Transient Increases in Cytosolic Free Calcium Appear To Be Required for the Migration of Adherent Human Neutrophils

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Abstract. Human neutrophils exhibit multiple increases in cytosolic free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) spontaneously and in response to the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). The function of these repetitive increases in [Ca\textsuperscript{2+}]\textsubscript{i}, as well as the role of Ca\textsuperscript{2+} in human neutrophil migration, remain unresolved. We have used microspectrofluorometry to measure [Ca\textsuperscript{2+}]\textsubscript{i} in single fura-2-loaded human neutrophils as they moved on poly-D-lysine-coated glass in the presence of serum. To investigate the role of Ca\textsuperscript{2+} in human neutrophil migration, we examined cells in the presence and absence of extracellular Ca\textsuperscript{2+}, as well as intracellular Ca\textsuperscript{2+}-buffered and Ca\textsuperscript{2+}-depleted cells. In the presence of extracellular Ca\textsuperscript{2+}, multiple increases and decreases in [Ca\textsuperscript{2+}]\textsubscript{i} occurred in every moving cell during chemokinesis, chemotaxis, and phagocytosis. In addition, neutrophils that extended pseudopodia and assumed a polarized morphology after plating onto a surface were always observed to exhibit [Ca\textsuperscript{2+}] transients even in the absence of chemoattractant. In contrast, a [Ca\textsuperscript{2+}] transient was observed in only one of the nonpolarized stationary cells that were examined (n = 15). Although some cells exhibited relatively periodic increases and decreases in [Ca\textsuperscript{2+}], resembling the regular oscillations that have been observed in some cell types, many others exhibited increases and decreases in [Ca\textsuperscript{2+}], that varied in their timing, magnitude, and duration. Buffering of [Ca\textsuperscript{2+}], or removal of extracellular Ca\textsuperscript{2+} damped out or blocked transient increases in [Ca\textsuperscript{2+}], and reduced or inhibited the migration of neutrophils. Under these conditions, polarized cells were often observed to make repeated attempts at migration, but they remained anchored at their rear. These data suggest that transient increases in [Ca\textsuperscript{2+}] may be required for the migration of human neutrophils on poly-D-lysine-coated glass in the presence of serum by allowing them to release from previous sites of attachment.

Cell motility is an integral part of such processes as development, wound healing, and immune defense. Directed cell migration involves the recognition of extracellular gradients, the transduction of these gradients into appropriate intracellular signals, and the generation of motion. Although different cell types migrate in response to different stimuli and travel at different rates, similar mechanisms may apply in transducing stimuli and in generating motion.

One commonly studied motile cell has been the neutrophilic polymorphonuclear leukocyte (neutrophil) (Wilkinson, 1982; Devreotes and Zigmond, 1988). When plated onto an appropriate surface, neutrophils rapidly adhere and spread (Boyles and Bainton, 1979). The homogeneous addition of chemoattractant to the cells results in stimulation of randomly directed migration (chemokinesis). Neutrophils are also capable of directed migration up concentration gradients of chemoattractant (chemotaxis) at rates of up to 10-20 \mu m/min (Maher et al., 1984). These cells have been reported to sense gradients in chemoattractant concentration of \~1% over cell length (\~10 \mu m) (Zigmond, 1977). However, whether a spatial, temporal, or spatial/temporal mechanism is involved in the perception of gradients remains unresolved (Haston and Wilkinson, 1987; Lauffenberger et al., 1988; Vicker, 1989).

The involvement of cytosolic free calcium concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in mediating migration is suggested by the fact that chemoattractants such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) lead to the release of Ca\textsuperscript{2+} from intracellular stores via the production of inositol-1,4,5-trisphosphate (Snyderman et al., 1986). The increased [Ca\textsuperscript{2+}],...
by directly affecting Ca²⁺-sensitive components of the cytoskeleton (Stossier et al., 1985). Recently, spontaneous and fMLP-induced oscillations in [Ca²⁺], in human neutrophils were reported by Jacouti et al. (1988). However, no correlation between the oscillations and any cellular property was observed. Additionally, aside from these observations of changes in [Ca²⁺], the Ca²⁺ requirement for neutrophil motility remains controversial: a number of conflicting reports have been published on the intracellular and extracellular Ca²⁺ requirements of neutrophils for migration.

Using the modified Boyden chamber, in which neutrophils crawl through pores in a cellulose filter, Wilkinson (1975) found that the migration of human neutrophils occurred to a slightly reduced extent in the nominal absence of extracellular Ca²⁺. Boucek and Snyderman (1976) used the same apparatus to assess the migration of human neutrophils in the presence of lanthanum chloride, which acts as an inhibitor of Ca²⁺ influx across the plasma membrane. Based on their observations, they concluded that Ca²⁺ influx was required for migration. Elfferink and Deierkauf (1985), looking at the effects of buffering [Ca²⁺], also used the modified Boyden chamber assay and observed decreased migration in quin-2-loaded rabbit neutrophils. However, stimulated migration, although decreased, was still observed in these cells even in the absence of extracellular Ca²⁺ and Mg²⁺. Meshulam et al. (1986) found a similar result in human neutrophils: Ca²⁺-depleted cells were still capable of migration through filters, although a significant inhibition of migration was observed in the absence of extracellular Ca²⁺. Zigmond et al. (1988), using both modified Boyden chambers to observe populations of cells and time-lapse video microscopy to observe individual cells migrating on a surface, reported that migration of rabbit neutrophils was essentially independent of extracellular Ca²⁺. In addition, they found that even cells that had been Ca²⁺ permeabilized with ionophore in the presence of EGTA could still migrate.

