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Localised and global cytosolic Ca^{2+} changes in neutrophils during engagement of Cd11b/CD18 integrin visualised using confocal laser scanning reconstruction

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SUMMARY

A confocal laser scanning technique was used to provide optical sections in the vertical plane through living neutrophils during engagement of integrin and shape change. This has permitted the visualisation of cytosolic free Ca^{2+} rises localised to the sites of integrin immobilisation. These localised Ca^{2+} changes resulted from the release of Ca^{2+} from intracellular stores, and were not inhibited by removal of extracellular Ca^{2+} or by the Ca^{2+} channel blocking nickel ions. After integrin engagement and initial cell shape changes, a second rapid phase of cell spreading occurred, which was accompanied by global Ca^{2+} signalling bursts that continued sporadically after maximum spreading. This global signalling was driven mainly by Ca^{2+} influx from the extracellular medium. We propose that the localised and global Ca^{2+} signalling triggered by integrin engagement results from a common underlying mechanism and these signals are important for neutrophil shape change and extravasation.

Key words: Confocal imaging, Cytosolic Ca^{2+}, Integrin, Neutrophil, Signalling

INTRODUCTION

Adhesion molecules on neutrophils are important for the process of extravasation to occur during the inflammatory response (Springer, 1990; Hynes, 1992). Although selectins are involved in slowing neutrophils from the circulation and inducing 'rolling' along the endothelial cell layer (Bevilacqua, 1993), it is only after integrin engagement at sites of ICAM-1 expression that neutrophils are signalled to change shape, become motile and extravasate (Springer, 1990; Hynes, 1992). Ca^{2+} signalling is triggered by cross-linking antibodies bound to integrin CD11b/CD18 on the neutrophil surface (Richter et al., 1990; Ng-Sikorski et al., 1991; Petersen et al., 1993) and results from localised increase in Ca^{2+} at the periphery of the cell (Petersen et al., 1993). The major problem with the interpretation in previous studies has been the inability to determine the time and position at which integrin engagement occurred. Furthermore, it has not been possible to correlate integrin engagement induced Ca^{2+} signalling with cell shape change and adherence.

Here, we have used a confocal laser scanning microscope to reconstruct a vertical (*z* plane) view through fluo3-loaded neutrophils. This provides an optical slice in which the contact points between the cell and the underlying substrate can be clearly defined and their relationship to cytosolic free Ca^{2+} concentration changes determined. We show here that localised Ca^{2+} signalling occurs at specific contact sites, where integrin engagement occurred.

MATERIALS AND METHODS

Neutrophil preparation

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously (Hallett et al., 1990). Following dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia) and hypotonic lysis of red cells, neutrophils were washed and resuspended in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM Hepes and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH).

Integrin binding surface

Antibodies (Dako, High Wycombe, UK) to the integrin subunits α (CD11b) or β (CD18) were adhered to glass surfaces in sufficient density to ensure that integrin engagement on neutrophils occurred (Pettit and Hallett, 1994). The amount of adherent antibody, quantified using an ECL second antibody technique, was estimated at approx. 3×10^4 molecules/μm^2. This was sufficient to cause cross-linking and oxidase activation (Pettit and Hallett, 1994).

Confocal imaging and measurement

Neutrophils were loaded with fluo3 by incubation with acetoxymethyl ester (Molecular Probes, Oregon) as previously described (Minta et al., 1989), to give an estimated cytosolic concentration of 100 μM, resulting in an increase in Ca^{2+} buffering capacity of the cytosol of approximately 5-10% (Al-Mohanna and Hallett, 1988). Confocal *z*-plane images, which permitted the timing of neutrophil 'touch-down' onto the integrin engaging surface to be determined were obtained using a CLSM-Fluovert confocal laser scanning microscope (Leica,

Heidelberg, Germany), by stepping the microscope stage through the confocal laser line scanning in the *x* direction to produce transverse images at a total rate of 0.08/second (i.e. the interval between successive images was 12.5 seconds). The time to acquire a single image was 4 seconds. In order to prevent photobleaching of fluo3 during the experiment, the laser power was attenuated. The loss of signal was offset by increasing the voltage of the photomultiplier detectors with a subsequent increase in the noise on the images. This is apparent in unprocessed images (see Fig. 1), but was reduced by a nearest neighbour smoothing algorithm. A similar procedure was used to acquire images of integrin distribution in neutrophils, pre-coated with fluorescein-conjugated antibody to the β-subunit (CD18, Dako High Wycombe, UK) before sedimentation on to anti-α-subunit coated glass. The *z*-plane imaging rate was sufficiently fast to capture events during 'touch-down' of fluo3-loaded and integrin labelled neutrophils onto surfaces coated with anti-CD11b antibody.

