Cytosolic free Calcium Increases before and Oscillates during Frustrated Phagocytosis in Macrophages

Benjamin A. Kruskal and Frederick R. Maxfield*

Department of Pharmacology, New York University School of Medicine, New York 10016; and
*Department of Pathology, Columbia University, College of Physicians and Surgeons, New York 10032

Abstract. When macrophages and neutrophils are allowed to settle onto an appropriate surface, they attach and spread in a frustrated attempt to phagocytose the substrate. Spreading is associated with extensive rearrangements of the actin cytoskeleton which resemble those occurring during phagocytosis. We have previously shown that spreading in human neutrophils is preceded by an increase in cytosolic-free calcium concentration ($[Ca^{2+}]_i$) (Kruskal, B. A., S. Shak, and F. R. Maxfield. 1986. *Proc. Natl. Acad. Sci. USA.* 83:2919–2923). To assess the generality of this signal, we measured $[Ca^{2+}]_i$ in single thioglycollate-elicited mouse peritoneal macrophages as they spread on an immune complex-coated surface, using fura-2 microspectrofluorometry. A $[Ca^{2+}]_i$ increase always precedes spreading. This increase can involve several (up to 8) $[Ca^{2+}]_i$ spikes, with an average peak value of 387 ± 227 nM (mean ± SD, n = 92 peaks in 24 cells), before spreading is detected. Neither spreading nor the magnitude of these spikes is significantly altered by removal of extracellular calcium.

Many of the spreading macrophages exhibit periodic $[Ca^{2+}]_i$ increases before and during spreading. The proportion which does so varies among experiments from 0 to 90%, but it is frequently greater than 40%. The largest number of cells (~25%) exhibited only a single peak. In 13 cells that showed more than 10 peaks, the median period was 29 s (range 19–69 s). The average peak $[Ca^{2+}]_i$ was 385 ± 266 nM (mean ± SD, n = 208 peaks in 14 cells). The calcium producing these increases is derived from intracellular pools. The oscillations occur with spreading on either opsonized or nonopsonized surfaces. The function of these oscillations is not clear, but the large number of cells which exhibit them suggest that they may be important to macrophage function.

MACROPHAGES and polymorphonuclear leukocytes (PMN)[1] will adhere and spread on a variety of substrates in a frustrated attempt to phagocytose the surface (Boyles and Bainton, 1979; Rabinovitch and DeStefano, 1973). Spreading is accompanied by major changes in the actin cytoskeleton which are quite similar to those accompanying phagocytosis (Boyles and Bainton, 1979, 1981). In both PMN and macrophages, a network of thin filaments (of the correct size to be actin microfilaments) linked by globular centers is rapidly formed adjacent to the plasma membrane on the adherent side of the cell (Boyles and Bainton, 1979, 1981; Trotter, 1981).

The cytosolic free calcium concentration ($[Ca^{2+}]_i$) has long been postulated as an important regulator of cytoskeletal structure and motile functions, such as phagocytosis, in nonmuscle cells (Campbell, 1983). A number of important actin-associated proteins which are found in macrophages and neutrophils (as well as many other cell types), such as gelsolin and myosin (Southwick and Stossel, 1983), are regulated by $[Ca^{2+}]$ (Pollard and Cooper, 1986).

The fluorescent calcium-sensitive dyes quin2 and fura-2, designed and synthesized by Tsien (Tsien et al., 1982; Grynkiewicz et al., 1985), have made it possible to measure $[Ca^{2+}]_i$ during phagocytosis in small cells such as neutrophils and macrophages, and thus to test this hypothesis. Various studies conducted thus far have yielded conflicting results. Lew et al. (1985), using quin2 to measure average $[Ca^{2+}]_i$ from a population of cells, reported an increase during the ingestion by PMN of particles opsonized with Ig or C3bi, but suggest that C3bi-mediated phagocytosis is calcium independent. A moderate increase in $[Ca^{2+}]$ localized to the cytoplasm surrounding the nascent phagosome, with little or no change in whole-cell average calcium, was observed by Sawyer et al. (1985), who used quin2 fluorescence-ratio imaging on single PMN. Using aequorin to measure the average $[Ca^{2+}]_i$ from a dish of ~10^5 thioglycollate-elicited peritoneal macrophages simultaneously, McNeil et al. (1986) found no change in $[Ca^{2+}]_i$ during spreading on a substrate or during ingestion of opsonized sheep red blood cells except when the extracellular pH was increased beyond the normal physiologic range. Our previous studies, using fura-2 ratio microspectrofluorometry on individual PMN, showed a large transient increase in $[Ca^{2+}]_i$ (derived from intracellular stores) immediately preceding spreading on a substrate

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, cytosolic free calcium concentration; PMN, polymorphonuclear leukocyte(s).

(Kruskal et al., 1986). By inhibiting actin polymerization with cytochalasin B, spreading could be blocked without blocking the Ca^{2+} spike. We did not find any conditions which permitted spreading without a preceding increase in $[Ca^{2+}]_i$.

The apparent conflict among these results may be explained by the differing indicators and measurement methods used. For example, at concentrations sufficient to produce a reliable signal in single cells, quin2 buffers calcium changes markedly (Tsien et al., 1985). Fura-2, with a 30-fold higher fluorescence on a molar basis (Grynkiewicz et al., 1985), can be used at concentrations which distort cellular calcium changes less. Quin2 is also quite labile to photobleaching (Tsien et al., 1985), whereas fura-2 is so photostable that hundreds of measurements can be made in a single cell. Aequorin provides a more accurate measure of changes in $[Ca^{2+}]$ at higher $[Ca^{2+}]$ than either of the fluorescent dyes (Blinks, 1985). Measurement of $[Ca^{2+}]_i$ in populations of cells suffers from two important drawbacks when compared with single cell methods: (*a*) changes which do not occur simultaneously in the population will appear artefactually low or may not even be detected; (*b*) the signal from sick or dying cells will be averaged in, whereas with single cell measurements, morphologic criteria can exclude at least some unhealthy cells. These technical considerations led us to believe that a single-cell study using fura-2 as the indicator might help resolve whether an increase in $[Ca^{2+}]_i$ occurs during phagocytosis.

We chose to study spreading as a less complicated model of phagocytosis because there is a single event per cell which is much easier to detect with precise timing. To determine whether a $[Ca^{2+}]_i$ increase precedes spreading in macrophages as it does in PMN, we measured $[Ca^{2+}]_i$ in individual macrophages during spreading. We consistently observe that $[Ca^{2+}]_i$ rises before spreading begins in thioglycollate-elicited peritoneal macrophages. In a large number of cells, we also observe periodic spikes of $[Ca^{2+}]_i$ occurring with a frequency of ~1–3 per min, continuing in some cases for more than 30 min. Oscillations in membrane potential of similar frequency have been observed in macrophages and fibroblasts (Gallin et al., 1975; Okada et al., 1977).

