Multiple Elevations of Cytosolic-free Ca^{2+} in Human Neutrophils: Initiation by Adherence Receptors of the Integrin Family

M. E. E. Jaconi,* J. M. Theler,‡ W. Schlegel,§ R. D. Appel,‖ S. D. Wright,¶ and P. D. Lew*

*Division of Infectious Diseases, Geneva University Hospital, Geneva, Switzerland; ‡Division de Biochimie Clinique, Centre Médical Universitaire, Geneva, Switzerland; §Fondation pour Recherches Médicales, Geneva, Switzerland; ‖Unité d'Imagerie Numérique, Geneva University Hospital, Geneva, Switzerland; and ¶Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

Abstract. Multiple spontaneous transient elevations of cytosolic-free calcium ($[Ca^{2+}]_i$) are observed in single human neutrophils during adherence. The interrelation between adherence and spontaneous $[Ca^{2+}]_i$ transients was analyzed by simultaneous monitoring of $[Ca^{2+}]_i$ and cell morphology. Fluorescent images of fura 2-loaded neutrophils attached to albumin-coated glass were recorded with a high sensitivity CCD camera while $[Ca^{2+}]_i$ was assessed with a dual excitation microfluorimetry. The majority of the initially round cells studied showed changes in shape which started either before or at the same time as the onset of the $[Ca^{2+}]_i$ transients.

These data suggested that a rise in $[Ca^{2+}]_i$ is not a prerequisite for shape change. This conclusion was confirmed by observation of movement and spreading in cells whose $[Ca^{2+}]_i$ transients were abolished by chelation of extracellular Ca^{2+}. Instead, our data suggest that spreading or adhesion itself initiates the $[Ca^{2+}]_i$ activity. In keeping with this hypothesis, cytochalasin B, which prevents both cell movement and adhesion, completely inhibited generation of $[Ca^{2+}]_i$ transients. To determine if the movement alone or adhesion alone is responsible for $[Ca^{2+}]_i$ activity, we treated cells with antibodies against the β chain (CD18, β_2) or the α subunit (CD11b, α_m) of the dominant leukocyte integrin (CR3). Antibody-treated cells showed normal extension of pseudopods but impaired ability to adhere. Inhibition of adhesion in this way inhibited $[Ca^{2+}]_i$ activity.

Taken together these results suggest that following sequence of events after contact of neutrophils with surfaces: (*a*) cell movement and shape change lead to enhanced contact of integrins with the surface; and (*b*) integrins-mediated adhesion generates multiple $[Ca^{2+}]_i$ transients. The $[Ca^{2+}]_i$ transients may then control exocytic events associated with movement and may provide a link between adherence and activation or priming of neutrophils to other stimuli.

CYTOSOLIC-free calcium ($[Ca^{2+}]_i$) is a key intracellular messenger involved in several responses of neutrophils. Highly sensitive and specific fluorescent Ca^{2+} probes have allowed assessment of $[Ca^{2+}]_i$ in single adherent neutrophils by microfluorimetric methods. Sawyer et al. (28) and Murata et al. (21) have shown that during chemotaxis and phagocytosis, $[Ca^{2+}]_i$ increases occur mainly in the anterior part of the cell and around the phagocytic vesicle. Kruskal et al. (13) have shown that upon attachment of neutrophils to polylysine-coated glass there is a rapid and transient (<1 min) $[Ca^{2+}]_i$ elevation.

Using a rapid monitoring system we have described spontaneous and chemoattractant-triggered, multiple $[Ca^{2+}]_i$ elevations in neutrophils adherent both to albumin or fibronectin-coated surfaces (11). More recently Marks and Maxfield (19) have shown that such $[Ca^{2+}]_i$ transients appear to be linked to neutrophil movement during chemotaxis on poly-D-lysine–coated glass in the presence of serum. Neutrophils that extended pseudopods and assumed a polarized morphology were always observed to exhibit $[Ca^{2+}]_i$ transients even in the absence of chemoattractants. While $[Ca^{2+}]_i$ transients are associated with cell spreading and locomotion, it is not clear whether transients in some way cause the movement, or if they are the result of cell movement.

Spreading and locomotion are mediated by receptors that bridge the cell to the substrate. The numerically and functionally dominant adhesion receptor of neutrophils is the integrin CR3 (also known as Mac-1, Mol, CD11b/CD18, and $\alpha_m\beta_2$) (36). Blockade of this receptor with mAbs prevents spreading and/or chemotaxis on a variety of substrates including albumin (6), fibrinogen (38), complement protein C3bi (39), as well as on endothelial cells in vitro (9, 17) and in vivo (4, 26, 33). CR3 is likely to make a major contribution to cell locomotion since the chemoattractant stimuli C5a (17), tumor necrosis factor (TNF)α (17), interleukin (IL)-8 (7), and PMA (35) cause dramatic enhancement of the ca-

pacity of this receptor to mediate adhesion. Moreover, adhesion mediated by CR3 is transient and can be readily broken (17, 35), thus, allowing the cycles of adhesion and detachment necessary for cell locomotion.

To assess the link between transient $[Ca^{2+}]_i$ elevations and movement in attached neutrophils we monitored in parallel both $[Ca^{2+}]_i$ and morphological parameters. In addition, we used mAbs to block adherence receptors. Our results indicate that changes in cell morphology precede or are concomitant with $[Ca^{2+}]_i$ elevations and that functional CR3 is necessary for the generation of the $[Ca^{2+}]_i$ transients.

Materials and Methods

Preparation of Human Neutrophils

Human neutrophils were purified as previously described (16). In brief, blood was obtained from healthy adult volunteers, using citrate-containing syringes. Whole blood was allowed to sediment on Dextran T500 after which the neutrophils were isolated in a Ficoll gradient (Pharmacia Fine Chemicals, Piscataway, NJ). The contaminating erythrocytes were eliminated by a 15–20-s hypotonic lysis in distilled water. The neutrophils were washed twice before resuspension in "Ca^{2+} buffer" (containing 138 mM NaCl, 6 mM KCl, 1mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM $NaHPO_4$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4). Cells were suspended at a final concentration of 10^7 cells/ml in Ca^{2+} buffer containing 0.1% human serum albumin (HSA 20%; Albuman Berna, Institut Sérothérapique et Vaccinal Suisse, Berne, CH).