Faster time resolution Ca^{2+} measurements were made by positioning the laser scanning line in the *x* direction approximately 1 μm above the antibody-coated surface, parallel to the plane of the surface. Neutrophils were then permitted to sediment onto the surface. Cells which fell into the scanning line were consequently imaged with *x* direction spatial information and with a temporal resolution of 12.5 milliseconds. By angling the antibody-coated surface relative to the laser scanning direction, rapid scanning, with the same resolution was also acheived through a plane which sectioned both the cell and its underlying substrate.

All measurements were performed with cells maintained at 37°C, using a specially constructed heating chamber. The cytosolic free Ca^{2+} concentration was calculated using the standard equation with a K_d for fluo3 of 864 nM (Merritt et al., 1991).

RESULTS

Localised Ca^{2+} signalling during integrin engagement

As neutrophils contact the anti-CD11b-coated surface, they rapidly deform and spread out (Fig. 1a). The cytosolic free Ca^{2+} concentration closest to the deformed regions of membrane, at the integrin-engaging contact points, was transiently elevated to about 1 μM (Fig. 1a). As cell spreading continued, and new contact points were formed, further localised and transient elevations in cytosolic free Ca^{2+} concentration were triggered (Fig. 1a). Blocking the neutrophil surface integrins by pre-treatment with anti-integrin antibody prevented both cell shape change and Ca^{2+} signalling (Fig. 1b), demonstrating their dependency on integrin engagement. At a higher time resolution, by fast laser scanning in a single line approximately 1 μm above the antibody-coated surface and parallel to the plane of the surface, cells which fell into the scanning line were imaged with *x* direction spatial information with a temporal resolution of 12.5 milliseconds (Pettit and Hallett, 1995). This technique demonstrated that the localised Ca^{2+} transients had an up-phase of about 3 seconds and recovery of about 4-6 seconds (Fig. 2c). The elevated Ca^{2+} was localised to approximately 4-5 μm^3 of cytosol over this time scale.

Integrin clustering at the contact surface was visualised by fluorescently staining the β chain (CD18) on neutrophils before contacting an anti-α-chain (CD11b). This revealed the appearance of asymmetrical patterns at the contact surface (Fig. 2a,b) with a similar time course as initial Ca^{2+} signalling. This was consistent with the hypothesis that localised Ca^{2+} signalling resulted from localised integrin cross-linking.

The localised Ca^{2+} signalling at the contact site continued in cells in which shape change was prevented by cytochalasin B (Fig. 3a). This demonstrated that Ca^{2+} signalling was not a consequence of cell shape change. Buffering the localised Ca^{2+} changes with intracellular BAPTA (concentration estimated to be approx. 5 mM) prevented both firm adherence and the subsequent cell shape change (Fig. 3b). These results were, therefore, consistent with the localised Ca^{2+} signalling resulting from integrin engagement at the surface and being responsible for mediating an early stage of neutrophil adherence.