Materials and Methods

Preparation of Cells and Loading with Fura-2

Swiss-Webster mice of either sex were injected interperitoneally with 3 ml of 3% sterile fluid thioglycollate medium (Difco, Detroit, MI). Cells were harvested 3–4 d later and stored in RPMI 1640 (Gibco, Grand Island, NY) with 10% heat-inactivated FCS (Gibco) with 100 U/ml penicillin and 100 μg/ml streptomycin at 4°C. We found that the percentage of cells which spread varies from day to day. Experiments were not done on a day when this fraction was less than 10%. Each day's experiments were performed on a single batch of cells obtained from a single animal. Cells were loaded with fura-2 by slowly tumbling 1–5 × 10^6 cells/ml for 15 min at room temperature in fura-2 loading medium. (Fura-2–loading medium was prepared by mixing the following items in this order: 4 μl of 5 mM fura-2 pentakis [acetoxymethyl ester; Molecular Probes, Junction City, OR] in DMSO, 1 μl of 25% (wt/vol) Pluronic F-127 [Molecular Probes] in DMSO, 30 μl of heat-inactivated FCS [Gibco], and 1.4 ml of incubation medium [150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, pH 7.4]). Pluronic F-127 is a nonionic dispersing agent which helps solubilize the fura-2/acetoxymethyl ester in aqueous solution (Poenie et al., 1986). The cells were then washed twice with incubation medium, and stored at 4°C. (We performed some experiments with cells that were stored at room temperature. We obtained results identical to those found with cells stored at 4°C. We always observed $[Ca^{2+}]_i$ oscillations in spreading cells. Resting $[Ca^{2+}]_i$ values were similar whether cells were stored at 4°C or at room temperature. However, the rate of dye leakage is much higher at room temperature than at 4°C, so we routinely stored the cells at 4°). Fura-2–loaded cells were only used if their fluorescence intensity was greater than 10 times the autofluorescence and were not used at all if any punctate fluorescence was observed. Less than 10% of fura-2–loaded batches of cells were rejected for this reason. Cells which had been loaded with fura-2/acetoxymethyl ester were lysed with 0.1% Triton X-100. The excitation spectrum of the lysate at low or high $[Ca^{2+}]$ was identical to that obtained by mixing fura-2 pentapotassium salt with lysed unloaded cells. This demonstrates that the fura-2/acetoxymethyl ester is essentially completely converted to the carboxylate form inside the cell. By comparison of the lysate with various concentrations of fura-2 pentapotassium salt mixed with lysed unloaded cells, we determined that the average intracellular [fura-2] was 180 μM. This concentration is comparable to that used in other fura-2 studies, but the effect on $[Ca^{2+}]_i$ buffering in macrophages is not known. Thus, the peak $[Ca^{2+}]_i$ values may slightly underestimate the true peak, and time courses of transients may be somewhat slower than those in an unperturbed cell.

Measurement of $[Ca^{2+}]_i$

Calcium concentrations were determined from the ratio of fura-2 fluorescence at 340 nm excitation and 380 nm excitation as previously described (Kruskal et al., 1984, 1986). Calibration of the 340:380 ratio as a function of $[Ca^{2+}]$ and fluorescence microscopy and microspectrofluorometry were performed as described (Kruskal et al., 1984, 1986), with the following exception: to detect exceedingly brief $[Ca^{2+}]$ transients, we designed and constructed a system with much higher temporal resolution, which is described in detail elsewhere (Kruskal, B. A., F. R. Maxfield, and P. Marks, manuscript in preparation). Briefly, two mercury arc lamp fluorescence illuminators (model No. 100Z with Osram HBO 100 lamps; E. Leitz Inc., Wetzlar, Federal Republic of Germany), each with an electronically controlled shutter, and each with its own narrow-band (10 nm full width at half maximum, greater than 40% transmission; Oriel, Stamford, CT) interference filter (one at 340 nm, one at 380 nm), are attached to a housing containing a 40% transmitting, 40% reflecting mirror at 45° to the optical axis. One beam (coincident with the optical axis) is transmitted through the mirror, the other (perpendicular to the optical axis) is reflected by the mirror onto the opical axis (see Fig. 1). Switching between the two excitation wavelengths is accomplished by closing one shutter and opening the other (controlled by a computer in a pre-set sequence) and thus is limited only by the shutter speed. We can attain speeds of 20–100 s^{-1}.

The experimental chamber consisted of a 35-mm tissue culture dish which had a 1.2-cm diameter hole punched out of the bottom, with a No. 1 glass coverslip attached beneath the hole. Coverslips were prepared by soaking 2 h in Chromerge, followed by thorough rinsing. They were then coated with polylysine and the polylysine was derivatized with DNP groups according to Michl et al. (1979). Most experiments used an opsonized surface, which was prepared from DNP-derivatized dishes by incubating for 0.5 h at 37°C with a 1:20 dilution of antiserum to dinitrophenyl-bovine serum albumin (DNP-BSA; Miles, Elkhart, IN) in PBS. Experiments were performed at 34–37°C, maintained by an air-curtain incubator. A dish was filled with medium and pre-warmed to 34–37°C, and then placed on the microscope stage. Three μl of cell suspension containing 3,000–10,000 cells were pipetted gently onto the surface of the medium in the dish. A single cell was centered in the measuring field of the microscope photometer before attachment. Measurements began before or just at the moment of attachment. The focal plane was set at the lower surface of the cell just as the cell touched the substrate. The illumination and measurement fields were coincident and were ~30 μm in diameter. We routinely acquired one 340:380 pair of measurements every 2.4 s, with 1.1 s between each wavelength. Photometric measurements were made for 0.1 s at each wavelength. Each measurement bleached less than 0.05% of the fura-2. The dye leakage rate averaged ~0.02% per s. We continued to acquire data until the cell was fully spread, or oscillations had ceased for more than 2 min, or neither oscillations nor spreading were observed for more than 8 min. After an experiment was completed, the cell was moved out of the measuring field and background intensities were measured at each wavelength from an adjacent empty area of the culture dish. These background values, which were usually less than 10% of the cell intensities, were used to correct the cellular fluorescence values before the 340:380 ratio was calculated.

For experiments performed under "Ca^{2+}-free" conditions, incubation medium was prepared omitting the $CaCl_2$ and adding 1 mM EGTA and an additional 1 mM $MgCl_2$.

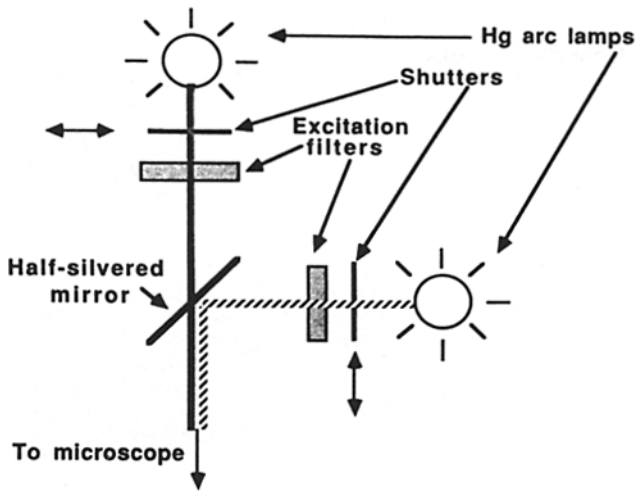

Figure 1. Diagram of the dual wavelength fast alternation illumination system. The excitation wavelength is changed by alternately opening the electronic shutters.