Loading with Fura 2 and Attachment Procedure for Single Cell Analysis

The procedure for homogeneously loading neutrophils with fura 2 has been described previously (11). Briefly, the cells at the concentration of 10^6/ml were loaded with 2 μM of the fluorescent indicator fura 2/AM (Molecular Probes Inc., Eugene, OR) in Ca^{2+} buffer containing 0.1% HSA for 45 min at 37°C. 25 μl of cell suspension were plated onto glass coverslips and were allowed to attach to the surface for 3–5 min at 37°C; nonattached cells were removed by washing with 1 ml of buffer. The coverslips were mounted on a perfusion chamber and the cells were studied thereafter in the microscope for a maximum of 30 min.

Selection of Cells for Single Cell Analysis

The morphology of the cell population attached to the coverslips was initially screened and two morphologies were defined under phase-contrast microscopy: (a) round, stationary, phase-bright cells; and (b) spread (both stationary and moving) cells, easily defined by the grey contrast over the cell surface. Consistent with nomenclature employed by other authors (31) attachment was defined by the inability to wash neutrophils off with gentle buffer changes, whereas the term spread was used for cells that adhere actively to a various extent ranging from pseudopod extensions and movement to complete flattening. After an initial 3–5-min attachment phase, most of the cells appeared round, and after 20 min virtually all the cells on the coverslip had adhered, spread, or were moving. This transition allowed monitoring of the onset of cell shape changes during adherence, spreading, or initial random movement and correlation of these morphological parameters with $[Ca^{2+}]_i$ values. Thus, for most of the studies reported here, the round, phase-bright cells were initially chosen and studied over time.

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

$[Ca^{2+}]_i$ measurement and cell morphology monitoring were performed simultaneously by modifying our dual excitation microfluorimetry equipment. As previously described (11, 29), single cell microfluorimetry was performed with a modular spectrofluorimeter, Fluorlog 2 (SPEX Instrument Inc., London, UK) and a Nikon inverted fluorescence microscope. The emitted fluorescence by the cells was divided: 50% of the light was measured by the photomultiplier tube permitting evaluation of the total $[Ca^{2+}]_i$ changes, while the other 50% of fluorescence intensity was monitored with a TV CCD-camera (Photonics Science, Turnbridge Wells, UK). Fluorescent images result from a mean over 5 s of the emitted light during excitation by both 340 and 380 nm. Neutrophils were observed for a maxi-

mum of 15 min, a time during which the loss of dye due to leakage and photodestruction was <25% (11). Images were digitalized point by point (512 × 512 pixels) over a selected part of the microscopic field and stored by the image processor (Imagine, Synoptic, Cambridge, UK). Images were acquired at 30-s intervals. This time interval proved to be convenient because it was on the time scale both of the $[Ca^{2+}]_i$ transients (average 28 ± 5 s, see reference 11) and the observed changes in cell morphology.

Analysis of Morphology

Digitalized images were analyzed on a SUN workstation 3/60 with an image analysis program named MELANIE (2, 3, 24). The program, which was originally used for the analysis of several thousand protein spots on 2D-gel electrophoresis, was adapted in order to permit smoothing of images and measurement of the area and perimeter of the cells from a gray level threshold selected for optimal matching of the resulting calculated contours with the original fluorescent images. This procedure permitted observation of the kinetics of shape changes even after spreading or pseudopodal extension in cells well labeled in fura 2. Given the limits of camera sensitivity and resolution, the finest pseudopodal extensions observed with conventional microscopy (interference modulation contrast [10]) are not considered in the shape factor (SF)[1] analysis. Accordingly, the minimal SF values resulting from the present analysis (around 0.6) were higher than SF values described for neutrophils previously (18).

Cell morphology was defined by the SF determined according to the equation $SF = 4 \times \pi \times area/(perimeter^2)$, with a perfect circle resulting in a shape factor of 1.0, whereas elongation resulted in a decrease in SF (a flat line would result in SF approaching 0) (18). Thus, when the cell moves and projects pseudopods the SF decreases. Since the SF is a ratio between area and perimeter, it takes into account decreases in cell fluorescence which can be due to the fact that cells leave the area of optimal fluorescence excitation.

Round, phase-bright cells appeared to be more fluorescent than flat adherent cells. For convenience, the grey level map has been reequilibrated (but only after morphological parameter measurement) for replotted images in which fluorescence of the cell decreased exceedingly, due to cell flattening or movement out of the optical center of the microscope.

Modulation of Adherence Conditions

Adherence and spreading of human neutrophils was modified by mAbs against adhesion receptors. mAb IB4 (purified IgG2a) directed against the β subunit (CD18, β_2) of CR3 was as described (37). This antibody also recognizes the β_2 integrins LFA-1 (CD11a/CD18) and p150,95 (CD11c/CD18), but these two receptors are 10–20-fold less prevalent on neutrophils than CR3 (36). Ascites fluid containing mAb 44a directed against the α chain (CD11b, α_m) of CR3 was also used (a generous gift of R.F. Todd III, Ann Arbor, MI). Control antibodies included OKM1 (purified IgG2b) directed against an epitope on the α chain of CR3 that is not involved in adhesion (17, 37, 40), and ascites containing IgG1, IgG2a and IgG2b antibodies of unknown specificity (a generous gift of Dr. Isui Shozo, Geneva).

10^6 cells/ml were incubated with the mAb in suspension for 10–15 min at room temperature and then added to the coverslip in the microfluorimetry system as described above. Concentrations of mAb or ascites dilutions are indicated in figure legends.

For studies to determine the effect of mAbs only on morphology of the cells, a more simplified procedure was adopted. Cells were incubated with the mAb for 10–15 min at room temperature. Incubations with mAb performed at 0°C yielded similar results. Evaluation of the population morphology was performed by depositing 10^5 cells/100 μl on glass slides containing three-wells of 14 mm in diameter (Dynathec, Embrach, CH) for 10 min at 37°C. Nonadherent cells were then removed by washing the lamella with Ca^{2+} buffer and round or spread cells were counted in 10 microscopic fields (40×) in the continuous presence of various mAbs.