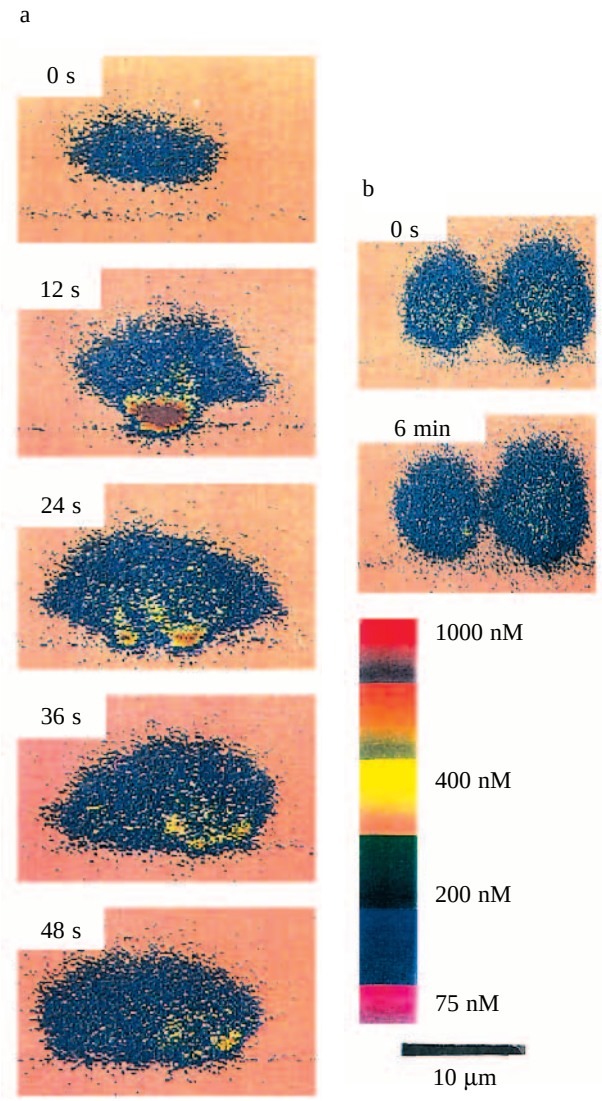

Fig. 1. Transverse confocal images of cytosolic free Ca^{2+} in neutrophils during integrin engagement. Images show the cytosolic free Ca^{2+} concentration, pseudo-coloured according to the colour scale, in transverse sections through a neutrophil contacting an anti-CD11b antibody coated surface: (a) an untreated neutrophil (0-48 seconds); (b) a neutrophil pre-coated with anti-CD11b to block cell surface integrin and prevent CD11b engagement (0 and 6 minutes). Both sets of images are 'raw' data, showing the photon noise and light scatter from the contacting surface. The photon noise originated from the low laser power and high voltage on the photomultipier detectors. In the first image there was distortion during sedimentation as a result of the movement of the cell relative to the scanning line. The data shown are typical of similar experiments using either anti-CD11b or anti-CD18 coated glass (*n*=52).

Localised Ca^{2+} signalling as a result of store release

The localised Ca^{2+} signalling spikes were observed both in the absence of extracellular Ca^{2+} (Fig. 4a) and in the presence of the transmembrane blocking ion Ni^{2+} (Fig. 4b). These data demonstrated that localised Ca^{2+} signalling was the release from Ca^{2+} stores and that there was no requirement for transmembrane influx of extracellular Ca^{2+}. Furthermore, no quenching of fluo3 occurred in the presence of extracellular Mn^{2+} (Fig. 4c), suggesting there was no contribution from local channel opening.

Global Ca^{2+} bursts and cell spreading

After contact with the surface, a second rapid stage of cell shape change was seen as contact with the surface became more intimate (Fig. 5a). In approximately 60% of cells (n=200), this was preceded by a burst of high Ca^{2+} throughout the whole cell (Fig. 5a). This probably represents an underestimate of the correlation between global Ca^{2+} signalling and rapid spreading, as the relatively long inter-image interval (12.5 seconds) would not have permitted detection in all cases. Fast time resolution single line scanning enabled more accurate determination of the relationship between the initial Ca^{2+} burst and spreading (Fig. 5b). Once firmly attached, bursts of elevated Ca^{2+} continued sporadically (Fig. 6a,b,c). The interval between these bursts was variable up to 140 seconds (104±30 seconds, n=15). There was no evidence that these bursts were the result of a soluble factor diffusing from neighbouring cells, as Ca^{2+} bursts in neighbouring cells occurred asynchronously (Fig. 6b), suggesting that there was no communication between adjacent cells.

The Ca^{2+} bursts were mediated by additional integrin engagement, as they were inhibited by blocking cellular integrin by adding anti-CD11b antibody to adherent neutrophils (Fig. 7d). Furthermore, localised but not global Ca^{2+} signalling was triggered in cytochalasin B-pretreated cells (Fig. 3a). However, after adherence, subsequent cytochalasin B treatment did not prevent global Ca^{2+} bursts. These data suggested that the generation of the Ca^{2+} burst were insensitive to cytochalasin B, but required a large contact area.

Global Ca^{2+} burst as a result of Ca^{2+} influx

Unlike the localised Ca^{2+} signal seen during initial contact, global Ca^{2+} burst responses were inhibited by lack of extracellular Ca^{2+} (Fig. 7b) and by Ni^{2+} (Fig. 7c), suggesting transmembrane Ca^{2+} influx. Furthermore, although a small elevation in cytosolic free Ca^{2+} concentration was observed in the presence of extracellular Mn^{2+}, quenching of fluo3 rapidly accompanied these elevations bursts.