A peak is defined as a $[Ca^{2+}]_i$ maximum to which there has been a consistent increase and from which there is a consistent decrease, involving reciprocal changes at 340 and 380 nm, and at which the $[Ca^{2+}]_i$ is greater than that at the adjacent minima by 10% or 10 nM, whichever is greater. In a quiescent cell, the SD of single $[Ca^{2+}]_i$ measurements over time was 3.5% or 7 nM.

Nomarski Microscopy

Nomarski differential interference-contrast microscopy was performed on a Leitz Diavert microscope with Zeiss Nomarski optics and a Hamamatsu Photonics (Oak Brook, IL) C1965-01 chalnicon tube contrast-enhancing camera.

Results

Calibrated $[Ca^{2+}]_i$ recordings were made for 5–30 min in 29 thioglycollate-elicited mouse peritoneal macrophages as they spread on an anti-DNP/DNP-poly-lysine-coated surface. The minimum $[Ca^{2+}]_i$ observed during these recordings was 140 ± 97 nM (mean ± SD, n = 29). This is somewhat greater than the values obtained in thioglycollate-elicited peritoneal macrophages of 84 ± 38 nM (McNeil et al., 1986) or the J774 cell line of 87 ± 15 nM (Young et al., 1984), but is not outside the range of resting values reported in some cell types (Campbell, 1983). The minimum $[Ca^{2+}]_i$ we observe may be higher than a true resting value since measurements on many cells did not start until the moment when cells contacted the surface. For many of these cells, the minimum was observed near the end of the measurement period.

$[Ca^{2+}]_i$ and Spreading

When macrophages in suspension are plated onto an opsonized surface, they rapidly settle and attach to the substrate. They gradually flatten, and eventually begin to extend lamellipodia. Unlike neutrophils, macrophages do not usually spread in one concerted motion, but rather seem to spread in several discrete steps. The cell extends a number of small lamellipodia asynchronously. Each lamellipod and its motion look like a small version of PMN spreading, with extension of an area of hyaline cytoplasm followed a short while later by migration of organelles into the lamellipod. After the most rapid phase of its motion, a lamellipod will often begin to ruffle (see Fig. 2). A single macrophage may spread in up to 20 individual steps, over a period of up to 12 min.

To measure $[Ca^{2+}]_i$ during spreading, we loaded macrophages with fura-2. The morphology of spreading in fura-2 loaded cells was no different from that in untreated cells. We placed a drop of cell suspension into a pre-warmed dish containing warm incubation medium, allowed the cells to settle, chose one cell, and started to measure its $[Ca^{2+}]_i$ as it attached. In all of the cells which we observed from the moment of attachment and which subsequently spread, we observed an increase in $[Ca^{2+}]_i$ before spreading. This increase could take one of several forms. The most common involved several (up to eight) $[Ca^{2+}]_i$ spikes occurring before spreading was visible. The median number of spikes preceding spreading was four. The average $[Ca^{2+}]_i$ at the peak was 387 ± 227 nM (mean ± SD, n = 92 peaks before spreading in 24 cells). Fig. 3 *a* depicts the time course of $[Ca^{2+}]_i$ changes in a representative cell. Less often (~15%), a single $[Ca^{2+}]_i$ spike preceded spreading. Rarely (3 out of 101 cells), spreading was preceded by a very slow gradual increase to $[Ca^{2+}]_i$ levels as high as those attained during spikes, followed by spreading and slow recovery of $[Ca^{2+}]_i$ to resting levels.

Extracellular calcium is not required for spreading or its prior $[Ca^{2+}]_i$ increase. Macrophages plated into "Ca^{2+}-free" medium containing EGTA (see Materials and Methods) had the same median number of peaks before spreading, with the same mean $[Ca^{2+}]_i$ level (372 ± 128 nM, mean ± SD, n = 25 peaks in 6 cells) as cells plated into normal Ca^{2+}-containing medium. Thus, the calcium producing these spikes is also derived from intracellular stores. A typical time course is shown in Fig. 3 *b*.

Ligation of the Fc receptor is not required for either spreading or the antecedent $[Ca^{2+}]_i$ increase. 16 out of 40 cells plated onto unopsonized surfaces spread, and all showed a preceding rise in calcium (data not shown). However, the proportion of cells which spread is strongly influenced by the presence or absence of immune complex on the substrate, as illustrated in Table I. We have never observed a cell which spread without a prior calcium increase (more than 100 cells observed). About ⅓ of the cells which did not spread did exhibit a calcium increase, but the mean number of peaks exhibited was much less than in spreading cells (14.2 ± 11.2, mean ± SD, n = 24, spreading cells, vs. 4.07 ± 3.8, mean ± SD, n = 28; $p < 0.001$ by Student's t-test).

$[Ca^{2+}]_i$ Oscillations

Many cells exhibited periodic $[Ca^{2+}]_i$ spiking behavior. The proportion of cells which behaved in this fashion varied from 0 to 90% from experiment to experiment, but was frequently greater than 40%. A similar oscillatory behavior of the membrane potential in activated peritoneal macrophages was reported by Gallin et al. (1975), as was the variation among animals in the proportion of cells exhibiting oscillations. A histogram of the number of peaks observed in individual cells is shown in Fig. 4. The largest number of cells exhibited only a single $[Ca^{2+}]_i$ peak. The time course of a typical cell showing repetitive periodic $[Ca^{2+}]_i$ spikes is shown in Fig. 3 *a*.