Results

Cell Surface Interactions and Changes in $[Ca^{2+}]_i$

A previous study demonstrated that interaction of a neutrophil with a polylysine-coated surface resulted in marked

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1. *Abbreviations used in this paper*: KLH, keyhole limpet hemocyanin; SF, shape factor.
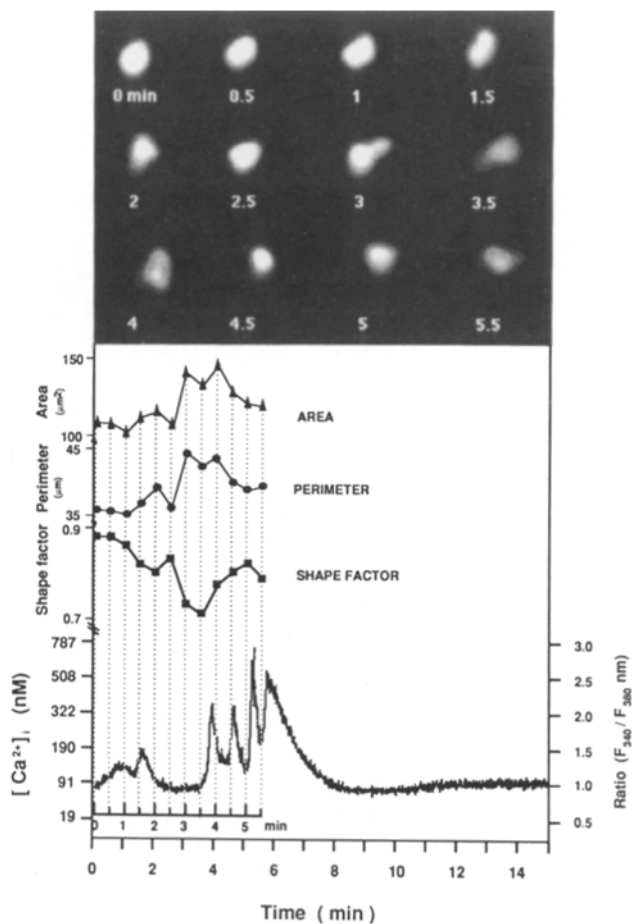
Figure 1. Relation between changes in $[Ca^{2+}]_i$ and morphological parameters in the first minutes of adherence, random movement and/or spreading of a single neutrophil deposited on a coverslip in a Ca^{2+} buffer containing albumin. $[Ca^{2+}]_i$ was monitored by a dual excitation spectrofluorimeter equipment; morphologic parameters (area and perimeter) were measured with the MELANIE image analysis program. The dotted lines are plotted to better indicate the points in the $[Ca^{2+}]_i$ trace in which images are acquired for the calculation of the morphological parameters. See Materials and Methods for details of SF calculation.

changes in $[Ca^{2+}]_i$. The neutrophils proceeded rapidly to spreading which was complete within 2 min, and during this period a marked rise in $[Ca^{2+}]_i$ occurred (13). We found using noncoated glass (in the presence of 0.1% human albumin) that only a small fraction of neutrophils showed such a rapid progression into spreading, whereas the majority stayed round for several minutes. In the present study we have chosen to observe the latter type of behavior, since the slower progression permitted a correct acquisition of the fluorescent images required for the morphological analysis. Visual observation on the rapid spreading population were made by continuously monitoring the cell during landing. As with the slowly spreading cells (see below), and in agreement with an earlier report (13), a marked rise in $[Ca^{2+}]_i$ concomitant with the spreading was observed (data not shown).

Correlation between $[Ca^{2+}]_i$ Transients and Changes in Cell Morphology

Fura 2-loaded neutrophils were observed in the absence of

chemotactic or phagocytic stimuli with a dual excitation microfluorimetry equipment coupled to an image acquisition system. This equipment allowed acquisition of fluorescence images in digital form and simultaneously measurement of $[Ca^{2+}]_i$ changes averaged over a single fura 2-loaded neutrophil. From the images, morphological parameters such as area and perimeter were derived by numerical image processing.

An example of the data obtained in such experiments is given in Fig. 1. All cells observed were round at the start of the recording period. Area, perimeter, and the calculated SF were obtained from images taken at 0.5-min intervals (*dotted lines*) and shown in the top panel. During the first 2 min the observed neutrophil showed a modest elongation resulting in a slight decrease in the SF that was concomitant with two small $[Ca^{2+}]_i$ peaks. The cell became slightly rounder (time 2.5 min) and thereafter it polarized extending a pseudopod (3 min). This second change in morphology caused a very marked drop in the SF. This alteration in SF clearly preceded a series of $[Ca^{2+}]_i$ elevations reaching peaks of 200-300 nM. During the period of the $[Ca^{2+}]_i$ transients (>4 min) the cell spread; finally $[Ca^{2+}]_i$ returned to a stable baseline.

To define precisely the temporal relation between the onset of $[Ca^{2+}]_i$ transients and shape changes, we studied a large number of neutrophils. Cells were classified according to the temporal relationship between the first shape change and the first $[Ca^{2+}]_i$ transient detected upon initiation of monitoring these two parameters in round-attached cells. The cell in Fig. 1 shows a first shape change concomitant with the onset of a rise in $[Ca^{2+}]_i$ and was classified accordingly, although later in the recording a marked further decrease in SF precedes the onset of a series of subsequent $[Ca^{2+}]_i$ transients. As illustrated on Table I the majority of the cells (18 out of 25) show visible shape changes that start before (9 out of 25) or at the same time (9 out of 25) as the onset of the $[Ca^{2+}]_i$ transients. Most of the round cells that did not move during the monitoring period and nonmoving cells did not change their $[Ca^{2+}]_i$.

Representative examples of cells from the three major groups described in Table I are shown in Fig. 2, *A-C.* Fig. 2 *A* illustrates a round cell with no changes in $[Ca^{2+}]_i$ (SF ~ 0.93). In at least 70% of the cell population the onset of $[Ca^{2+}]_i$ activity either was preceded by or was simultaneous with changes in morphology. Examples of these two types of relations are shown in Fig. 2, *B* and *C.* In Fig. 2 *B* a neutrophil extended a pseudopod during the first minute resulting in a marked lowering of SF. No changes in $[Ca^{2+}]_i$ were

Table I. Temporal Relationship between Onset of $[Ca^{2+}]_i$ Transients and Changes in Cell Morphology of Single Attached Neutrophils

| | $[Ca^{2+}]_i$ transients | | Onset of $[Ca^{2+}]_i$ transients | |
	No	Yes	Coincident with ΔSF	After ΔSF
Adherent and spreading	1	18	9	9
Round and attached	5	1	NA	NA

