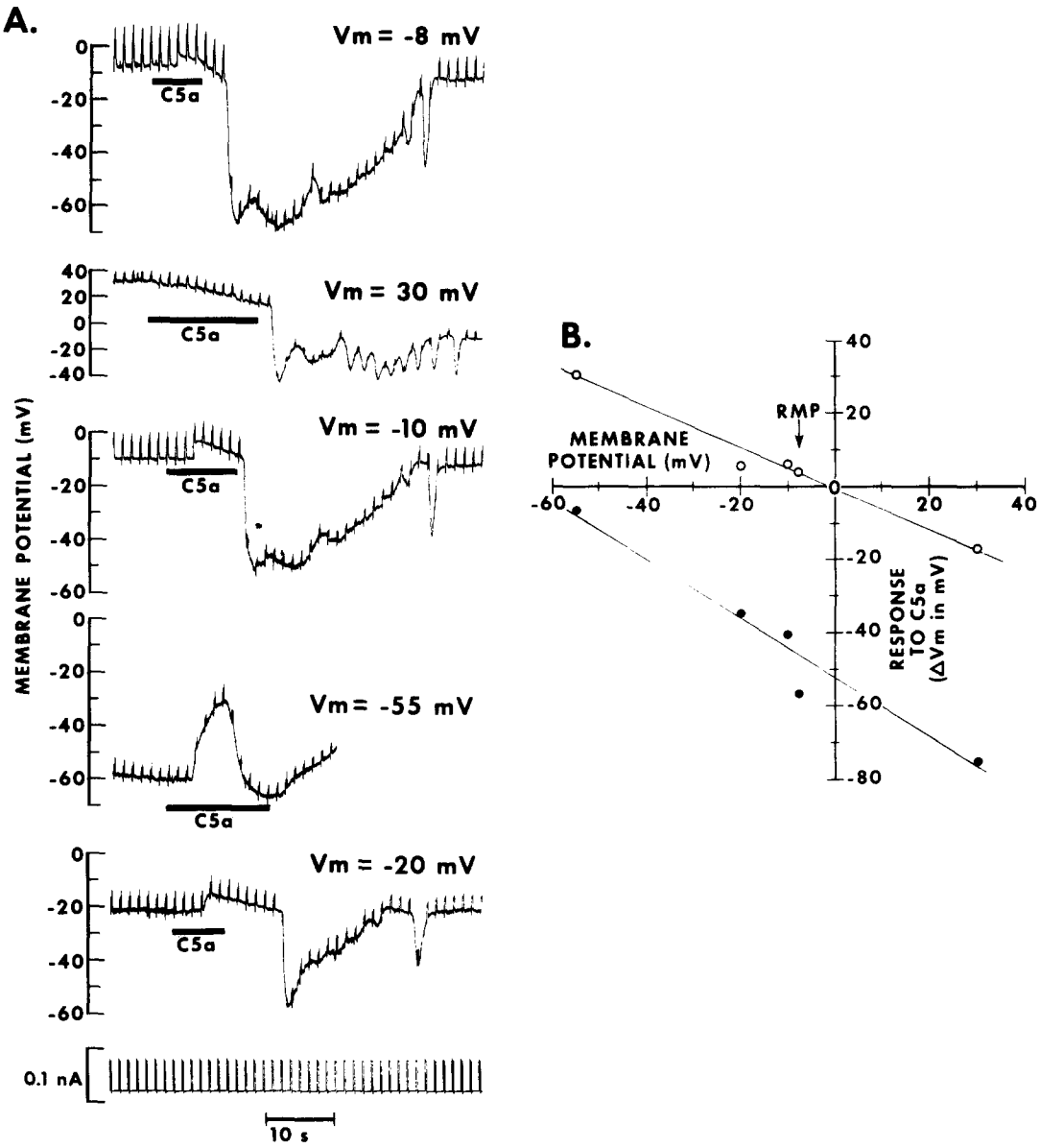

FIGURE 7 (A) Voltage changes produced in a macrophage during five consecutive exposures to C5a at different membrane potentials (Vm). The membrane potential was altered by applying a steady DC current through the microelectrode. C5a was applied through a blunt microelectrode. Resting membrane potential = −8 mV. (B) The peak amplitudes of both the depolarizing responses (open circles) and the subsequent hyperpolarizing responses (closed circles) shown in 7 A as a function of steady membrane potential (Vm) in a human macrophage. Resting membrane potential indicated in the graph.

hpf in response to buffer. When blunted electrodes containing f-met-leu-phe (10^{-3} M – 10^{-5} M) were brought adjacent to cultivated macrophages, a hyperpolarization resulted. An example of this response is shown in Fig. 8 in which a cell with a −8 mV resting potential was exposed to both C5a and f-met-leu-phe by bringing separate blunt electrodes filled with either of these agents near the cell surface at different times. Each agent induced a membrane hyperpolarization. Repeated

stimulation with f-met-leu-phe resulted in desensitization of the response as was noted with C5a (Fig. 6).

DISCUSSION

The data presented are the first direct evidence that molecules which are capable of initiating directed migration (chemotaxis) of human leukocytes transiently alter the membrane potential of those cells. Initially, we noted that endotoxin-activated normal human serum, which was chemotactic for cultured macrophages, induced large membrane hyperpolarizations, whereas endotoxin-activated human serum deficient in C3 neither possessed chemotactic activity nor evoked membrane potential changes. Both properties were restored by addition of purified C3. These initial experiments suggested that chemotactically active products of complement activation caused the membrane potential changes.