The mean $[Ca^{2+}]_i$ of all the peaks (before and after spreading) is 385 ± 266 nM (mean ± SD, n = 208 peaks in 14 cells). The lowest $[Ca^{2+}]_i$ reached between peaks is often above the minimum $[Ca^{2+}]_i$ for that cell. There was no

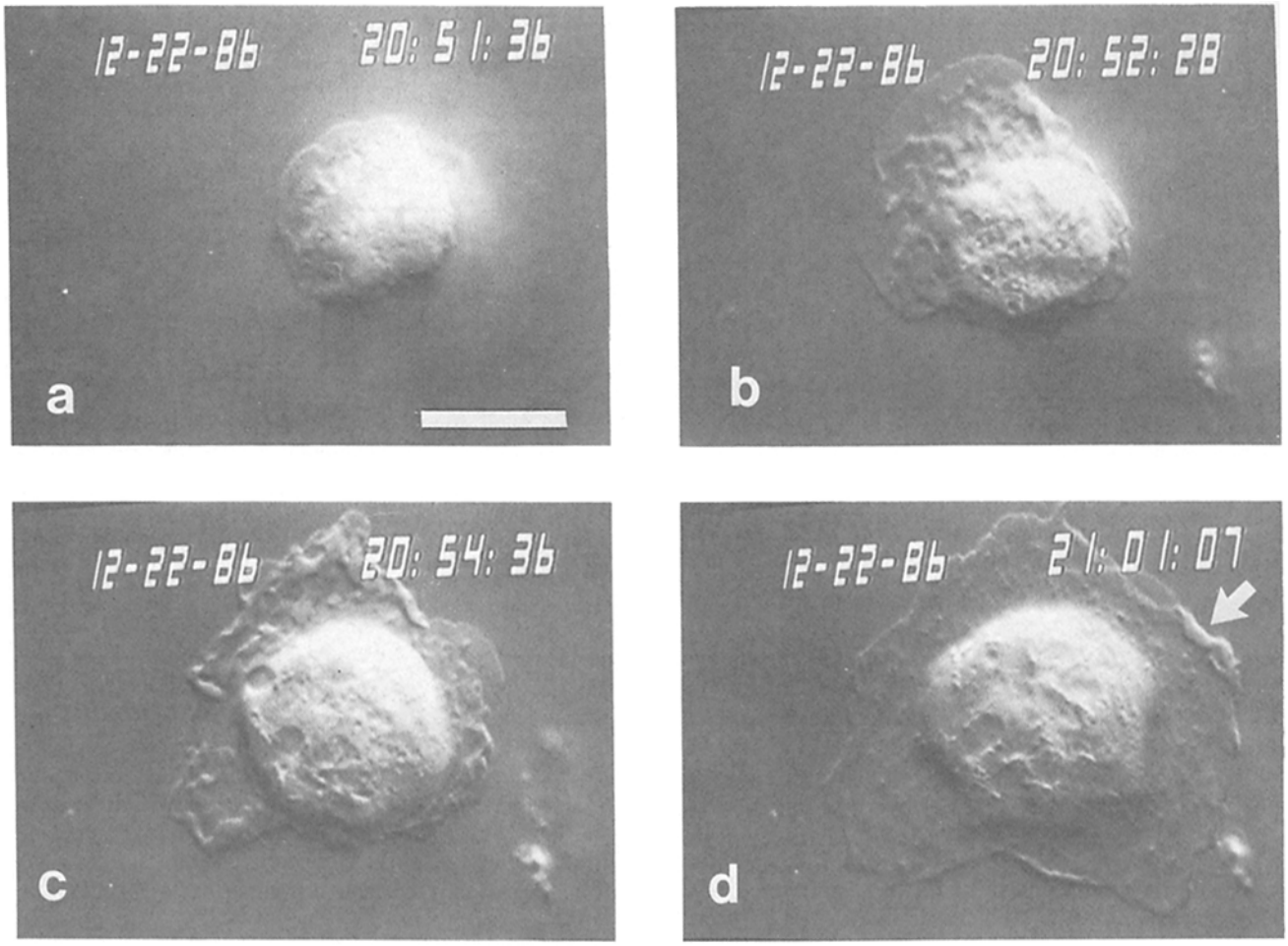

Figure 2. Morphology of cell spreading. Nomarski micrographs of a spreading macrophage. Cells were plated onto an opsonized surface as for $[Ca^{2+}]_i$ measurements, and observed with differential interference contrast optics. One image per second was recorded on a Panasonic Optical Disc Video Recorder (Matsushita Electronics Corp., Osaka, Japan), and photographs were later taken from a video monitor. (*a*) The cell has just settled on the surface. (*b*) 1 min later, the cell has begun to spread. (*c*) By 3 min, the cell has spread around most of its circumference. (*d*) Approximately 10 min after plating, spreading is complete. Note the ruffles in *c* and *d* (one ruffle is indicated in *d* by the arrow). Bar, 10 μm.

significant difference between mean peak $[Ca^{2+}]_i$ before and after spreading began. No autocorrelation of peak $[Ca^{2+}]_i$ (e.g., tall peak followed by a short one or vice versa) was observed. A strong correlation is seen between the minimum $[Ca^{2+}]_i$ for a cell and the mean peak $[Ca^{2+}]_i$ for that cell, as illustrated in Fig. 5. This is unlikely to be due to a simple calibration artifact, as cells from a single day (calibration) were spread out across the range. These two $[Ca^{2+}]$ values might be correlated because they both depend on the abundance of calcium in the intracellular storage pools.

The timing of $[Ca^{2+}]_i$ peaks is apparently periodic, as demonstrated by a clustering of the intervals between peaks (periods) about a central value (illustrated for a typical cell in Fig. 6). In 13 cells that showed more than 10 peaks, the median period in individual cells ranged from 19 to 69 s, with a median over all cells of 29 s (Fig. 7). There was no autocorrelation of periods (e.g., long period followed by a short one or vice versa).

The oscillations are not dependent on occupancy of the Fc receptor, as we observed oscillations in several cells plated on a plain polylysine-coated coverslip without DNP-derivatization or coating with antiserum (data not shown).

The calcium producing each spike is derived from intracellular stores. Lowering the external $[Ca^{2+}]$ to less than 10^{-7} M during the oscillations did not halt them. 80% of oscillating cells to which we added EGTA produced at least one more calcium spike, and 70% produced at least three more spikes. In one case, illustrated in Fig. 8, ten more spikes occurred after addition of EGTA. Individual cells continued spiking for varying amounts of time after the decrease in extracellular calcium. This variation may represent variations in the calcium content of their intracellular stores at the moment of EGTA addition. We did not find a statistically significant difference in the rate at which the oscillations decayed when EGTA was added. However, this could be due to our small sample size.

Discussion

A role for $[Ca^{2+}]_i$ in the control of phagocytosis is suggested by its well-known effects on isolated cytoskeletal assemblies in vitro (Campbell, 1983). In agreement with this hypothesis, we found that spreading in activated mouse peritoneal macrophages is always preceded by a rise in $[Ca^{2+}]_i$ (more