Since the global Ca^{2+} signal was dependent on integrin binding and the site of integrin engagement was only at the contact surface, the possibility existed that Ca^{2+} influx occurred only through this contact surface. However, no evidence was found, by high time resolution (12.5 milliseconds) scanning, that Ca^{2+} near the contact surface, i.e. the site of integrin engagement, rose before Ca^{2+} at the membrane distant from the contact site (Fig. 6d). It was therefore concluded that the local engagement of integrin at the contact surface could signal Ca^{2+} channel opening at other sites.

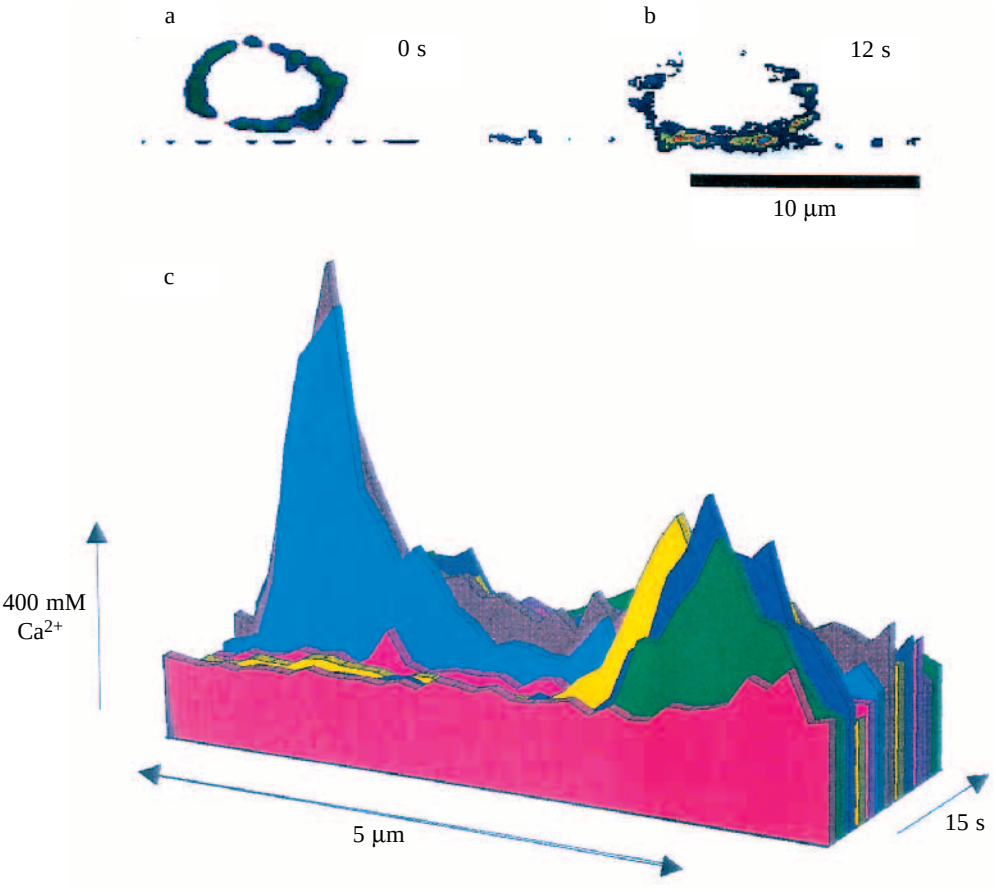

Fig. 2. Integrin distribution and the time course and spatial spread of two initial localised Ca^{2+} events. Images of fluorescein conjugated anti-CD18, as a marker of the distribution of cell surface integrin, are shown (a) before and (b) after contact with an anti-CD11b coated surface. (c) The 3-D graph shows the time course of two localised Ca^{2+} events immediately after 'touch-down' onto an anti-CD11b coated surface. The data were captured by rapid laser scanning at a plane 1 μm above the contacting surface. The x axis shows the cell dimension, the y axis time and the z axis, cytosolic free Ca^{2+} concentration.