To further explore the relationship between chemoattractants and changes in transmembrane potential, endotoxin-activated serum was fractionated by molecular sieve chromatography (G-75 Sephadex). Only chemotactically active fractions eluting with an estimated molecular weight of 12,500 daltons initiated membrane potential changes. The active moiety was identified as the small molecular weight cleavage product of C5, C5a, on the basis of its stability at 56°C (30 min) and its inactivation by goat antisera to human C5 but not C3. Additional evidence for an association between chemotactic activity and membrane potential changes was provided by experiments in which pure synthetic f-met-leu-phe produced membrane potential changes similar to those noted with C5a. Furthermore, the desensitization of the electrical response with repeated exposure to C5a and f-met-leu-phe may be the physiological correlate of "deactivation" of leukocyte-directed locomotion by pretreatment of leukocytes with chemotactic factor (22).

In addition to activating macrophage movement, chemotactic factors stimulate enzyme secretion (1, 2, 14, 20). Our results do not exclude the possibility that the potential changes produced by C5a may be related to secretion or other events triggered by chemotactic factors that are not directly related to chemotaxis. In leukocytes, both secretion and directed locomotion required calcium (2, 12) as do the membrane potential changes produced by C5a. Modulation of leukocyte locomotion and/or secretion by chemotactic factors may be controlled by the amount of cal-

TABLE III

Effect of Agents Which Inhibit Chemotaxis on C5a-Induced Potential Changes

Agent	Responding cells*	
	Before treatment	After treatment
Colchicine (10^{-6} M):		
Exp. 1	4/4 (100)	5/5 (100)
Cytochalasin B (5 μg/ml):		
Exp. 1	0/1 (0)	5/6 (63)
Exp. 2	5/5 (100)	4/5 (100)
Exp. 3	3/3 (100)	1/2 (50)
Exp. 4	4/4 (100)	4/4 (100)
Hydrocortisone sodium succinate (0.5 mg/ml):		
Exp. 1	3/3 (100)	1/4 (25)
Exp. 2	5/5 (100)	8/8 (100)
Exp. 3	4/4 (100)	6/8 (75)
Exp. 4	3/4 (75)	1/3 (33)
Exp. 5	4/7 (57)	2/4 (50)

* Number of cells responding with potential changes per number of cells tested; percent of responding cells in parentheses.

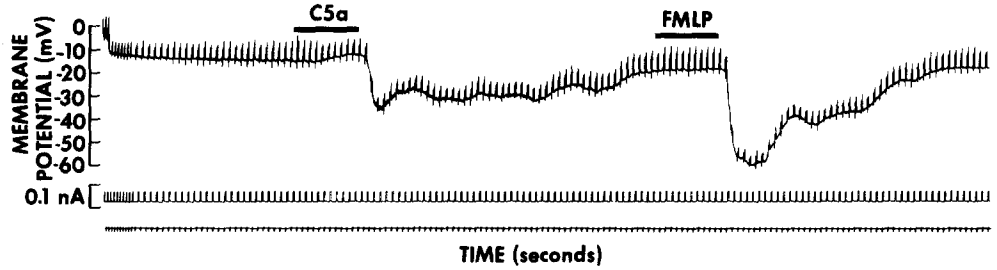

FIGURE 8 Response of macrophage to both C5a (5 μg/ml) and f-met-leu-phe (10^{-4} M) applied with blunt microelectrodes. Resting membrane potential = −10 mV.

cium being displaced from the membrane or entering the cell during stimulation.

To determine whether morphological changes occurred during or subsequent to the membrane potential changes, simultaneous electrical and photomicroscope analyses were done. Morphological changes could be detected in over half the cells examined after C5a-induced potential changes. During constant C5a stimulation, the electrical response did not recur once the cell recovered from the initial hyperpolarization even though morphological changes continued. However, when the stimulus was removed and reintroduced and a new gradient of stimulus established, a second electrical response was observed. This suggested that only one electrical response was associated with each new source of stimulus (chemotactic factor) presented to the cell.

The data indicate that changes in the ionic permeability of the macrophage membrane occur during cell activation with chemotactic factors and provide the basis for speculation as to how ionic events may modulate chemotactic factor-induced morphologic changes. The decrease in membrane resistance during C5a-induced potential changes indicates an increased membrane permeability to one or more ions. Most likely, C5a initially produces an influx of cations (calcium or sodium), causing the observed depolarization. This would be in agreement with the calcium influx observed during neutrophil chemotaxis (5). A rise in the intracellular calcium ensues, followed by an increased potassium permeability, producing a hyperpolarization. Potassium permeability has been shown to be related to intracellular calcium levels in a number of cells (15, 16), and we have previously presented evidence supporting calcium-sensitive potassium permeability changes in macrophages (9). It is of interest that A23187, which produces potential changes similar to those produced by C5a in macrophages, also produces a large potassium efflux in erythrocytes (8) and probably in leukocytes (9).

Other ionic events, perhaps more delayed, would be expected to occur as a consequence of increased intracellular ionized calcium. A calcium-extrusion or sequestering mechanism would be necessary for recovery. In other cells, calcium extrusion is accomplished by a sodium-calcium exchange (4) or activation of an ATP-dependent calcium pump (18). A large sodium influx in leukocytes stimulated with chemotactic factors was recently reported (17), and we have previously shown that chemotactic factors also induce calcium release (12). An efflux of calcium and an influx of sodium along with water would be expected if, as a result of chemotactic factor stimulation, a 3 Na^+:1 Ca^{++} electrogenic exchange (as reported in preparations in which the kinetics of the exchange have been studied [4]) or a 2 Na^+:1 Ca^{++} electroneutral exchange were initiated. The resulting decrease in ionized calcium could favor microtubule assembly which has been implicated as being important for maintaining cell orientation during chemotaxis (12). Future investigations of ion fluxes in conjunction with electrophysiological, biochemical, and ultrastructural studies will clarify the physiology of leukocyte activation by chemotactic factors.

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