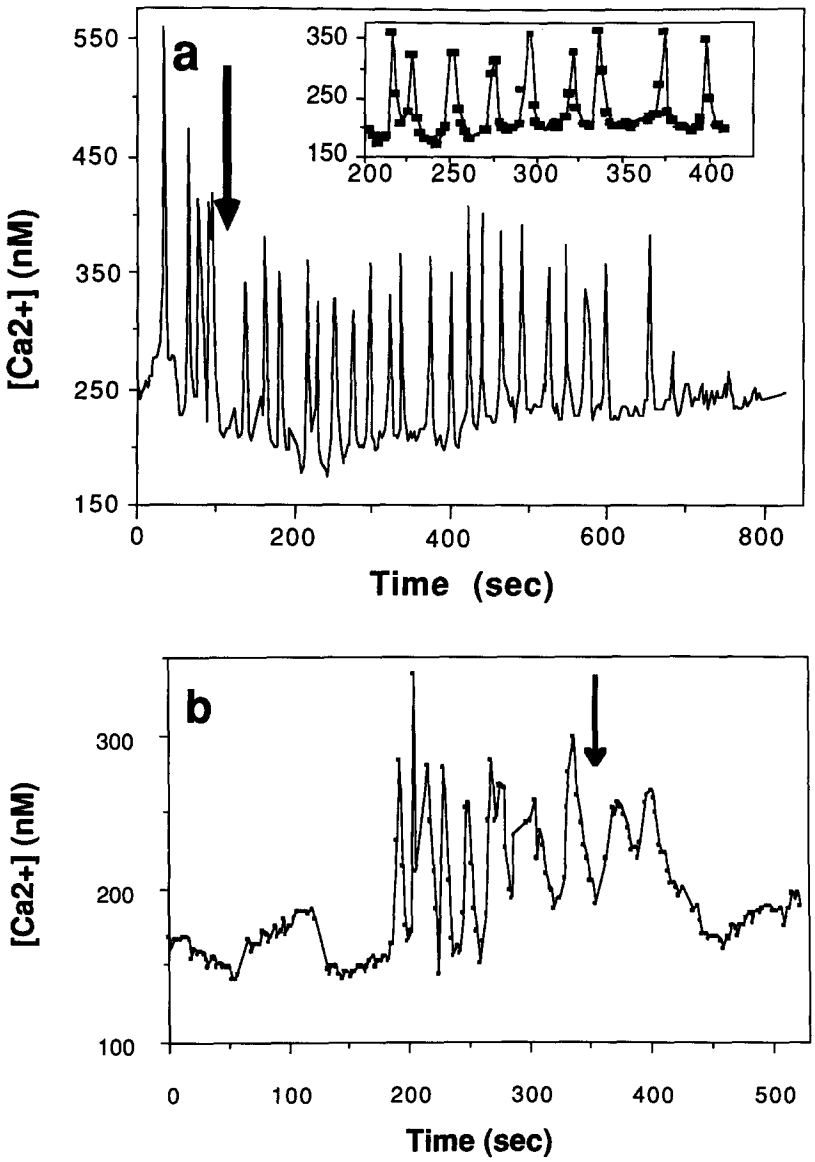

Figure 3. (*a*) Time course of $[Ca^{2+}]_i$ in a typical oscillating spreading cell. Cells were plated onto an opsonized surface as described in Materials and Methods. A cell was selected while still in suspension and its $[Ca^{2+}]_i$ was measured every 2.4 s, except for occasional 4.8 s gaps to obtain better morphologic information. (*Inset*) Detail of the $[Ca^{2+}]_i$ from 210 to 410 s, allowing better appreciation of the periodicity of the $[Ca^{2+}]_i$ spikes. (*b*) Spreading in the absence of extracellular calcium. Conditions were as in *a* except the cells were plated into "Ca^{2+}-free" incubation medium (see Materials and Methods). At the time indicated by the solid arrow, spreading was first detected. The SD of $[Ca^{2+}]_i$ over time in a quiescent cell was 7 nM or 3.5%.

than 100 cells observed). The calcium producing this increase comes from intracellular storage sites. Spreading and the calcium increase do not require specific binding to the Fc receptor.

Spreading of phagocytes on a surface has long been thought of as frustrated phagocytosis (North, 1968). A persuasive argument in favor of the homology of these two processes is the similarity in cytoskeletal rearrangements which occur just inside the plasma membrane next to the stimulatory surface (Boyles and Bainton, 1979, 1981).

The cytoskeletal mechanisms underlying spreading are not well understood. Several types of processes may be pictured as taking part in spreading: (*a*) release of tension stored in the plasma membrane, allowing it to bleb outward; (*b*) active force generation, as by actomyosin contraction or actin polymerization; and (*c*) fusion of intracellular membranes with the plasma membrane, increasing membrane surface area available for attachment. Any one or combination of these processes could be involved in spreading. Calcium regulation of these processes has been described in some systems.

The pattern of $[Ca^{2+}]_i$ increase preceding spreading dif-

Table I. Dependence of Spreading on Substrate

	% of cells which spread	
Substrate treatment	+Ab	−Ab
Chromerge clean, Polylysine coat, DNP-derivatize	45	14
Chromerge clean, Polylysine coat	3	3
Chromerge clean	6	7

Cells were plated onto coverslips treated as indicated above (see Materials and Methods for details), pre-warmed to 35.5 ± 1.5°C and maintained at that temperature by an air-curtain incubator. Spreading was allowed to go on for 10 min, and then cells were fixed with 4% formaldehyde in PBS for 2 min. After rinsing, spreading was scored visually as an increase of greater than 1.5-fold in cell diameter, as significant flattening, and the presence of a hyaline lamellipodium. Greater than 140 cells were counted for each condition. These are the results of a single experiment representative of several performed on different days.

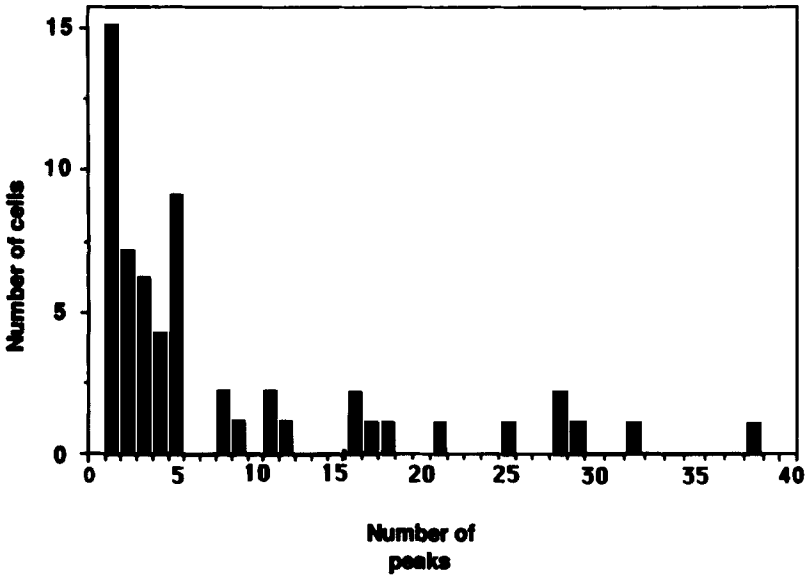

Figure 4. Histogram of total number of $[Ca^{2+}]_i$ peaks in individual cells. Cells were plated onto an opsonized surface as described in Materials and Methods. A cell was selected while still in suspension and its $[Ca^{2+}]_i$ was measured every 2.4 s until it exhibited no more $[Ca^{2+}]_i$ peaks. Peaks (indicating an increase in $[Ca^{2+}]_i$ of 10% or 10 nM, which ever was greater) were counted, and the number of cells in which each number of peaks occurred was plotted.