Neutrophils attached to uncoated glass were subjected to morphometric combined with $[Ca^{2+}]_i$ activity analysis ($n = 25$) as shown in Fig. 1 and described in Materials and Methods. Cells showing changes in shape were classified as adherent and spreading; the temporal relationship between the onset of $[Ca^{2+}]_i$ transients and the initiation of adherence (seen as a marked change in shape factor (ΔSF)) is given for the first ΔSF observed. NA = nonapplicable.
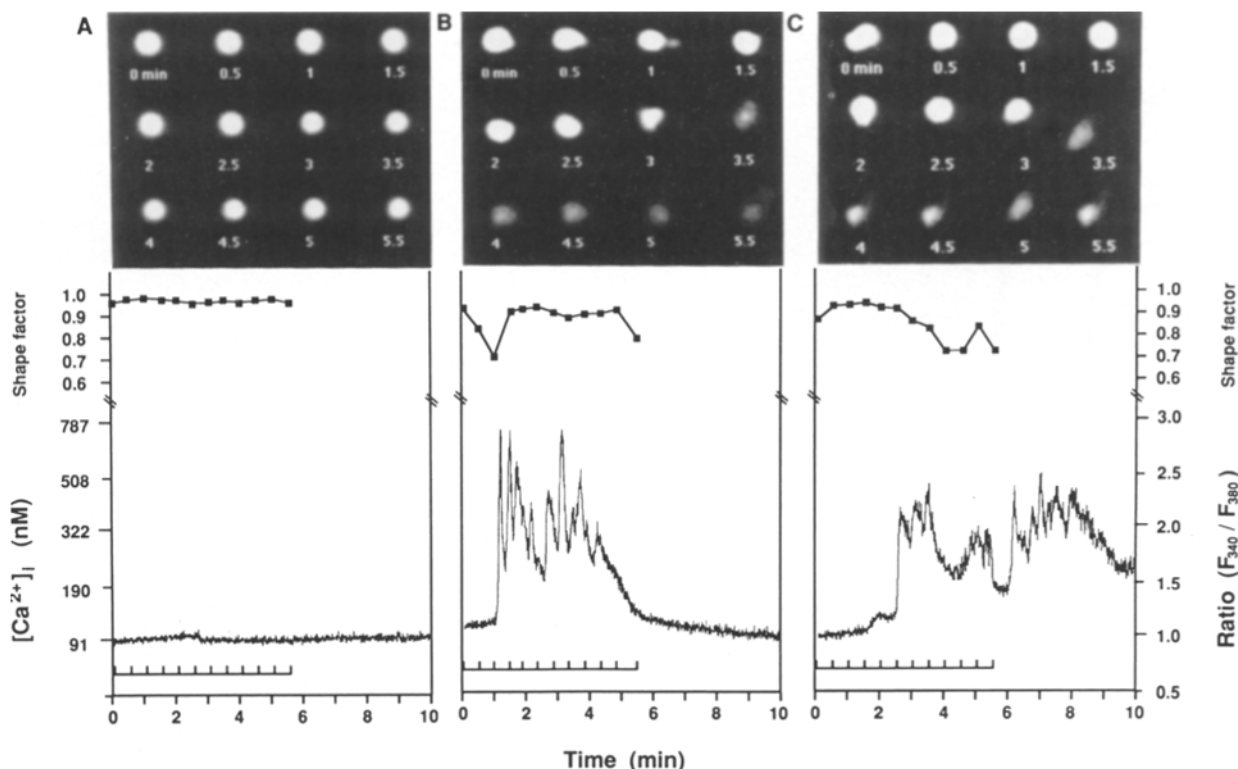
Figure 2. Representative examples of the correlation between $[Ca^{2+}]_i$ transients occurring in single attached neutrophils during adherence, random movement and/or spreading. Morphology changes are expressed by the SF as described in Materials and Methods. (*A*) Round cell with no $[Ca^{2+}]_i$ changes; (*B*) cell with changes in SF preceding the onset of the $[Ca^{2+}]_i$ transients; and (*C*) cell with changes in SF starting in parallel to $[Ca^{2+}]_i$ transients. The ruler indicates the acquisition time of the fluorescent images of the cell (every 30 s) which are pasted and printed in the black panel.

recorded in this period, but a series of $[Ca^{2+}]_i$ transients were detected in the following 5 min, after polarization was completed. In this and many other examples (see Fig. 7 and Fig. 3) a major shape change was possible at stable baseline $[Ca^{2+}]_i$. After the polarization, the cell rounded up (1.5–2 min) and started to spread (3–5.5 min). The multiple $[Ca^{2+}]_i$ elevations are associated with the onset of the spreading. It appears that $[Ca^{2+}]_i$ returns to a stable baseline before the spreading is complete.

Within the limits of the time resolution (30 s) the onset of the changes in the SF coincided with the onset of the $[Ca^{2+}]_i$ activity in many cells. An example of this behavior is shown in Fig. 2 *C*. It shows a progressive decrease in the SF due to spreading of the cell (after 2 min) which is concomitant with the appearance of a series of $[Ca^{2+}]_i$ peaks. In sum, these studies fail to show that a rise in $[Ca^{2+}]_i$ precedes cellular movement thus suggesting that $[Ca^{2+}]_i$ transients do not trigger the motile events of spreading. Instead, our results demonstrate that shape changes precede or are concomitant with the onset of $[Ca^{2+}]_i$ transients.

Influence of Extracellular Ca²⁺

To study the effect of omission of extracellular Ca^{2+} on cell morphology, cells presenting repetitive Ca^{2+} transients were rapidly superfused with a Ca^{2+}-free buffer containing 1 mM EGTA, while images of the cell were continuously recorded as indicated in the example of Fig. 3. As we have previously shown (11), removal of extracellular Ca^{2+} progressively

abolished $[Ca^{2+}]_i$ transients in a time-dependent manner: in this example three $[Ca^{2+}]_i$ peaks occur after removal of extracellular Ca^{2+} and $[Ca^{2+}]_i$ stabilizes at a lower steady-state value. However, this does not impair the ability of the cells to adhere, to polarize (image time 9.8), nor to spread over the following few minutes (see images between 12.3 and 13.8 min). The latter observation confirms the studies of Zigmond et al. (42) who observed no alteration in polarization or chemotaxis in Ca^{2+}-depleted cells in Ca^{2+}-free medium. It is also consistent with the fact that although removal of extracellular Ca^{2+} decreases CR3 function, the Mg^{2+} in our buffer (1 mM) allows significant CR3 functional activity (40).