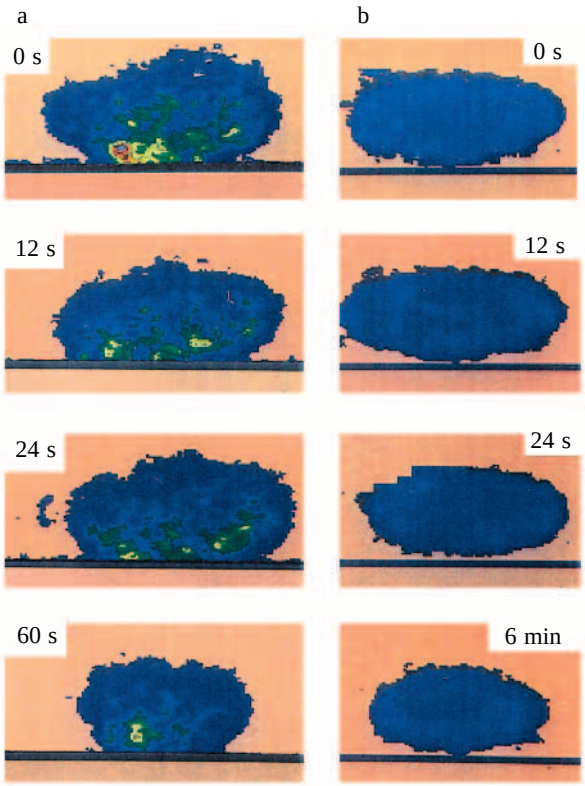

Fig. 3. Ca^{2+} signalling within neutrophils during integrin engagement. The cytosolic free Ca^{2+} concentration changes occurring during contact with anti-CD18 antibody coated surfaces within neutrophils (a) pretreated with cytochalasin B (5 μg/ml) to prevent cell spreading and (b) BAPTA (intracellular concentration 5 mM) to prevent cytosolic free Ca^{2+} signalling. The pseudo-colour scale converting colour to cytosolic free Ca^{2+} concentration is shown in Fig. 1. The examples shown are typical of replica experiments (n>10).

DISCUSSION

The data shown here demonstrate two aspects of integrin mediated Ca^{2+} signalling in neutrophils, namely localised Ca^{2+} signalling as a result of limited integrin engagement during the initial contact, and the subsequent global Ca^{2+} signalling after further integrin engagement. The global Ca^{2+} signalling demonstrated here may correspond to previously reported global rises in cytosolic free Ca^{2+} concentration and sudden change in shape accompanying 'down-touch' (Kruskal et al., 1986), and the sporadic global spiking of cytosolic free Ca^{2+} after integrin engagement (Jaconi et al., 1991), and which also occurs during chemotaxis, as the result of engagement of integrin molecules (Marks and Maxfield, 1990; Jaconi et al., 1988; Hendry and Maxfield, 1993). However, the initial localised Ca^{2+} signalling reported here (Fig. 1) has not previously been observed.

The localised Ca^{2+} signalling, as a result of limited integrin engagement, resulted from localised release of Ca^{2+} from intracellular stores. These Ca^{2+} stores were distinct from the single focal juxta-nuclear store released by the seven transmembrane domain receptors, such as f-met-leu-phe (Hallett et al., 1990; Davies et al., 1991). The stores which liberate Ca^{2+} in response to integrin immobilization may, however, be related to the Ca^{2+}-ATPase organelles that cluster at sites at the periphery of phagocytotic vesicles (Stendahl et al., 1994). It has been suggested that emptying intracellular Ca^{2+} stores signals Ca^{2+} channel opening in the plasma membrane and Ca^{2+} influx by releasing a diffusible calcium influx factor (CIF) from the store itself (Randriamampita and Tsein, 1993; Parekh et al., 1993; Davies and Hallett, 1995). Localised Ca^{2+} signalling, in the absence of a global Ca^{2+} rise, may thus result from limited integrin engagement generating insufficient CIF to trigger plasma membrane Ca^{2+} channel opening. However, on further cell-surface contact, additional integrin engagement, perhaps of newly up-regulated molecules after the initial touch down (Springer, 1990; Hynes, 1992) would result in an increased CIF release and trigger the global Ca^{2+} change observed. This would also provide an explanation for the requirement of increased integrin binding and the large contact area for global Ca^{2+} bursts (Figs 5, 6). Although the global cytosolic free Ca^{2+} increases were driven by influx of extracellular calcium (Fig. 7a,b,c), part of the requirement for Ca^{2+} influx may be related to maintaining the concentration of Ca^{2+} within the stores. However, the liberation of a diffusible factor (CIF) could also result in the opening of Ca^{2+} channels at sites remote from the integrin engagement sites. The differences in the density of integrin cross-linking may thus explain both localised and global integrin-mediated Ca^{2+} signalling by a single underlying mechanism.