We wanted to determine the properties and significance of the [Ca²⁺] transients which have been observed in adherent human neutrophils. Using the Ca²⁺-sensitive indicator fura-2 (Grynkiewicz et al., 1985) and microspectrofluorometry, we measured [Ca²⁺], in human neutrophils after plating onto a surface and during chemokinesis stimulated by human serum. [Ca²⁺] during chemotaxis and phagocytosis was studied using a system in which neutrophils migrate in response to the chemoattractant C5a generated by the addition of a dilution of serum to immunoglobulin-coated fixed erythrocytes covalently attached to a surface (Pytowski et al., 1990). We found that there were transient increases in [Ca²⁺], in all migrating neutrophils and that increases in [Ca²⁺] were very rare in nonpolarized stationary cells. These increases appeared to be required for migration on poly-L-lysine-coated glass in the presence of serum since manipulations that blunted the [Ca²⁺] increases slowed or prevented migration. Observation of cells by time-lapse video-enhanced differential interference contrast (DIC) microscopy showed that the inhibition in migration resulted from an inability to release from previous sites of attachment to the surface.

**Materials and Methods**

**Cell Preparation**

Human whole blood was obtained from healthy volunteers. Blood was collected using a syringe, and a portion was immediately transferred to a sodium-heparin-containing polypropylene tube. The remaining blood was placed into a siliconized glass tube containing no additive for use in preparing fresh serum. Polymorphonuclear leukocytes were isolated by ficoll-hypaque discontinuous gradient centrifugation (Engel and Andersen, 1974) followed by 30-40 s hypotonic lysis of remaining erythrocytes. Cells were then washed with and resuspended in PBS. After serum was prepared as kept on ice. Although the polymorphonuclear cell fraction also contains a small number of basophils and eosinophils, we only examined migration in neutrophils. Once attached to a surface, neutrophils were distinguished from the other cell types by their smaller, less conspicuous granules.

**Loading with Fura-2, Quin-2, and 1,2-bis(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA)**

For loading with fura-2 alone, polymorphonuclear leukocytes were washed once with incubation medium (150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Hepes, 10 mM glucose, pH 7.4). Cells were then resuspended in a solution of 5 μM fura-2-acetoxyethyl ester (fura-2/AM; Molecular Probes Inc., Eugene, OR) in loading medium (incubation medium containing 5 mg/ml fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO) and tumbled for 30 min at room temperature. After this, the cells were washed twice with incubation medium and kept in the same medium on ice until use. Experiments were performed on cells within 4 h after loading. During this time, cells maintained their ability to spread on a surface, migrate, and phagocytose.

In Ca²⁺ buffering experiments, cells were tumbled for 30 min with either 50 μM quin-2/AM (Molecular Probes Inc.) or 75 μM BAPTA/AM (Molecular Probes Inc.) in loading medium, then washed twice with incubation medium, and placed on ice. For Ca²⁺ depletion experiments, polymorphonuclear leukocytes in PBS were washed once with Ca²⁺-free incubation medium containing 1 mM EGTA and then tumbled for 30 min with either 50 μM quin-2/AM or 75 μM BAPTA/AM in Ca²⁺-free loading medium containing 1 mM EGTA, and placed on ice. For both buffering and depletion experiments, control cells were tumbled in loading medium alone and otherwise treated similarly. Similar results were obtained with BAPTA- and quin-2-loaded cells, and the use of BAPTA permitted the simultaneous measurement of [Ca²⁺] using fura-2 in some experiments. To measure [Ca²⁺], in BAPTA-loaded neutrophils, 5 μM fura-2/AM was included in the loading solution containing BAPTA/AM. The fura-2 loading was significantly diminished in the presence of high concentrations of BAPTA, but an adequate fura-2 signal was obtained for photometry.

Stock solutions of fura-2/AM, quin-2/AM, and BAPTA/AM were prepared at 5, 50, and 50 mM, respectively, in anhydrous DMSO. The optimization of the procedure for homogeneously loading neutrophils with fura-2 has been described previously (Marks and Maxfield, 1989). The loading solutions described above were always centrifuged 5 min at 10,000 g before use to remove any undissolved particles, and this step was essential for the homogeneous loading of fura-2 into the cytosol. In addition, the use of fura-2/AM at concentrations >5 μM often resulted in Ca²⁺-insensitive punctate fluorescence. Such cell-associated Ca²⁺-insensitive fura-2 fluorescence has been described (Scandol et al., 1988). By loading neutrophils with 5 μM fura-2/AM and using a homogenization procedure described previously (Ratan et al., 1986), <5% of Ca-sensitive fluorescence was found in membrane-bound organelles. Cleavage of the intracellular indicator was confirmed by comparison of the excitation spectrum of the homogenate supernatant with that of free-fura-2 in solution. The cytosolic fura-2-free concentration determined by this method was ~25 μM. The homogenization procedure used for fura-2 was also used to determine the amount of BAPTA loaded into cells. Since BAPTA has been reported to contain a significant amount of water as an impurity (Harrison and Bers, 1987), we dried the BAPTA that we used for making standard solutions at 85°C for 3 h. The average concentration of BAPTA loaded into neutrophils was 4.8 mM.