Effect of Prevention of Adherence on [Ca²⁺]ᵢ Activity

Effect of Cytochalasin B. Useful means to block adherence is to pretreat cells with cytochalasin B. This drug both prevents microfilament assembly (5, 27, 32) and blocks the activity of CR3 (Detmers, P. A., E. Olsen, and Z. A. Cohn. 1988. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:A1450–6703). Pretreatment of the cells with cytochalasin B (5 μg/ml) for 5 min in suspension did not impair cell attachment to the glass surface as indicated in Fig. 4 *A*. However, both cellular spreading and repetitive $[Ca^{2+}]_i$ transients are significantly prevented in cytochalasin B-treated cells ($n = 25$ cells, performed in two different experiments). Most of the attached and treated cells are unable to spread. It should be noted that in ~50% of the round cytochalasin B-treated cells, isolated
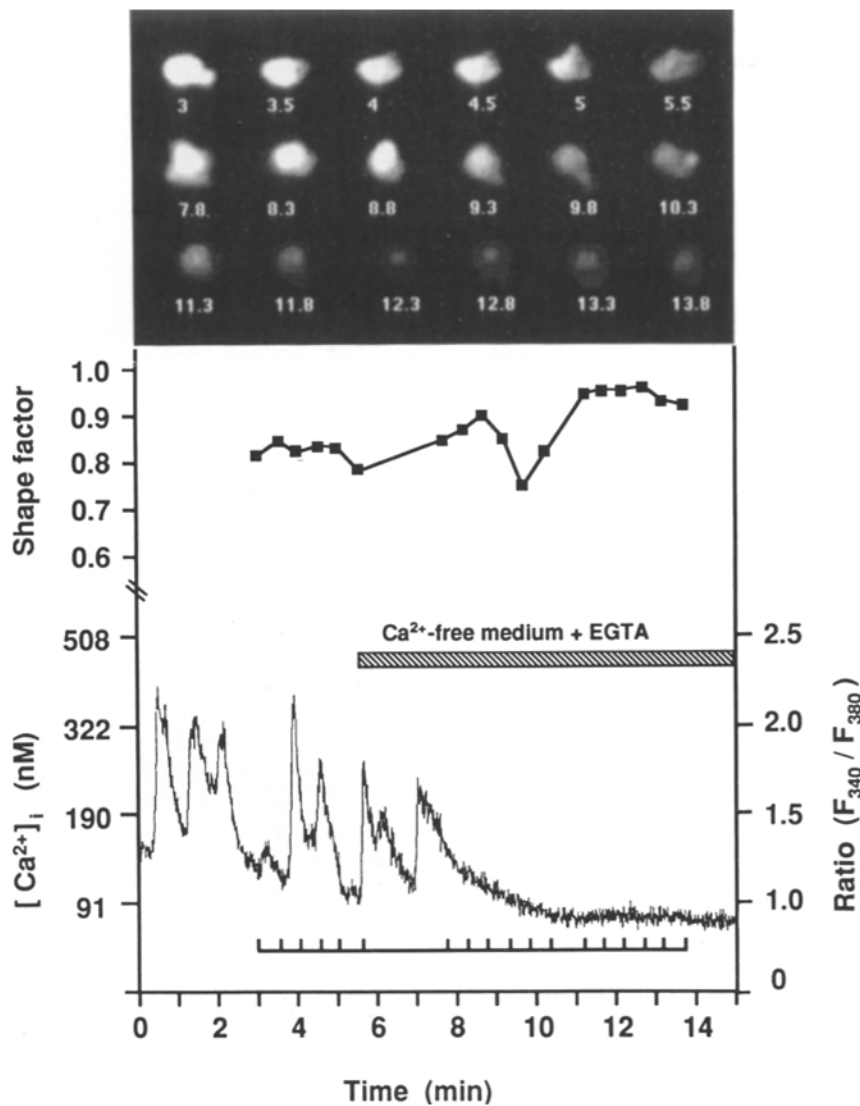
Figure 3. Effect of removal of extracellular Ca^{2+} on $[Ca^{2+}]_i$ transients and morphological changes in a single neutrophil during adherence, random movement, and/or spreading. Extracellular Ca^{2+} was removed by changing the medium both by addition into the chamber of 0.5 ml of Ca^{2+}-free medium containing 2 mM EGTA and by a perfusion with Ca^{2+}-free medium containing 1 mM EGTA. Note that since the cell moved out of the field of optimal illumination it was recentered at time 5.5 min, leading to an increase in overall fluorescence.

$[Ca^{2+}]_i$ peaks of reduced amplitude could still be detected (Fig. 4 *B*).

Cytochalasin B treatment did not alter the $[Ca^{2+}]_i$ responses of attached neutrophils to fMLP: 8 cells out of 13 respond to fMLP 10^{-8} M and 8 cells out of 10 respond to fMLP 10^{-6} M (see one example in Fig. 4 *C*). This is consistent with results obtained previously on neutrophils in suspension (30).

Effect of mAbs Directed against the CR3 Receptor. We postulated that functioning adherence receptors trigger $[Ca^{2+}]_i$ transients. To test this hypothesis, we employed mAb 44a against the α chain and IB4 against the β chain of the integrin CR3, the major adhesion-promoting receptor of neutrophils. In agreement with published reports (6, 38, 39), preincubation of cells in either of these antibodies prevented spreading and locomotion (see Fig. 7). Specificity of the inhibition is indicated by the observation that irrelevant murine IgG2a (Fig. 5), IgG1, or IgG2b (not shown) caused no inhibition of spreading. More importantly, mAb OKM1, directed against an epitope on the α chain of CR3 which is not involved in adhesion, caused no blockade of spreading or locomotion (Fig. 5).

Blockade of CR3 prevented not only spreading but also prevented the generation of $[Ca^{2+}]_i$ transients (Fig. 6 and Fig. 7). In the presence of 44a, most cells remained round and only 1 of 13 round cells showed $[Ca^{2+}]_i$ transients. In the control preparation the majority of cells showed both shape changes and $[Ca^{2+}]_i$ transients (14/20). A similar effect on the reduction of $[Ca^{2+}]_i$ transients was also observed in cells treated with IB4 while cells treated with control antibody OKM1 showed normal responses. In IB4-treated cells, 13/14 cells remained round and three of those showed only one $[Ca^{2+}]_i$ transient; in OKM1-treated cells, 10/12 cells spread concomitant with multiple $[Ca^{2+}]_i$ transients. An example of the correlation between $[Ca^{2+}]_i$ and morphology in anti-CR3-treated cells is shown in Fig. 7. The cell apparently attempted to spread, but in spite of multiple, tentative extensions of pseudopods it remained round. Under these conditions there was no significant $[Ca^{2+}]_i$ activity.