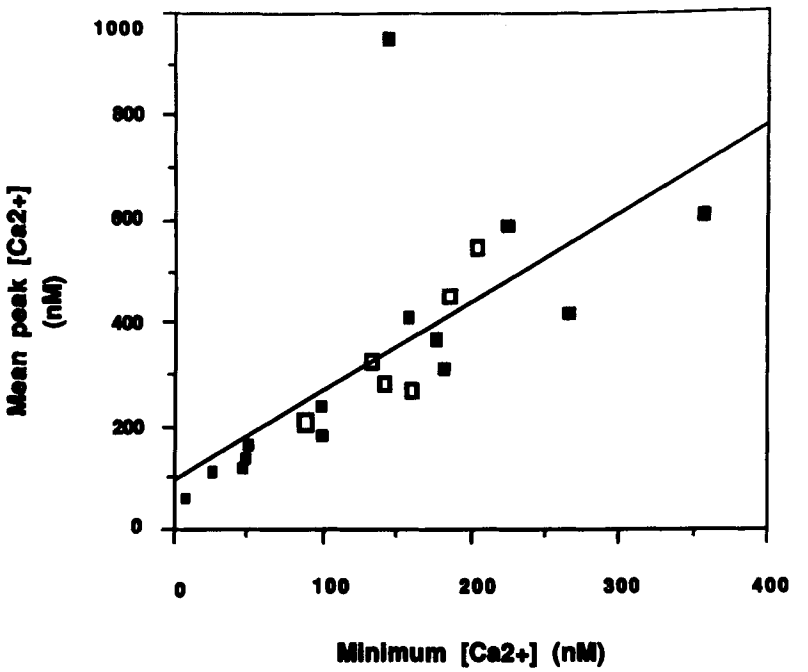

Figure 5. Correlation of minimum $[Ca^{2+}]_i$ with mean peak $[Ca^{2+}]_i$ for individual oscillating cells. The closed symbols indicate cell oscillating in calcium-containing incubation medium, while the open symbols indicate cells in "Ca^{2+}-free" conditions (free $[Ca^{2+}]$ less than 10^{-7} M). The line is a least-squares fit to all of the points, with and without extracellular Ca^{2+}.

fers between neutrophils and macrophages. In PMN, the $[Ca^{2+}]_i$ increase always consists of a single spike and precedes spreading by a fixed time (Kruskal et al., 1986). The situation in the macrophage is more complicated. The $[Ca^{2+}]_i$ increase is not followed immediately by spreading, and the time separating the two events varies from cell to cell. Differences between the two cell types may reflect differences in the signaling mechanisms or in the effector mechanisms. It is important to note that signals other than calcium are almost certain to be involved in the control of spreading and phagocytosis as well.

Although the conclusions of previous studies on the role of $[Ca^{2+}]_i$ in phagocytosis disagree, we believe they can be reconciled. By measuring $[Ca^{2+}]_i$ in individual cells during spreading, we have filled the gap between measurements on populations of cells and localization of $[Ca^{2+}]_i$ changes within cells. Our previous study in PMN (Kruskal et al., 1986) and the data presented here for macrophages provide support for the hypothesis that an increase in $[Ca^{2+}]_i$ is an important signal in phagocytosis. Young et al. (1984) showed that a large increase in $[Ca^{2+}]_i$ occurred when J774 cells (a macrophage-like cell line) in suspension were incubated with opsonized particles or immune complexes. Lew et al. (1985) using population average techniques with quin2 on PMN in suspension found that $[Ca^{2+}]_i$ increased when cells were phagocytosing particles opsonized with either Ig or C3bi. Using digital image-processing techniques to examine the intracellular distribution of $[Ca^{2+}]_i$, Sawyer et al. (1985) found a moderate increase around the forming phagosome in PMN. Using aequorin as an indicator and measuring a population average $[Ca^{2+}]_i$, McNeil et al. (1986) saw no change in $[Ca^{2+}]_i$ during ingestion of opsonized particles or during spreading on an opsonized surface. By averaging together the signal from thousands of cells, however, changes which oc-

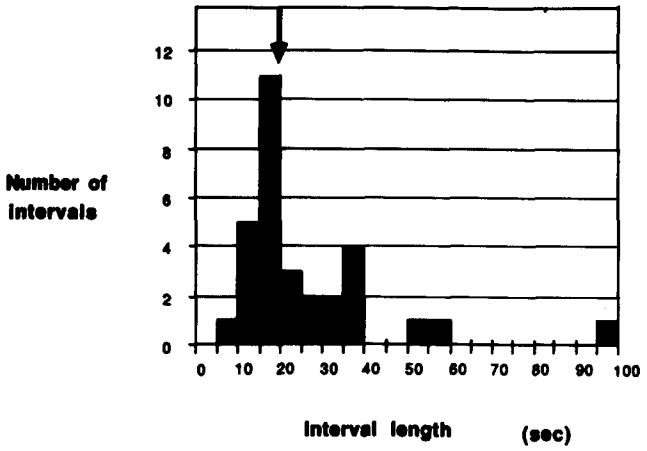

Figure 6. Distribution of length of intervals between peaks in a typical cell. $[Ca^{2+}]_i$ was measured in a single cell as in Fig. 3 *a*, and the time from peak to peak $[Ca^{2+}]_i$ was measured and plotted as a histogram. Arrow indicates the median period in this cell.

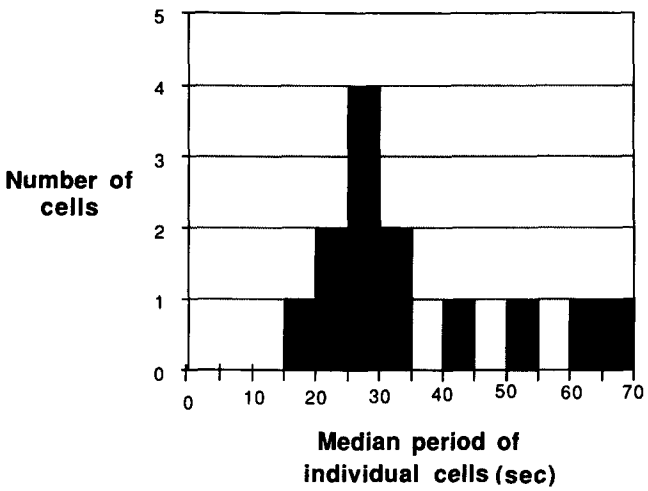

Figure 7. Distribution of median period for 13 individual cells. Medians were determined from data like that of Fig. 6.

cur asynchronously throughout the population may be averaged out to undetectability. We have calculated that as many as 75% of the cells in their experiment could be oscillating with the typical period and magnitude that we have observed and the population average $[Ca^{2+}]_i$ would still be within 1 SD of their mean resting value.[2] Our observations at the single-cell level provide strong support for the hypothesis that $[Ca^{2+}]_i$ increases may be important in initiating phagocytosis.