This work also raises the question of the identity of the intracellular messenger responsible for releasing Ca^{2+} from the peripheral Ca^{2+} stores. With f-met-leu-phe, it is thought that IP_3 mediates Ca^{2+} release (Thelen et al., 1993). Signalling Ca^{2+} release from a remote site near the nucleus at the centre of the cell (Hallett et al., 1990; Davies et al., 1991) is theoretically feasible as IP_3 diffuses rapidly in the cytosol (Allbritton et al., 1992). However, as the peripheral Ca^{2+} store is not triggered by f-met-leu-phe, the messenger responsible for the integrin triggered release site at the cell periphery, may thus be different. Recently, it has been shown in cardiac cells, that the Ca^{2+} sparks are generated as the result of the increase in Ca^{2+} near the Ca^{2+} store (Lopez-Lopez et al., 1995; Cannell et al., 1995). This

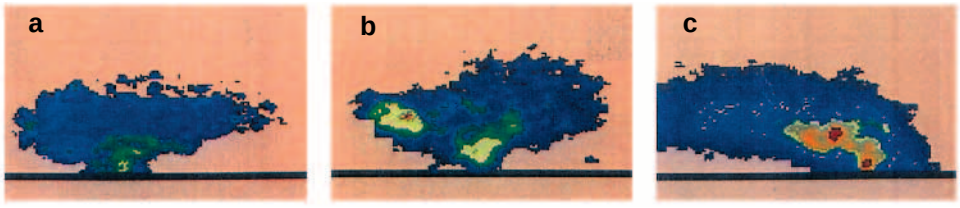

Fig. 4. Localised Ca^{2+} signalling during 'touch-down' of neutrophils onto anti-CD18 coated surfaces: (a) in the absence of extracellular Ca^{2+} (EGTA, 1 mM); (b) in the presence of the Ca^{2+} channel blocking ion nickel ions (Ni^{+}, 1 mM); and (c) in the presence of the fluo3 quenching ion Mn^{2+} (0.1 mM). The pseudo-colour scale converting colour to cytosolic free Ca^{2+} concentration is shown in Fig. 1. The examples shown are typical of replica experiments (EGTA, n=10; Ni, n=12; Mn, n=25).