The effect of anti-CR3 was a selective suppression of $[Ca^{2+}]_i$ transients associated with adherence. Anti-CR3 did not alter $[Ca^{2+}]_i$ responses induced by fMLP. Fig. 6 *B* shows the $[Ca^{2+}]_i$ response of one anti-CR3-treated cell stimulated with 10^{-8} and 10^{-6} M of fMLP. $[Ca^{2+}]_i$ transients were ob-

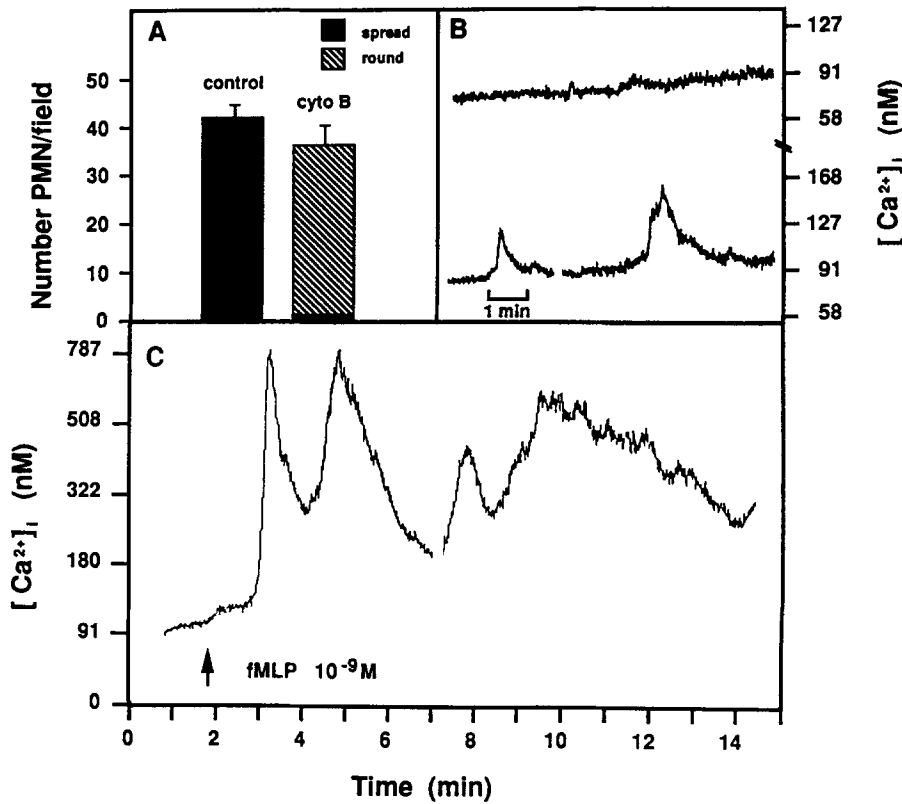*Figure 4.* Effect of cytochalasin B treatment (5 μg/ml) on (A) spreading and (B) spontaneous [Ca^{2+}]$_i$ activity in single neutrophils. Representative [Ca^{2+}]$_i$ traces of two round cells ($n = 25$ cells) treated by cytochalasin B are shown in this panel. (C) [Ca^{2+}]$_i$ activity of one cytochalasin B-treated cell in response to 10^{-9} M fMLP.

served but no spreading occurred. In eight cells treated with anti-CR3 and stimulated with 10^{-8} M fMLP, the maximal amplitude (650 $\pm$ 95 nM) and duration (113 $\pm$ 32 s) of the [Ca^{2+}]$_i$ transients were statistically not different from those obtained in the absence of anti-CR3 (11) (amplitude of 431 $\pm$ 139 nM and duration of 123 $\pm$ 32 s, $p = 0.3$ by variance analysis). These results argue against a nonspecific inhibitory effect of anti-CR3 on [Ca^{2+}]$_i$ activity.
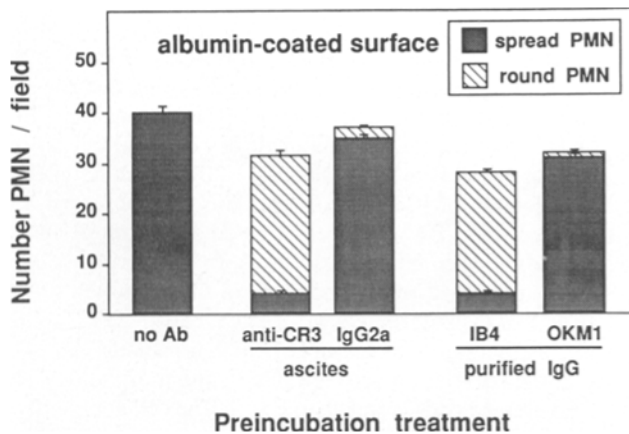

Figure 5. Effect of mAb directed against the α or β subunits of CR3 on cell morphology of attached neutrophils. Gray bars represent adherent and spread neutrophils, black bars represent round cells, observed by phase-contrast microscopy. Cells were preincubated in the presence of the indicated antibodies (ascites or purified IgG) and subsequently attached to a glass coverslip in the presence of albumin (see Materials and Methods). Anti-CR3 and IgG2a, dilution 1:1,000; IB4 and OKM1, 10 μg/ml.

The link between adherence receptor activation and [Ca^{2+}]$_i$ oscillations was further strengthened by experiments in which the adherence substrate was modified or the kinetics of adherence was enhanced. Of the various coating procedures that may prevent spreading (30), keyhole limpet hemocyanin (KLH) was found most effective. As is shown in Fig. 8, coating of glass coverslips with KLH solutions (0.5 mg/ml or higher) prevented attachment of 90% of the neutrophils (Fig. 8 A). Those cells that attached stayed round and maintained stable baseline [Ca^{2+}]$_i$ (Fig. 8 B). We also searched for means of speeding the kinetics of adherence and for this purpose we used phorbol esters (which increase adherence but do not cause [Ca^{2+}]$_i$ transients [14, 23]). In over 16 cells treated with PMA (50 nM for 10 min in suspension), 14 spread within 1 min and concomitantly presented [Ca^{2+}]$_i$ transients of similar amplitude and duration than control cells during spreading.