2. Assume a period of 30 s (our median), peaks of triangular shape with width 7 s and height 400 nM (our mean). If cells are oscillating with no synchrony (i.e., random phase), then this increase would be distributed over the entire population. If 100% of the cells were oscillating, the increase above resting levels would be 47 nM; if 75% were oscillating, the increase would be 35 nM, with McNeil et al. SD of 38 nM (1986).

During the course of our experiments on spreading, we unexpectedly found that many cells showed periodic increases in $[Ca^{2+}]_i$. The period of these oscillations ranged from 19 to 69 s. The calcium producing the increases comes from intracellular pools, and the period does not depend on extracellular calcium. These oscillations do not require occupancy of the Fc receptor. We have been unable to observe any periodic motion during spreading that we could correlate with the oscillations in $[Ca^{2+}]_i$. However, the best morphologic information we can obtain in rapid alternation with fluorescence measurements (by brightfield optics) is less than ideal, leaving open the possibility that the oscillations may be related to the incremental steps of spreading or to ruffling, both of which we observed with differential interference contrast microscopy. In hepatocytes, $[Ca^{2+}]_i$ oscillations have

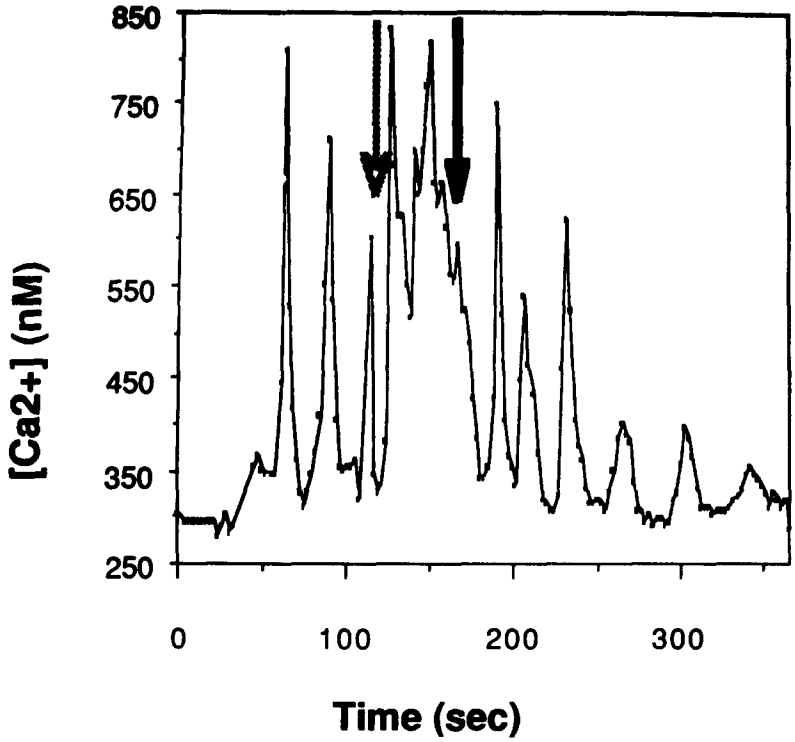

Figure 8. Oscillation continues even when extracellular $[Ca^{2+}]$ is reduced to less than 10^{-7} M. Conditions were as in Fig. 3 *a*. At the time indicated by the cross-hatched arrow, EGTA was added to a final concentration of 5 mM and the medium was well mixed. Spreading was first detected at the time indicated by the solid arrow.

been observed in response to hormonal stimuli (Woods et al., 1986). These may be related to Ca^{2+}-stimulated rhythmic contractions which these cells undergo to propel fluid along bile canaliculi (Watanabe et al., 1985).

Oscillations of the membrane potential with frequency similar to our $[Ca^{2+}]_i$ oscillations have been observed in macrophages (Gallin et al., 1975) and fibroblasts (Okada et al., 1977). Okada's group has extensively characterized these oscillatory hyperpolarizations using giant fibroblasts (produced by X-irradiation or polyethylene glycol (PEG) fusion), because of the difficulty of impaling normal cells. Ueda et al. (1986) have shown that oscillations of $[Ca^{2+}]_i$ (measured with an ion-selective microelectrode) probably underlie the periodic hyperpolarizations. The membrane potential oscillations can be blocked without blocking the $[Ca^{2+}]_i$ oscillations, but conditions which inhibit the $[Ca^{2+}]_i$ oscillations cause the potential oscillations to cease (Ueda et al., 1986). Correlations between membrane potential oscillations and phagocytosis of inert particles have been observed in macrophages (Kouri et al., 1980) and in fibroblasts (Okada et al., 1981). Gallin and Gallin (1977) also reported potential oscillations in macrophages, induced by soluble stimuli, such as activated complement.

Our observations of $[Ca^{2+}]_i$ oscillations in activated peritoneal macrophages suggest that, as in L-cells, spontaneous oscillations in membrane potential are caused by an underlying oscillation in $[Ca^{2+}]_i$. Our data suggest that the source for this calcium is intracellular, in agreement with the interpretation of Nelson and Henkart (1979), whereas Okada et al. (1982) suggests that in fibroblasts the source is outside the cell.

The mechanism by which $[Ca^{2+}]_i$ increases periodically is not known. The oscillations could be driven by some underlying oscillation (e.g., in pH) or periodic phenomenon, such as pulsatile release of inositol triphosphate. Another possible mechanism would be a "push–pull" oscillation involving two different signals. For example, a transient (i.e., self-limited) change in cytosolic pH could stimulate a transient increase in $[Ca^{2+}]_i$, which could in turn stimulate a cytosolic pH transient. With an appropriate time delay between the peak of a transient in one parameter and the beginning of a peak in the other, oscillations in both would result. Interactions of cytosolic pH changes and $[Ca^{2+}]_i$ changes have been reported recently by Siffert and Akkerman (1987) and Ives and Daniel (1987).

The oscillations in $[Ca^{2+}]_i$ could instead result from the intrinsic properties of the cell's calcium regulatory mechanism. A hysteresis in the $[Ca^{2+}]$ dependence of a Ca^{2+} removal mechanism or of a calcium channel (in either the plasma membrane or an internal membrane) could cause $[Ca^{2+}]_i$ to oscillate. However, the shape of the $[Ca^{2+}]_i$ transients which we observe cannot be explained by a simple hysteresis mechanism. Similarly, a time delay in the response of a Ca^{2+} removal mechanism to changes in $[Ca^{2+}]$ could produce oscillations. (This situation is frequently encountered in feedback control systems.) A time delay alone, however, like hysteresis, is not sufficient to produce $[Ca^{2+}]_i$ oscillations with the characteristics of those we observe. The mechanism which is simplest and most consistent with our data is one involving two interacting signals such as $[Ca^{2+}]_i$ and cytosolic pH.