**Experimental Chambers**

For studying neutrophils plated onto a surface, the experimental chamber consisted of a 35-mm tissue culture dish that had a No. 1 glass coverslip attached beneath a 1.2-cm hole that had been punched in the bottom. The surface of the glass was coated with 100 μg/ml poly-L-lysine. Chemotaxis chambers with covalently attached erythrocytes were prepared as follows, according to the method of Pytowski et al. (1990). Nochromix (Godax Laboratories Inc., New York)-cleaned coverslips were attached to the tissue culture dishes. The glass surface was treated with a 100 μg/ml poly-D-lysine.
solution in water for 30 min, washed three times with water, and then reacted 30 min with a 1% glutaraldehyde solution in PBS (Electron Microscopy Sciences, Fort Washington, PA). The dishes were then rinsed three times with PBS, and 4 ml of a solution of sheep erythrocytes (Diamedix, Miami, FL) that had been washed three times and resuspended in PBS at 0.8% hematocrit was immediately added to each. After 1 h, the dishes were rinsed extensively with PBS to remove unattached erythrocytes. The attached erythrocytes were fixed overnight with a solution of 0.5% formaldehyde in PBS, and then the dishes were washed with running water. A solution of 0.1% l-lysine in water was then added for 1 h to block free aldehydic groups. The dishes were then rinsed with water and allowed to air dry. Before chemotaxis experiments, the dishes were treated for 1 h with a 1:30 dilution of rabbit anti-sheep red blood cell IgG and IgM (Diamedix) in PBS and rinsed three times with PBS; the surface of the dish was covered with PBS until use. All of the above steps were performed at room temperature.

Polymorphonuclear leukocytes (10³-10⁴ cells) were plated into the coverslip area of the chamber and allowed to attach and spread for 3–5 min. A 10% solution of human serum in incubation medium was then added to initiate chemotaxis. For measurement of chemokinetics, a similar dilution of serum was added to neutrophils plated onto a chemotaxis chamber that had not been treated with immunoglobulins. The ability of human serum in which the complement cascade has not been activated to act as a chemottractant has been characterized (Jungi, 1977). Experiments in the absence of extracellular Ca²⁺ were performed using serum diluted into Ca²⁺-free incubation medium containing 5 mM EGTA. In all experiments, measurements were made on the microscope stage at 37°C between 3 and 20 min after the addition of serum. The loss of fura-2 fluorescence from the cytosol during this time was <25% of the initial fluorescence.

Measurement of [Ca²⁺].

A microscope (Diavert; E. Leitz, Inc., Wetzlar, FRG) and photometry system (KineteK, Yonkers, NY) equipped for rapidly switching between two illumination wavelengths has been described previously (Marks et al., 1988). The stability of the illumination provided by the two lamps used in this system has been characterized. Measured photometrically every 1 s over 5 min, the standard deviation of the illumination intensity from each lamp varies <2% from the mean illumination intensity. For studying [Ca²⁺] changes in neutrophils, 0.2-s photometric measurements at 340 and 380 nm wavelengths were made at a rate of one pair every 2.15 s using a 100× UV objective (Nikon Inc., Garden City, NY). After subtracting the background fluorescence, which was determined by making measurements of an area with no cells at each wavelength, the 340-to-380 nm ratio was calculated. In experiments using serum, the contribution of the fluorescence from 10% human serum was usually <10% of the total signal at each wavelength. Ratio values were converted to [Ca²⁺] levels by constructing a calibration curve based on the fluorescence of fura-2–free acid in solution as described previously (Kruskal et al., 1986). The Kᵦ for fura-2 at 37°C calculated using this method was in good agreement with the published value of 224 nM (Gryniewicz et al., 1985).

Analysis of Motility

To examine neutrophil motility under various conditions, a microscope (Diavert; E. Leitz, Inc.) equipped for phase contrast with a 25× objective was used. Single frames were recorded every 10 s with an optical memory disc recorder (Panasonic; Matsushita Electronics Corp., Osaka, Japan) using an image intensifier (VideoScope, Washington, D.C.) coupled to a tube camera (Newvicon; Dage-MTI Inc., Michigan City, IN). Other experiments examining motility were performed using a microscope (Axiovert; Carl Zeiss, Inc., Thornwood, NY) equipped with DIC optics. Single frames were recorded every 2 s onto an optical memory disc using a Chalnicon tube contrast-enhancing camera (Hamamatsu Photonics K. K., Oak Brook, IL). Migrating cells were defined as those in which both the leading edge and tail underwent vectorial movement.

Results

To measure [Ca²⁺], microspectrofluorometrically using fura-2 during chemotaxis and phagocytosis, we used a system in which sheep red blood cells were covertly attached to poly-D-lysine–coated glass coverslips (Pytowski et al., 1990). The red blood cells can then be coated with IgG and/or IgM antibody, and a dilution of fresh serum can be added to initiate chemotaxis. In the presence of opsonizing immunoglobulin, the enzymes of the complement cascade present in serum are activated at the surface of the red blood cells, rapidly generating microgradients of the chemotactant C5a, as well as leading to the opsonization of the erythrocytes with C3b. Neutrophils migrate up these gradients of C5a and are capable of engulfing the red blood cells in a manner similar to that of the phagocytosis of a free particle. In the absence of extracellular Ca²⁺ and in the presence of Mg²⁺, this system generates significantly less C5a via activation of the alternative pathway of complement since the classical pathway is inhibited (Whaley, 1987). Chemokinesis stimulated by serum alone can be studied in these chemotaxis chambers by simply omitting the step of coating the erythrocytes with antibody. Unopsonized sheep erythrocytes do not activate the human complement cascade to a significant extent (Whaley, 1987), and the neutrophils migrate randomly on the surface in response to the chemoattractant present in human serum in which the complement cascade has not been activated (Jungi, 1977).