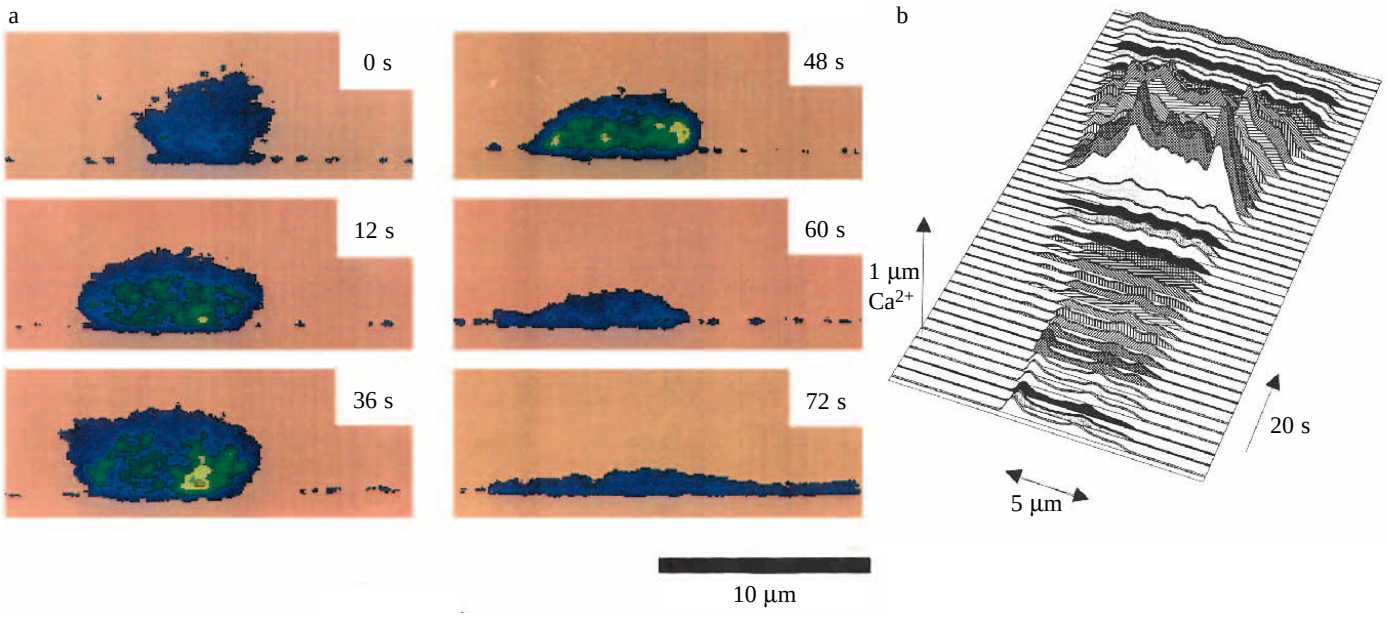

Fig. 5. A global Ca^{2+} rise after integrin engagement showing subsequent cell spreading (a) visualised by *z* sectioning, and (b) visualised by rapid laser scanning, shown as a 3-D plot where the horizontal axis is the dimension across the cell, the receding axis is time and the vertical axis is cytosolic free Ca^{2+} concentration. The pseudo-colour scale converting colour to cytosolic free Ca^{2+} concentration for the images in a is shown in Fig. 1. The examples shown are typical of replica experiments (*n*= 200).

would seem an unlikely mechanism in neutrophils, because no CICR can be demonstrated and agents which interact with CICR, such as caffeine and ryanodine have no effect of Ca^{2+} signalling. Furthermore, the Ca^{2+} spikes reported here persisted in the absence of transmembrane influx. The identity of the messenger for the release of Ca^{2+} from peripherally located Ca^{2+}

