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Does cytosolic free Ca^{2+} signal neutrophil chemotaxis in response to formylated chemotactic peptide?

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SUMMARY

Cytosolic free Ca^{2+} concentration was measured and imaged in human neutrophils moving towards a source of formylated peptide in a micropipette held close to the cells. Under these conditions, neutrophils changed shape and displayed chemotaxis without significant or persistent global or localised elevations in cytosolic free Ca^{2+}. A rear-to-front persistent Ca^{2+} gradient of less than 0.5 nM/μm was present in the migrating neutrophils, until they reached the zone of higher peptide concentration, when an abrupt rise in cytosolic free Ca^{2+} concentration was triggered and chemotaxis stopped. Small localised rises in cytosolic free Ca^{2+}, which were occasionally observed during neutrophil manoeuvring, were attributed to the effect of local deformation of the neutrophil membrane, since deformation of the membrane with a blunt micropipette caused similar Ca^{2+} changes. These data suggest that neutrophil chemotaxis towards a source of formylated peptide occurs without significant changes in Ca^{2+} signalling.

Key words: neutrophil, chemotaxis, cytosolic free Ca^{2+} concentration, signalling

INTRODUCTION

Neutrophils play an important part in combating infection by phagocytosing and killing infecting bacteria. These cells leave the blood stream and migrate to the site of infection towards a source of chemotactic agents, including formylated peptides released from bacteria (Schiffmann et al., 1975). The neutrophil formylated peptide receptor is a classic seven-transmembrane spanning G-protein linked receptor (Boulay et al., 1990; Murphy, 1994), occupancy of which can cause a major increase in cytosolic free Ca^{2+} concentration (Hallett and Campbell, 1982; White et al., 1983; Pozzan et al., 1983) both by release of Ca^{2+} from an intracellular store and by inducing Ca^{2+} influx (Hallett et al., 1990; Thelen et al., 1993). However, the role of cytosolic free Ca^{2+} in chemotaxis induced by agonists of this receptor, such as fMet-Leu-Phe, remains unresolved (Maxfield, 1993; Hendey and Maxfield, 1993).

Evidence for a role for cytosolic free Ca^{2+} concentration in neutrophil chemotaxis is inconsistent. Although in populations of neutrophils in suspension, cytosolic free Ca^{2+} changes occur during chemotactic stimulation (Hallett and Campbell, 1982; White et al., 1983; Pozzan et al., 1983), within individual migrating neutrophils, changes in cytosolic free Ca^{2+} have been reported to occur either only at the leading edge (Sawyer et al., 1985), transiently, but globally during turning (Marks and Maxfield, 1990a,b), or not at all (Perez et al., 1989). Also chemotaxis has been shown to be reduced by blocking Ca^{2+} influx with lanthanum (Boucek and Snyderman, 1976), whereas buffering cytosolic free Ca^{2+} concentration with BAPTA or quin2 has been reported to have no effect (Meshulam et al., 1986; Zigmond et al., 1988; Perez et al., 1989). One problem in interpreting the results of these studies arises from the role played by the surface. Recent studies have shown that some underlying substrata, which engage neutrophil integrin receptors, can provide signals to the cells (Ng-Sikorski et al., 1991; Petersen et al., 1993). It has been shown that neutrophils moving over such a surface are continually signalled by the engagement and disengagement of integrin (Marks and Maxfield, 1990a,b; Marks et al., 1991).

More recently, elegant work with giant newt eosinophils (Brundage et al., 1991, 1993; Gilbert et al., 1994), which are approx 100 μm long, has demonstrated persistent elevations in Ca^{2+} at the rear of the cell during chemotaxis, whether directed to a chemotactic source (newt serum) or randomly. This is similar to another large migrating non-mammalian cell, amoeba, in which aequorin was used to demonstrate that cytosolic free Ca^{2+} concentration was elevated at the rear (Taylor et al., 1980). We have therefore re-examined whether such a gradient can be demonstrated in human neutrophils.

In the present study, the problems of surface interaction have been avoided by measuring the cytosolic free Ca^{2+} within individual neutrophils during chemotaxis towards a source of fMet-Leu-Phe on plain glass. We demonstrate here that, under these conditions, neutrophils move rapidly up a concentration gradient of formylated peptide without global changes in cytosolic free Ca^{2+}, until reaching a zone of high peptide concentration, when a Ca^{2+} signal is triggered. Furthermore, unlike the giant newt eosinophil (Gilbert et al., 1994), a rear-to-front

Ca^{2+} gradient of less than 0.5 nM/μm existed. Small, localised changes in cytosolic free Ca^{2+} concentration were occasionally observed when neutrophils executed sharp turning manoeuvres, which could be replicated by local membrane deformation.

MATERIALS AND METHODS

Materials

fura-2/AM, pluronic F-127 and formylNle-Leu-Phe-Tyr-Lys-fluorescein derivative were purchased from Molecular Probes, Oregon, USA. fMet-Leu-Phe was from Sigma Chemicals, Poole, Dorset.

Neutrophil isolation

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously (Davies et al., 1991). Following dextran sedimentation, centrifugation through Ficoll-Hypaque (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).