[Ca²⁺]. Transients during Migration and Phagocytosis

Transient increases in [Ca²⁺], were observed in fura-2–loaded neutrophils during chemokinesis, chemotaxis, and phagocytosis (Figs. 1 and 2). For purposes of further analysis, a transient is defined here as an increase in [Ca²⁺], of at least 50 nM lasting at least 5 s. These transients corresponded to reciprocal changes in photometric measurements at 340 and 380 nm wavelengths, indicating that they were not an artifact of noise in the illumination or measurement systems. For comparison, the unstimulated [Ca²⁺], measured in fibroblasts had a standard deviation <5 nM over a number of minutes when measurements were made using this system (Marks et al., 1988).

Since addition of serum to cells in serum-free medium will stimulate chemokinetics, chambers in which the red blood cells were not opsonized were used to examine this serum-stimulated chemokinetics in the absence of C5a generation. Neutrophils were plated into the chamber and allowed 3–5 min to attach and spread over the surface. Upon the addition of a dilution of serum, a generalized membrane ruffling occurred, which was rapidly followed by the assumption of a polarized morphology in 40–60% of the cells. Although there was some day-to-day variability, ~25% of the cells then went on to randomly migrate in the chamber with no directional preference for nearby red blood cells. These nearby red blood cells were used as fixed spatial references for determining whether or not a given neutrophil was moving when [Ca²⁺] measurements were made. When cells were plated on a poly-D-lysine–coated surface without attached erythrocytes a similar percentage of cells underwent chemokinetics in response to the addition of a dilution of serum.

Fig. 1, a–c, shows the [Ca²⁺], measured in single neutrophils during serum-stimulated chemokinetics. It is apparent that the number of [Ca²⁺], transients observed in different cells during a 5-min period of measurement varied. In addition, the magnitude, duration, and time between transients were variable, both within a given cell and between cells. Although only a few [Ca²⁺], transients occurred in the majority of cells (Fig. 1, a and b), in some cells transients occurred more frequently (Fig. 1 c).
Chambers in which the red blood cells had been opsonized with antibody were used to measure \([\text{Ca}^{2+}]\) during chemotaxis and phagocytosis. In these chambers, addition of serum causes the generation of C5a at the erythrocyte surface. This initiates chemotaxis in neutrophils that have previously been allowed to attach to and spread on the surface of the chemotaxis chamber. Although a similar number of neutrophils assumed a polarized morphology as in the absence of a directional signal, those polarized cells in the vicinity (within 25 \(\mu\)m) of red blood cells rapidly extended pseudopodia and migrated toward them. \([\text{Ca}^{2+}]\) measurements were then made in cells that were undergoing directional migration toward and phagocytosis of erythrocytes. On the average, 40–50% of the neutrophils migrated, and many of these cells were observed to phagocytose the erythrocyte targets.

Fig. 1, d–f, shows \([\text{Ca}^{2+}]\) transients that were observed in single neutrophils during chemotaxis and illustrates the variability in the \([\text{Ca}^{2+}]\) transients observed during chemotaxis. Although the range of the number of \([\text{Ca}^{2+}]\) transients that occurred in a 5-min period during chemokinesis and chemotaxis was similar (1–10 transients), the average number of transients observed during chemotaxis (4.3) was slightly greater than the average number observed during chemokinesis (3.4) (Table I). This difference, however, was not statistically significant.

Fig. 2 shows the \([\text{Ca}^{2+}]\) transients that took place in a neutrophil undergoing chemotaxis that subsequently engulfed an IgG- and C3b-opsonized red blood cell. As phagocytosis occurs, the transients continue, and the baseline \([\text{Ca}^{2+}]\), upon which they occur increases. The average baselines during chemotaxis and phagocytosis were 215 \pm 75 and 347 \pm 157 nM, respectively (mean \pm SD; \(p < 0.01\) by \(t\) test). An increase in the number of transients occurring during a 5-min period and in the overall magnitude of the transients was also observed (Table I).

In addition to the \([\text{Ca}^{2+}]\) transients of \(\geq 50\) nM, which we included in our analyses, smaller transient changes in \([\text{Ca}^{2+}]\), of 25–50 nM were occasionally observed, which
were clearly distinguishable from noise in the system (see Fig. 1, e and f). These were more frequently observed during chemotaxis and phagocytosis than during chemokinesis.

[Ca\textsuperscript{2+}]. Transients after Plating onto a Surface
Since [Ca\textsuperscript{2+}] transients were observed during chemokinesis, chemotaxis, and phagocytosis, we examined whether such changes also occurred in neutrophils in the absence of chemoattractant. Neutrophils plated on poly-D-lysine-coated glass coverslips at 37°C were observed to rapidly spread over the surface. Some of the cells were then observed to assume a polarized morphology, extending pseudopodia in various directions without migrating. The number of cells assuming such a polarized morphology varied in different neutrophil preparations, but it was generally between 25 and 50%.