The ability to measure $[Ca^{2+}]_i$ in single cells has allowed us to detect asynchronous changes which could be averaged out to imperceptibility in populations and to obtain more accurate estimates of the magnitude and kinetics of changes in $[Ca^{2+}]_i$. Our data are consistent with the hypothesis that the $[Ca^{2+}]_i$ is a regulator of spreading (and by analogy, of phagocytosis). The measurement of $[Ca^{2+}]$ in living cells during motility provides an essential complement to in vitro studies of the effect of calcium on cytoskeletal proteins. Studies of the differences between $[Ca^{2+}]_i$ changes preceding spreading in PMN and macrophages may prove useful in elucidating the fundamental mechanisms which control phagocytosis.

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References

Blinks, J. R. 1985. Detection of Ca^{++} with photoproteins. *In* Bioluminescence and Chemiluminescence: Instruments and Applications. K. Van Dyke, editor. CRC Press, Boca Raton. Vol. II. pp. 185–226.

Boyles, J., and D. Bainton. 1979. Changing patterns of plasma membrane-associated filaments during the initial phases of polymorphonuclear leukocyte adherence. *J. Cell Biol.* 82:347–368.

Boyles, J., and D. Bainton. 1981. Changes in plasma membrane-associated filaments during endocytosis and exocytosis in polymorphonuclear leukocytes. *Cell.* 24:905–914.

Campbell, A. K. 1983. Intracellular Calcium: its universal role as regulator. John Wiley & Sons. Chichester. 206–256.

Gallin, E. K., and J. I. Gallin. 1977. Interaction of chemotactic factors with human macrophages. Induction of transmembrane potential changes. *J. Cell Biol.* 75:277–289.

Gallin, E. K., Wiederhold, M. L., Lipsky, P. F., and A. S. Rosenthal. 1975. Spontaneous and induced membrane hyperpolarizations in macrophages. *J. Cell. Physiol.* 86:653–662.

Grynkiewicz, G., Poenie, M., and Tsien, R. Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Ince, C., P. C. J. Leijh, J. Meijer, E. Van Bavel, and D. Ypey. 1984. Oscillatory hyperpolarizations and resting membrane potentials of mouse fibroblast cell lines. *J. Physiol. (Lond.).* 352:625–635.

Ives, H. E., and T. O. Daniel. 1987. Interrelationship between growth-factor induced pH changes and intracellular Ca^{2+}. *Proc. Natl. Acad. Sci. USA.* 84:1950–1954.

Kouri, J., M. Noa, B. Diaz, and E. Niubo. 1980. Hyperpolarisation of rat peritoneal macrophages phagocytosing latex particles. *Nature (Lond.).* 283:868–869.

Kruskal, B. A., C. H. Keith, and F. R. Maxfield. 1984. Thyrotropin-releasing hormone-induced changes in intracellular $[Ca^{2+}]$ measured by microspectrofluorometry on individual quin2-loaded cells. *J. Cell Biol.* 99:1167–1172.

Kruskal, B. A., Shak, S., and F. R. Maxfield. 1986. Spreading of human neutrophils is immediately preceded by a large increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 83:2919–2923.

Lew, D. P., T. Andersson, J. Hed, F. Di Virgilio, T. Pozzan, and O. Stendahl. 1985. Ca^{2+}-dependent and Ca^{2+}-independent phagocytosis in human neutrophils. *Nature (Lond.).* 315:509–511.

McNeil, P. L., J. A. Swanson, S. D. Wright, S. C. Silverstein, and D. L. Taylor. 1986. Fc-receptor-mediated phagocytosis occurs in macrophages without an increase in average $[Ca^{++}]_i$. *J. Cell Biol.* 102:1586–1592.

Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes of Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.

Nelson, P. G., and M. P. Henkart. 1979. Oscillatory membrane potential changes in cells of mesenchymal origin: the role of an intracellular calcium regulating system. *J. Exp. Biol.* 81:49–61.

North, R. J. 1968. The uptake of particulate antigens. RES. 5:203–229.

Okada, Y., Y. Doida, G. Roy, W. Tsuchiya, K. Inouye, and A. Inouye. 1977. Oscillations of membrane potential in L cells. I. Basic characteristics. *J.*

Membr. Biol. 35:319–335.

Okada, Y., W. Tsuchiya, T. Yada, J. Yano, and H. Yawo. 1981. Phagocytic activity and hyperpolarizing responses in L-strain mouse fibroblasts. *J. Physiol. (Lond.).* 313:101–119.

Okada, Y., W. Tsuchiya, and T. Yada. 1982. Calcium channel and calcium pump involved in oscillatory hyperpolarizing responses of L-strain mouse fibroblasts. *J. Physiol. (Lond.).* 327:449–461.

Poenie, M., J. Alderton, R. Steinhardt, and R. Y. Tsien. 1986. Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science (Wash. DC).* 233:886–889.

Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55:987–1035.

Rabinovitch, M., and M. J. DeStefano. 1973. Macrophage spreading *in vitro*. I. Inducers of spreading. *Exp. Cell Res.* 77:323–334.

Sawyer, D. W., J. A. Sullivan, and G. L. Mandell. 1985. Intracellular free calcium localization in neutrophils during phagocytosis. *Science (Wash. DC).* 230:663–666.

Siffert, W., and J. W. N. Akkerman. 1987. Activation of sodium-proton exchange is a prerequisite for Ca^{2+} mobilization in human platelets. *Nature (Lond.).* 325:456–458.

Southwick, F. S., and T. P. Stossel. 1983. Contractile proteins in leukocyte function. *Semin. Hematol.* 20:305–321.

Trotter, J. A. 1981. The organization of actin in spreading macrophages. The actin cytoskeleton of peritoneal macrophages is linked to the substratum via transmembrane connections. *Exp. Cell Res.* 132:235–48.

Tsien, R. Y., T. Pozzan, and T. J. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* 94:325–334.

Tsien, R. Y., T. J. Rink, and M. Poenie. 1985. Measurement of cytosolic free Ca^{2+} in individual small cells using fluorescence microscopy with dual excitation wavelengths. *Cell Calcium.* 6:145–157.

Ueda, S., S. Oiki, and Y. Okada. 1986. Oscillations of cytoplasmic concentrations of Ca^{2+} and K^+ in fused L cells. *J. Membr. Biol.* 91:65–72.

Watanabe, S., C. R. Smith, and M. J. Phillips. 1985. Coordination of the contractile activity of bile canaliculi. Evidence from calcium microinjection of triplet hepatocytes. *Lab. Invest.* 53:275–279.

Woods, N. M., K. S. R. Cuthbertson, and P. H. Cobbold. 1986. Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. *Nature. (Lond.).* 319:600–602.

Young, J. D.-E., S. S. Ko, and Z. A. Cohn. 1984. The increase in intracellular free calcium associated with IgGγ2b/γ1 Fc receptor-ligand interactions: Role in phagocytosis. *Proc. Natl. Acad. Sci. USA.* 81:5430–5434.