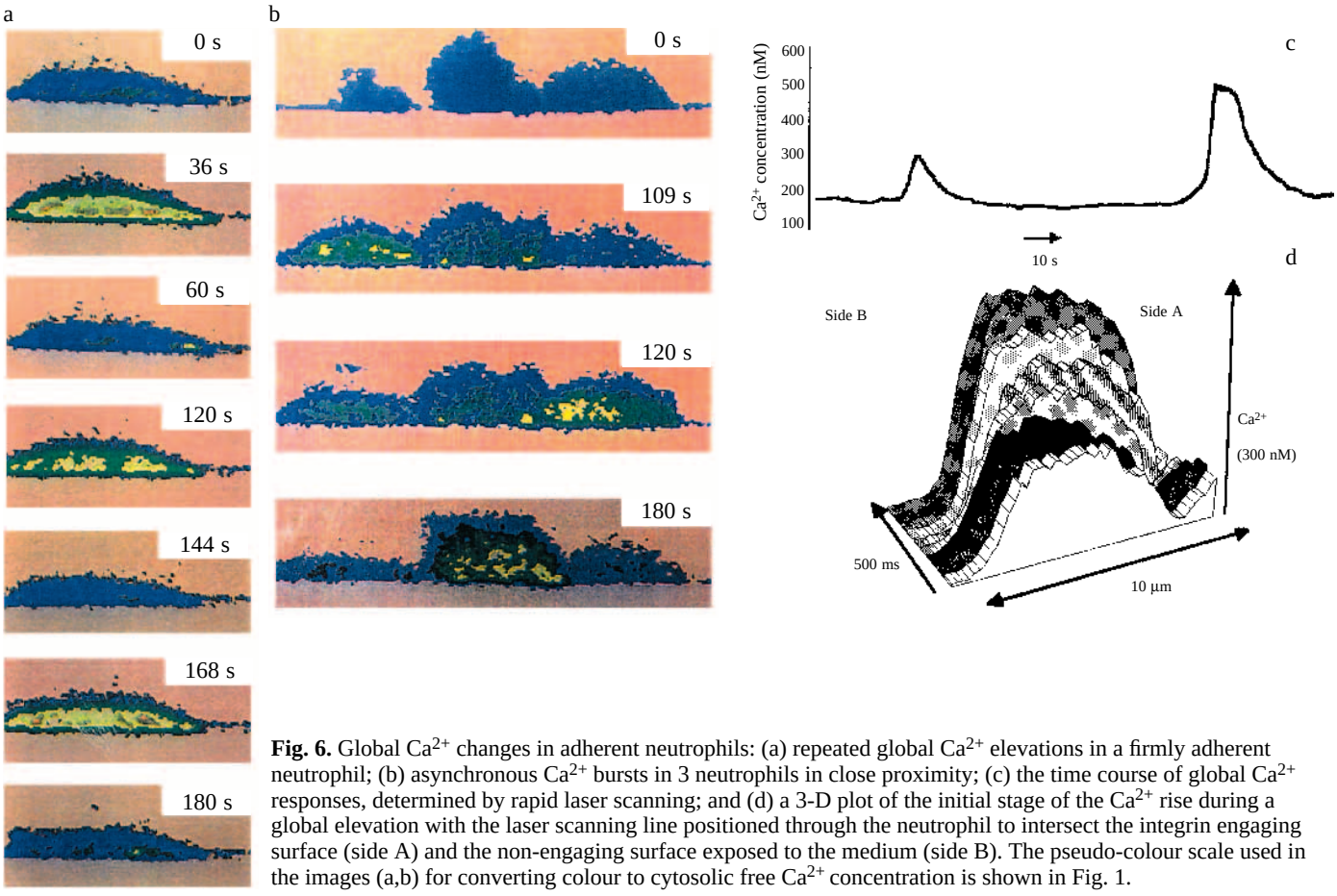

Fig. 6. Global Ca^{2+} changes in adherent neutrophils: (a) repeated global Ca^{2+} elevations in a firmly adherent neutrophil; (b) asynchronous Ca^{2+} bursts in 3 neutrophils in close proximity; (c) the time course of global Ca^{2+} responses, determined by rapid laser scanning; and (d) a 3-D plot of the initial stage of the Ca^{2+} rise during a global elevation with the laser scanning line positioned through the neutrophil to intersect the integrin engaging surface (side A) and the non-engaging surface exposed to the medium (side B). The pseudo-colour scale used in the images (a,b) for converting colour to cytosolic free Ca^{2+} concentration is shown in Fig. 1.

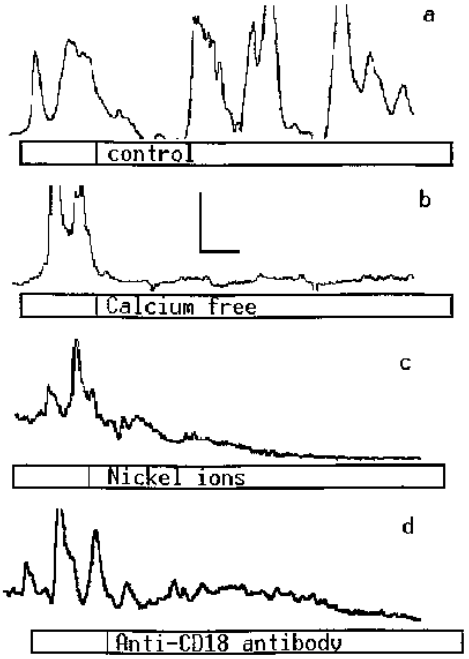

Fig. 7. Ca^{2+} elevations in neutrophils adherent to anti-CD18 coated surfaces before and after replacing the medium with: (a) incubation medium; (b) Ca^{2+} free medium (1 mM EGTA); (c) nickel containing medium (Ni^{+} ion concentration, 1 mM); and (d) soluble anti-CD18 antibody. The examples shown are typical of replica experiments (n>10).

stores in neutrophils thus remains unknown. The data presented here also provide evidence for a role of Ca^{2+} in the regulation of cell shape change. In cells in which the initial Ca^{2+} spike was suppressed by Ca^{2+} BAPTA, cell shape and firm adherence were prevented (Fig. 3b). Furthermore, a stage of rapid cell deformation, resulting in firm adherence, which is presumably a prelude to transendothelial migration in vivo, was preceded by a global cell Ca^{2+} flash. The possibility exists that, Ca^{2+} entering the cell via plasma membrane channels causes depolymerization of cortical actin (Downey et al., 1990; Al-Mohanna and Hallett, 1990) and thereby permits rapid shape change. The location of Ca^{2+} storage sites close to the plasma membrane may, therefore, have a strategic importance in this process.

Although the Ca^{2+} signalling events demonstrated here resulted from engaging integrin experimentally by using immobilised antibody, we propose that similar events also occur during physiological engagement by ICAM-1 on endothelial cell surfaces. In order to establish the relevance of these findings to the physiological process, it will be necessary to correlate the Ca^{2+} signalling triggered by CD11b/CD18 integrin engagement with trans-endothelial migration This may provide the key to understanding neutrophil emigration, and may lead to the development of novel therapies for inflammatory disease.

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