In all neutrophils observed that assumed a polarized morphology extending pseudopodia, at least one [Ca\textsuperscript{2+}] transient occurred. These transients were similar in magnitude and duration to those observed during chemokinesis and chemotaxis. However, fewer transients occurred during a 5-min period of observation. None of the round or slightly spread cells in incubation medium showed such transients (n = 8). The round cells were viable and could be induced to undergo a shape change by isotonically increasing extracellular potassium (Roberts et al., 1984). Accompanying this shape change was a rise in [Ca\textsuperscript{2+}], which was sometimes followed by transient increases in [Ca\textsuperscript{2+}].

Fig. 4 shows the [Ca\textsuperscript{2+}] in a neutrophil that had just been plated into incubation medium in a dish on the microscope stage at 37°C. The high value of [Ca\textsuperscript{2+}] at time 0 s represents the decay from the increase in [Ca\textsuperscript{2+}], which precedes spreading and has been described previously (Kruskal et al., 1986). During the time after spreading, the cell assumed a polarized morphology. This was accompanied by the initiation of repetitive increases in [Ca\textsuperscript{2+}]. At time 435 s, indicated by the arrow in Fig. 4, the medium was switched to Ca\textsuperscript{2+}-free incubation medium containing 1 mM EGTA. As shown, [Ca\textsuperscript{2+}] transients can still occur after the switch to Ca\textsuperscript{2+}-free medium. This was a consistent observation in cells examined in both the absence and presence of

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Table 1. Summary of Observed [Ca\textsuperscript{2+}], Transients

| Condition      | Sample | Transients | Duration | Increase in [Ca\textsuperscript{2+}] | 
|----------------|--------|------------|----------|--------------------------------------|
| Chemokinesis   | 13     | 3.4 ± 2.6  | 36 ± 13  | 117 ± 107                             |
| Chemotaxis     | 17     | 4.3 ± 2.8  | 33 ± 15  | 104 ± 48                              |
| Phagocytosis   | 5      | 5.2 ± 2.4  | 36 ± 22  | 197 ± 132                             |

Measurement of [Ca\textsuperscript{2+}] was performed as described for neutrophils plated into chemotaxis chambers on the microscope stage at 37°C. For the measurement of [Ca\textsuperscript{2+}], during chemokinesis, the sheep erythrocytes were not opsonized with antibody before the addition of a 10% dilution of fresh human serum. For measurement of [Ca\textsuperscript{2+}], during chemotaxis and phagocytosis, the erythrocytes were opsonized before use with anti-sheep red blood cell IgG and IgM, and a 10% dilution of serum was added to initiate directed migration. The mean number of transients occurring during a 5-min period of observation is given. Duration was measured as the time elapsed between the start of the rise of a transient and the end of its decay. Increase in [Ca\textsuperscript{2+}] was measured as the increase from the [Ca\textsuperscript{2+}], level just before the start of a transient to its peak. Values given represent the mean ± SD. Overall, during chemokinesis, chemotaxis, and phagocytosis, the magnitude of the transients increased from 50 to 700 nM, the duration ranging from 10 to 90 s, and the time between peaks ranging from 10 to 150 s. The difference in amplitude of the increase in [Ca\textsuperscript{2+}], between chemotaxis and phagocytosis was significant at the level p < 0.025 by t test.

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Figure 2. [Ca\textsuperscript{2+}], transients in a neutrophil observed during chemotaxis toward and phagocytosis of an opsonized target. [Ca\textsuperscript{2+}], measurements were performed as described in Materials and Methods on a single fura-2-loaded neutrophil migrating toward an erythrocyte coated with both anti-sheep red blood cell IgG and IgM. In this case, neutrophils can specifically recognize at least two opsonins on the surface of the red blood cells: IgG and C3b. During both chemotaxis and phagocytosis, [Ca\textsuperscript{2+}] transients occur; however, during the latter process these transients occur on a higher baseline [Ca\textsuperscript{2+}]. This cell was notable for the regularity of the transients observed.

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Figure 3. Absence of [Ca\textsuperscript{2+}], transients in stationary nonpolarized neutrophils after the addition of a dilution of human serum to chambers containing unopsonized red blood cells. [Ca\textsuperscript{2+}], was measured as described in Materials and Methods in stationary nonpolarized cells in the presence of a 10% dilution of serum in incubation medium. Measurements from two different cells are plotted on the axes. No [Ca\textsuperscript{2+}], transients were observed.
The role of Ca$^{2+}$ in neutrophil migration was investigated in a separate set of experiments using the chemotaxis chambers described above. Cells were loaded with buffering concentrations of the Ca$^{2+}$ chelators quin-2/AM or BAPTA/AM either in the presence or absence of extracellular Ca$^{2+}$, depending on whether Ca$^{2+}$ buffering or depletion was desired. The average intracellular concentration of BAPTA loaded into neutrophils was 4.8 mM. Control cells were similarly treated by incubating them with loading medium alone. The cells were then plated into chemotaxis chambers, and a dilution of serum in either incubum medium or Ca$^{2+}$-free incubation medium containing 5 mM EGTA was added. Optical memory disk recordings were then made of migration for later analysis. Similar results were obtained using either quin-2 or BAPTA to buffer [Ca$^{2+}$].