Discussion

Previous studies have shown transient increases in [Ca^{2+}]$_i$ associated with cell spreading (13) or locomotion (11, 19, 20). To determine if such [Ca^{2+}]$_i$ transients are necessary for spreading, we made simultaneous measurements of [Ca^{2+}]$_i$ and cell shape. We observed that in nearly one half of the cells, spreading preceded the first [Ca^{2+}]$_i$ transient. Thus, [Ca^{2+}]$_i$ transients are clearly not a prerequisite for movement of pseudopods across a substrate. This observation is consistent with the studies of Zigmond et al. (42) who showed normal rates of chemotaxis in Ca^{2+}-free buffers, a condition that rapidly blocks with time the generation of [Ca^{2+}]$_i$ transients.
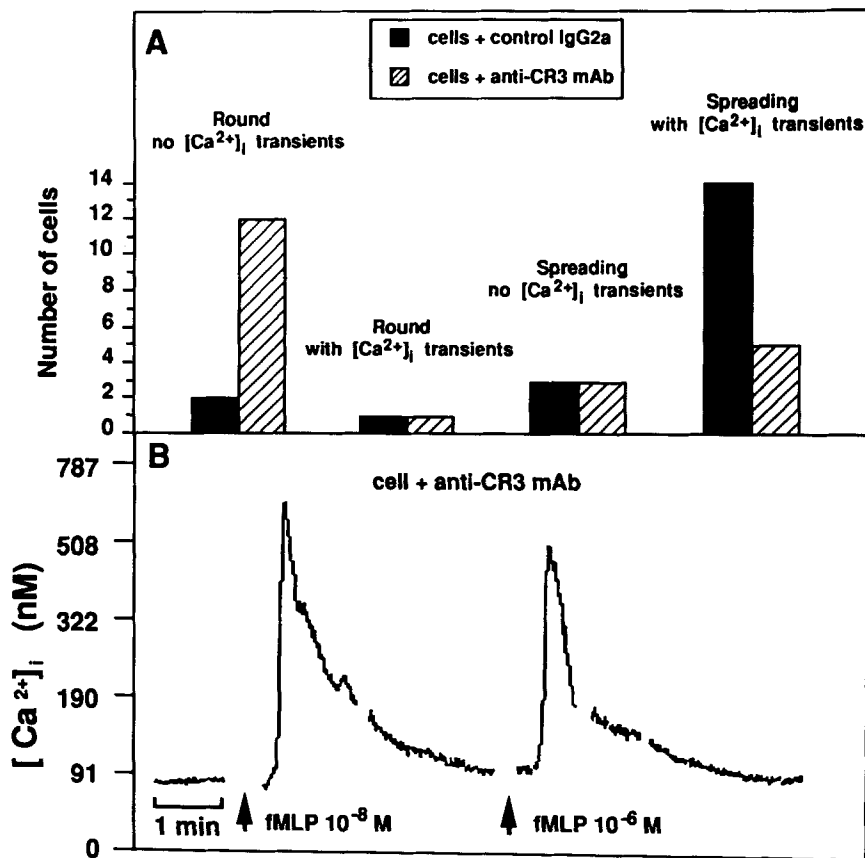
Figure 6. (A) Single cell analysis of the relationship between $[Ca^{2+}]_i$ activity and cell morphology in the presence of ascites-containing anti-CR3 or control mAb. Fura 2-loaded neutrophils were preincubated in the presence of anti-CR3 or control mAb IgG2a (dilution 1:1,000) for 10 min at room temperature in suspension and subsequently attached to a coverslip. $[Ca^{2+}]_i$ was monitored in the continuous presence of control or ascites-containing anti-CR3 mAb as described in Materials and Methods. Monitoring was interrupted at regular intervals (30 s) to observe the morphology (round or adherent and spreading) of neutrophils by phase contrast. $[Ca^{2+}]_i$ activity was defined as the presence of at least two $[Ca^{2+}]_i$ transients during a period of 5 min monitoring. The effects on anti-CR3 mAb are statistically significant as evaluated by a X^2 test ($p<0.001$). (B) $[Ca^{2+}]_i$ response of one round and silent anti-CR3-treated neutrophil to fMLP.

An alternative hypothesis is that events associated with motility cause the $[Ca^{2+}]_i$ transients. To test this hypothesis, we studied agents that block motility. Cytochalasin B blocks both the polymerization of actin filaments and the activation of the adhesion receptor, CR3 (Detmers, P. A., E. Olsen, and Z. A. Cohn. 1988. FASEB (Fed. Am. Soc. Exp. Biol.) J. 2:A1450–6703), without impairing attachment to the substrate. This agent caused complete cessation of spreading and of $[Ca^{2+}]_i$ transients, suggesting that movement may initiate $[Ca^{2+}]_i$ transients.

To further define the relation between spreading and $[Ca^{2+}]_i$ transients, we employed mAbs that specifically block one component of the motile apparatus. Antibodies against the integrin CR3 block spreading and locomotion by preventing the adherence of pseudopods to the substrate, but do not block polarization, degranulation, or actin polymerization in response to chemoattractants (1, 40). Blockade of CR3 prevented the generation of $[Ca^{2+}]_i$ transients (Fig. 6 and Fig. 7). This observation suggests that adhesion per se, and not the other cytoskeletal events associated with movement, is necessary for $[Ca^{2+}]_i$ transients. Consistent with this hypothesis is the observation that cells with blocked CR3 (Fig. 7) or cells in the continuous absence of extracellular Ca^{2+} may show considerable formation and movement of pseudopods with a stable $[Ca^{2+}]_i$ baseline. Furthermore, we observe no consistent pattern linking ongoing changes in shape and individual $[Ca^{2+}]_i$ transients.

A link between integrins and $[Ca^{2+}]_i$ has been suggested by other studies. Crosslinking of the integrin LFA-1 ($\alpha_l\beta_2$) on lymphocytes did not by itself cause measurable changes in $[Ca^{2+}]_i$, but cells with crosslinked LFA-1 showed exaggerated $[Ca^{2+}]_i$ responses to other agonists (34).