At 37°C, both Ca$^{2+}$-buffered and Ca$^{2+}$-depleted neutrophils were still capable of spreading when plated into incubation medium or Ca$^{2+}$-free incubation medium, respectively. Approximately 80% of control cells plated onto poly-D-lysine at 37°C in the presence of extracellular Ca$^{2+}$ spread within 10 min after plating. Only a small reduction in spreading was observed in Ca$^{2+}$-buffered neutrophils plated in the presence of extracellular Ca$^{2+}$: ~70% of these cells spread. A larger reduction in spreading was observed in both control cells and Ca$^{2+}$-depleted cells that were plated into Ca$^{2+}$-free incubation medium containing 1 mM EGTA. In both cases, spreading was reduced to a similar extent, with ~40% of the cells spreading.

As described above, ~40–50% of control cells would undergo chemotaxis upon addition of serum to chambers containing opsonized erythrocytes. In marked contrast, cells plated in Ca$^{2+}$-free incubation medium were never observed to migrate. This was found with or without buffering [Ca$^{2+}$]. Since C5a generation by the classical complement cascade is inhibited by removal of Ca$^{2+}$ (Whaley, 1987), reduction of the chemotactic response is not unexpected. Nevertheless, serum-stimulated chemokinesis comparable with the 25% observed in response to serum would be expected since this does not depend upon the activation of complement (Jungi, 1977). However, no migration whatsoever was observed. This was despite the fact that frequently cells appeared to be receiving a directional stimulus as judged by their polarization toward and repeated extension of membrane toward a nearby target. A complete inhibition of migration was also observed in neutrophils in the absence of extracellular Ca$^{2+}$ using chemotaxis chambers that had not been treated with immunoglobulin.

The inhibition of migration in the absence of extracellular Ca$^{2+}$ in control cells was reversible. Cells unable to migrate in response to serum in Ca$^{2+}$-free incubation medium containing 5 mM EGTA were capable of migration when external Ca$^{2+}$ was restored. In the absence of extracellular Ca$^{2+}$ and in the presence of 5 mM EGTA, the [Ca$^{2+}$], measured in separate experiments using fura-2 was reduced to a level between 25 and 50 nM, and no [Ca$^{2+}$] transients were observed. After the readddition of extracellular Ca$^{2+}$, transients in [Ca$^{2+}$], were observed in cells that migrated.

To examine the effects of damping [Ca$^{2+}$], transients in the presence of normal extracellular Ca$^{2+}$, quin-2- and BAPTA-buffered neutrophils were plated into incubation medium with 1 mM Ca$^{2+}$. These intracellular Ca$^{2+}$ buffers caused

![Figure 4. [Ca$^{2+}$] transients in a polarized neutrophil. Fura-2-loaded neutrophils were plated onto a poly-D-lysine-coated glass surface in a chamber containing incubation medium alone on the microscope stage at 37°C. Immediately after plating, [Ca$^{2+}$], measurements were made on a single adherent cell before its spreading. At time 435 s, indicated by the arrow, the medium was exchanged with Ca$^{2+}$-free incubation medium containing 1 mM EGTA. The elevation in [Ca$^{2+}$], at time 0 s represents the decay of the Ca$^{2+}$ increase before spreading which has previously been reported. During the first minutes, the cell assumed a polarized morphology at which time [Ca$^{2+}$], transients were observed. After the switch to Ca$^{2+}$-free medium, a sizable [Ca$^{2+}$], transient of ~100 nM occurred.]

### Table II. Effect of Altering Ca$^{2+}$ on Neutrophil Motility

| Condition | Ca$^{2+}$ | BAPTA | Sample | Migrating cells | Mean velocity |
|-----------|----------|--------|--------|----------------|--------------|
|           |          |        |        |                | μm/min       |
| +         | −        | 51     | 45     | 7.9 ± 3.4      |
| −         | −        | 91     | 0      | −              |
| +         | +        | 18     | 33     | 2.9 ± 1.4      |
| −         | +        | 22     | 0      | −              |

Assays of migration were performed in chemotaxis chambers on the microscope stage at 37°C, recording single frames onto videodisk for later analysis. In the −Ca$^{2+}$ conditions, cells were observed in the absence of extracellular Ca$^{2+}$ with 5 mM EGTA present. For the +BAPTA conditions, cells were loaded with 75 μM BAPTA/AM before observation. When cells were not observed to be migrating, recordings were made for 5 min. The velocity of migrating cells overall is given as mean ± SD. The difference in overall mean velocity between control and BAPTA-loaded cells in the presence of extracellular Ca$^{2+}$ was significant (p < 0.01 by t test).
partial inhibition of migration. In both cases, fewer cells were observed migrating, and those that did migrate moved more slowly. The mean velocity of BAPTA-loaded cells was $2.9 \pm 1.4 \mu m/min$ compared with $7.9 \pm 3.4 \mu m/min$ for control cells. A summary of the effects of $Ca^{2+}$ buffering and depletion on migration is presented in Table II.

To determine whether the partial inhibition of migration observed was an artifact of loading the cells with AM, neutrophils were loaded with quene-l/AM (Rogers et al., 1983), a pH-sensitive relative of quin-2, to an intracellular concentration of 3.9 mM. In the presence of extracellular $Ca^{2+}$, no effect on the number of cells migrating, their velocity, or their migratory morphology was found (data not shown). This indicates that high intracellular concentrations of quin-2 and BAPTA exerted effects on migration through their alteration of $[Ca^{2+}]$.

Differences in cell behavior became especially noticeable when the cells were viewed by video-enhanced DIC microscopy using a high-power objective.2 Fig. 5, a-f, shows a control cell undergoing directed migration toward and phagocytosis of an opsonized red blood cell in the presence of extracellular $Ca^{2+}$. The tail follows the rest of the cell relatively smoothly. In Fig. 5, g-l, a $Ca^{2+}$-depleted neutrophil plated in $Ca^{2+}$-free incubation medium is shown. Under these conditions, the $[Ca^{2+}]$ measured in separate experiments was <10 nM. Although the cell assumes a polarized morphology and is capable of membrane ruffling and pseudopod extension (Fig. 5 h, arrow), it remains anchored in place at its rear and does not undergo migration. The anchoring is especially apparent in Fig. 5 i in which a thin veil of cytoplasm remains attached to the surface (arrow). This behavior was typical of both control and $Ca^{2+}$-depleted cells plated in the absence of extracellular $Ca^{2+}$ and is similar to that observed in neutrophils strongly adherent to a substrate (Wilkinson, 1982).

Effects on migration were also seen in $Ca^{2+}$-buffered cells plated into $Ca^{2+}$-containing incubation medium. Frequently, cells appeared to have difficulty retracting their tails. An example of this is shown in Fig. 5, m-r, where the cell is unable to move close enough to the red blood cell to phagocytose it. Although the neutrophil extends a pseudopod toward the erythrocyte, apparently making contact with it (Fig. 5 n), the neutrophil does not continue on to engulf it. This type of behavior was rarely observed in control cells.

As summarized in Table II, only partial inhibition of migration was seen in BAPTA-loaded neutrophils in the presence of extracellular $Ca^{2+}$. To determine whether transients in $[Ca^{2+}]$, occurred in $Ca^{2+}$-buffered cells, $[Ca^{2+}]$ was measured by loading fura-2 along with the BAPTA. Fig. 6 shows representative tracings of the $[Ca^{2+}]$ measured with fura-2 in BAPTA-loaded neutrophils. The cell in Fig. 6 a migrated and went on to phagocytose a target, whereas the cell in Fig. 6 b remained stationary. Besides the higher baseline $[Ca^{2+}]$, a $[Ca^{2+}]$, transient occurred in the migrating cell. Only one or two $[Ca^{2+}]$, transients, such as the one shown in Fig. 6 a, were ever observed during a 5-min period in migrating cells loaded with BAPTA. This is significantly less than the number of transients seen under control conditions.

2. A VHS videocassette copy of the entire optical memory disk recordings used to produce the micrographs presented here along with additional data is available. Requests should be made to F. R. Maxfield with a check payable to Columbia University for $10 to cover costs.

**Discussion**

**[Ca^{2+}] Transients**

Transient increases in $[Ca^{2+}]$, were observed in polarized neutrophils during chemokinesis, chemotaxis, and phagocytosis, as well as in the absence of an added stimulus. In all of these cases, at least one $[Ca^{2+}]$, transient was observed during the time period of measurement. However, the number and character of the transients that were observed varied depending on the process that was examined. In contrast, only once was a $[Ca^{2+}]$, transient observed in stationary, nonpolarized cells ($n = 15$). These nonpolarized cells could be stimulated to undergo a shape change that was sometimes followed by $[Ca^{2+}]$, transients.

In describing our observations, we believe that it is more appropriate to call the repeated increases and decreases that have been detected in neutrophils $[Ca^{2+}]$, transients rather than oscillations. This distinction is warranted because oscillations imply periodic increases and decreases occurring with a similar magnitude and duration. $[Ca^{2+}]$, changes in which such criteria are fulfilled have been described in a variety of nonexcitable cell types, including hepatocytes (Woods et al., 1986), endothelial cells (Jacob et al., 1988), and macrophages (Kruskal and Maxfield, 1988). In contrast, in neutrophils, the spacing, magnitude, and duration of the increases are frequently variable within the same cell. To adequately consider the mechanism and function of these variable transients it is necessary to distinguish them from the regularity of oscillations. A review of the possible mechanisms leading to $[Ca^{2+}]$, oscillations has been published (Berridge and Gallione, 1988).

The magnitude and duration of the $[Ca^{2+}]$, transients that we have observed are similar to those reported as occurring spontaneously and in response to FMLP by Jacobi et al. (1988). In agreement with their findings, we found that the $Ca^{2+}$ increase of the transients that took place after plating onto a surface and in the presence of chemoattractant occurred from intracellular stores, and the removal of extracellular $Ca^{2+}$ resulted in the cessation of the transients within a few minutes. Our observations differ in that we find a clear correlation of $[Ca^{2+}]$, changes with migration. Every migrating cell exhibited at least one $[Ca^{2+}]$, transient. This correlation was facilitated by the use of chemotaxis chambers which permitted us to rapidly induce directional migration while measuring $[Ca^{2+}]$. The $[Ca^{2+}]$, transients that Jacobi et al. (1988) have reported as occurring spontaneously, and which we observe in spread neutrophils that assume a polarized morphology, could be the result of plating the neutrophils onto a surface. As described by Kruskal et al. (1986), contact with a surface triggers an increase in $[Ca^{2+}]$, and this may be sufficient to initiate subsequent $[Ca^{2+}]$, transients.