The precise role of $[Ca^{2+}]_i$ transients remains to be determined. Southwick et al. (31) have shown that while spreading occurs in the absence of extracellular Ca^{2+}, adherence-associated actin polymerization in the lamellipodia requires the presence of this cation in the extracellular medium. Marks and Maxfield (19) have suggested that transients may play a role in detachment of cells from the substrate, a suggestion consistent with our data. We have showed that $[Ca^{2+}]_i$ transients are involved in the control of exocytosis (15, 16) and that $[Ca^{2+}]_i$ transients are also necessary for phagosome-lysosome fusion (12). Ca^{2+}-proteins such as gelsolin may mediate fusion of granules with the phagosome or the plasma membrane (41). It is thus reasonable to speculate that the $[Ca^{2+}]_i$ transients caused by functioning adhesion receptors might lead to secretion of proteases. Proteolysis of receptors and/or extracellular matrix proteins might be important in detachment of cells and thus in the control of diapedesis and movement into tissues. An additional possibility is that $[Ca^{2+}]_i$ transients serve in the phenomenon of adhesion-dependent priming. While neutrophils in suspension show no oxidative response to TNFα, adhesion of neutrophils to substrates via leukocyte integrins enables the cells to mount a massive oxidative burst in response to TNFα (22). The intracellular signals that mediate this type of priming are unknown, and our results and data of Richter et al. (25) suggest that $[Ca^{2+}]_i$ transients may be a candidate signal.

Integrins are assumed to transmit intracellular signals since ligation of these receptors causes motility, phagocyto-
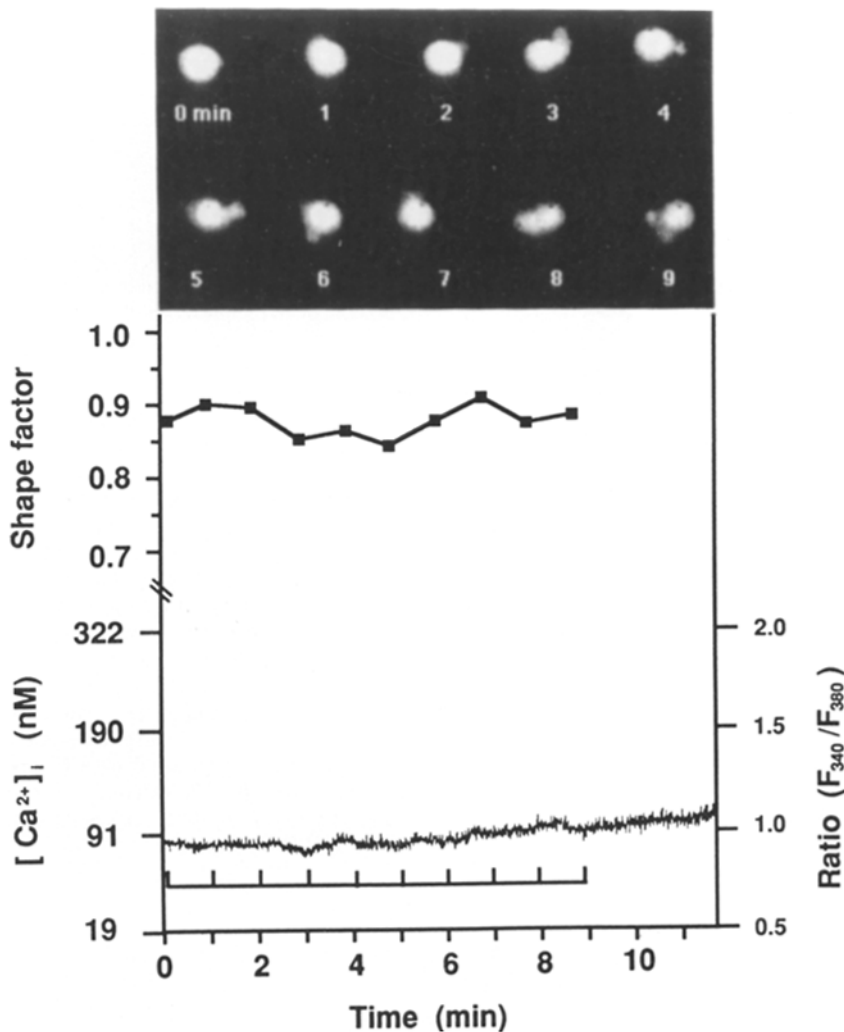
Figure 7. Effect of mAb anti-CR3 on [Ca²⁺]ᵢ and cell morphology in a single neutrophil attached to a coverslip in the continuous presence of ascites-containing anti-CR3 mAb (dilution 1:1,000).
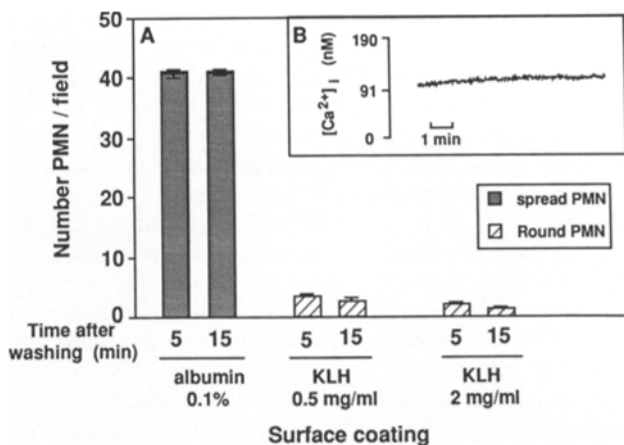

Figure 8. Effect of KLH- (keyhole limpet hemocyanin) surface on neutrophil morphology (*A*) and [Ca²⁺]ᵢ activity (*B*). Glass surfaces were coated with two concentrations of KLH: 0.5 to 2 mg/ml for 30 min at 37°C. Cell morphology was evaluated after 10 min of attachment time. (*gray bars*) Adherent and spread neutrophils, (*black bars*) round cells, observed by phase-contrast microscopy. B shows the [Ca²⁺]ᵢ activity observed in 17 out of 18 cells monitored on KLH-coated surface.

sis, priming, and altered paths or differentiation of embryonic cells (36). The nature of intracellular signal(s) triggered by receptors of the integrin family, however, has been obscure. While our observations suggest a role for integrins in control of [Ca²⁺]ᵢ, the precise mechanism of this control (whether it results directly from activation of the integrins or at different steps) and its generalization to other cellular systems remains to be determined.

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