In addition to the microspectrofluorometric studies described here, we have used video microscopy and image processing to examine whether localized increases or gradients of $Ca^{2+}$ occur in neutrophils undergoing chemotaxis and phagocytosis (Marks and Maxfield, 1990). During chemotaxis, although transient increases in $[Ca^{2+}]$ were observed which spread throughout the cytosol, no consistent localization of $Ca^{2+}$ gradients within the cells was detected. It remains possible that very small or highly transient gradients may not have been detected. $[Ca^{2+}]$, gradients were ob-
Figure 5. DIC micrographs illustrating the effect of altering Ca\(^{2+}\) on neutrophil migration. (a–f) Micrographs of a neutrophil undergoing chemotaxis and phagocytosis in the presence of extracellular Ca\(^{2+}\). Chemotaxis was initiated as described in Materials and Methods, and the neutrophil was viewed on the microscope stage at 37°C migrating toward an opsonized erythrocyte. Relatively smooth movement of the tail is apparent, and engulfment of the target occurs rapidly. (g–l) Micrographs of a Ca\(^{2+}\)-depleted neutrophil in the absence of extracellular Ca\(^{2+}\). Cells were Ca\(^{2+}\) depleted using quin-2 and observed after the addition of a dilution of serum in Ca\(^{2+}\)-free incubation medium containing 5 mM EGTA as described in Materials and Methods. In this series of micrographs, the cell appears to make an attempt at migration, extending a pseudopod (h, arrow), yet fails because it remains anchored to the dish at its rear (i, arrow). This anchoring is especially apparent in i where a thin veil of cytoplasm is seen which remains anchored to the surface. (m–r) Micrographs of a Ca\(^{2+}\)-buffered neutrophil in the presence of extracellular Ca\(^{2+}\). The cells were Ca\(^{2+}\) buffered using quin-2, and chemotaxis was initiated as described in Materials and Methods. This cell is able to extend itself to apparently touch the target, yet it is unable to release itself in order to engulf it. Chemotaxis chambers treated with both IgG and IgM were used for all three conditions. Time is indicated at the top of each micrograph. Bar, 10 μm.

The Role of [Ca\(^{2+}\)] in Chemotaxis

Our results are consistent with previous studies showing that shape changes and actin polymerization (Meshulam et al., 1986; Sha'afi et al., 1986) can occur without increases in [Ca\(^{2+}\)]. Ca\(^{2+}\)-depleted neutrophils plated in the absence of extracellular Ca\(^{2+}\) are still capable of spreading on a surface, assuming a polarized morphology, and repeatedly extending their plasma membrane. Thus, elevations in [Ca\(^{2+}\)] are not required for these processes. We have found, however, that there is a Ca\(^{2+}\) requirement for the migration of human neutrophils on poly-β-lysine–coated glass in the presence of serum. The intracellular Ca\(^{2+}\) buffering experiments which we have performed suggest that it is [Ca\(^{2+}\)] transients that are essential for continued migration and not extracellular Ca\(^{2+}\) or the resting level of [Ca\(^{2+}\)], since migration was inhibited in the presence of 1 mM extracellular Ca\(^{2+}\) and nearly normal [Ca\(^{2+}\)]. Video microscopy showed that the inhibition resulted from the difficulty which the cells had in releasing from sites of attachment to the substratum.

As summarized in the introduction, it has been found that migration of neutrophils on glass or through cellulose filters...
migration is likely explained by relatively tight adhesion to the substratum which occurs in our chambers when serum is added. The chambers we used have a poly-d-lysine coating that is necessary for the attachment of erythrocytes to the surface. When serum is added, fibronectin and perhaps other adhesive glycoproteins will bind to the poly-d-lysine (Klebe et al., 1981). Neutrophils express surface receptors which can then bind to these glycoproteins (Gresham et al., 1989). When attached to poly-d-lysine in the presence of serum, we have found that transient increases in \([Ca^{2+}]_i\) appear to be required for release from the substratum. Considering the composition of the extracellular matrix in vivo, the ability to release from sites of attachment may be an important component of neutrophil migration through tissue.

The mechanism by which increases in \([Ca^{2+}]_i\), would lead to release from the substratum is not known. One possibility is that increased \([Ca^{2+}]_i\), is necessary for the operation of a force-generating mechanism involved in the detachment of strongly adherent cells through a contractile action (Southwick and Stossel, 1983). Alternatively, though not necessarily exclusive of the above, elevated \([Ca^{2+}]_i\), could be required for the activation of a process leading to the disruption of specific sites of attachment to the substratum. Focal adhesion sites in other cell types have been shown to involve the actin cytoskeleton on the cytosolic side of the plasma membrane (Burridge et al., 1988), and elevated \([Ca^{2+}]_i\), would disrupt actin filaments through the action of Ca\(^2+\) binding proteins such as gelsolin (Stossel et al., 1985).

A role for \([Ca^{2+}]_i\), transients in disrupting specific attachment sites, although speculative, might not just be limited to neutrophils. Other migratory cell types such as fibroblasts form focal adhesions with linkage to the cytoskeleton (Burridge et al., 1988), and repeated disruption of these attachment sites is likely to be required for continued migration. The intracellular signal mediating this disruption has yet to be identified, and it is conceivable that \([Ca^{2+}]_i\), transients could serve in this function.

In addition to the relatively direct effects that \([Ca^{2+}]_i\), may have on attachment sites, \([Ca^{2+}]_i\), could also play a more indirect role in signaling by activating calmodulin-dependent processes. In this regard, although Ca\(^2+\) is evidently not required as the positive signal for actin polymerization and orientation, the transients we observe could function to facilitate re-orientation by “erasing” a previous positive signal. Further work is necessary to demonstrate such an indirect role for \([Ca^{2+}]_i\), during chemotaxis.

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