Measurement of cytosolic free Ca^{2+}

Neutrophils (2×10^7 cells/ml) were loaded with fura-2 as previously described (Al-Mohanna and Hallett, 1988). Ratio fluorescence measurements and ratio imaging of fura-2-loaded neutrophils adherent to glass coverslips were performed as previously described (Hallett et al., 1990, 1991; Davies et al., 1991). Excitation wavelengths were achieved using a Spex Fluorolog dual wavelength fluorimeter (Glen Spectra, Stanmore, UK), and ratio images acquired by an ISIS-M intensified CCD camera (Photonic Science, Tunbridge, UK) coupled to a Spex IM101 analysis system. Background subtraction, set at the autofluorescence level, avoided excluding the extreme cell periphery from subsequent ratio calculations. In our hands, this procedure generates artefactually low excitation ratios in a region 1-2 μm within the cell image (Hallett et al., 1991), but permits measurement of cytosolic free Ca^{2+} changes up to the apparent cell edge. Although this artefact can be removed by increasing the subtraction above the autofluorescence level, this was not done as it simply excluded visualisation of the periphery of the cell and may have masked localised Ca^{2+} changes near the front or trailing edges of the migrating neutrophil. Images were collected at a rate of 0.25/second. Ratio pixel values were converted to cytosolic free Ca^{2+} concentrations using the standard equation (Hallett et al., 1990). This approach would provide significantly different data for two regions of the image when the actual difference in cytosolic free Ca^{2+} concentration was greater than δ, where:

$$\delta > \{\sqrt{(2/n)}\}\{\sigma(Z_{2\alpha} + Z_{2\beta})\},$$

where n is the number of pixels in each of the two areas of the image, σ is the standard deviation of the distribution of Ca^{2+} values in the pixel arrays, Z_x is the standard normal deviate exceeded with probability x, α is the significance level for the test and (1–β) is the power of the test (Armitage and Berry, 1987). Areas of interest in which the number of pixels exceed n were, therefore, chosen, in order to statistically detect a Ca^{2+} difference of δ, where:

$$n > 2\{\sigma(Z_{2\alpha} + Z_{2\beta})/\delta\}^2.$$

This analysis sought to determine the presence of a Ca^{2+} gradient across the cell by taking test areas at defined distances apart. However, as seen from equations for n and δ, the ability to detect small and localised changes in Ca^{2+}, depends on both the magnitude of the Ca^{2+} change and the area its occupies. Therefore, the possibility of smaller and more localised Ca^{2+} changes *within* a test area cannot be excluded by the approach.

Stimulation of chemotaxis

Neutrophils were prepared from healthy human blood as previously described and allowed to adhere to glass coverslips in Krebs medium containing bovine serum albumin (0.1% w/v). A micro-pipette with tip diameter of 1-2 μm (Sterile Femtotips, Eppendorf, Hamburg, Germany) was filled with fMet-Leu-Phe (1 μM) in Krebs medium, previously filtered through a 0.2 μm sterile filter (Millipore). After

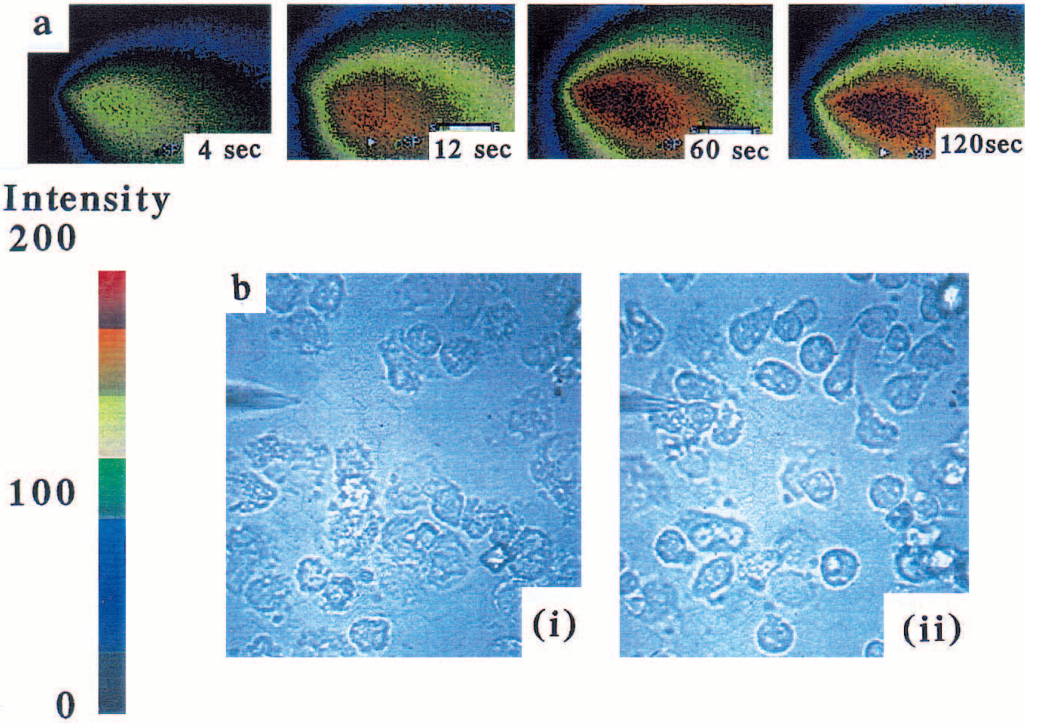

Fig. 1. Formation of a formylated peptide gradient to stimulate neutrophil chemotaxis. (a) The leakage of fluorescein-labelled formylated peptide from a micropipette is shown after the times indicated. The images show pseudo-coloured images of the fluorescence intensity given by the colour scale. By 60 seconds, the gradient was formed which remained constant. (b) A bright-field view of human neutrophils: (i) immediately after placing the micropipette containing fMet-Leu-Phe (1 μM) in position; and (ii) 2 minutes later, showing both the accumulation of neutrophils at the pipette tip and the effect on other neutrophils in the field.

placing the micropipette tip within the microscopic field, a constant pressure (40-50 mbar) was applied using a micro-injector pressure system (Micro-injector 5242, Eppendorf, Hamburg, Germany) to slowly eject the chemotactic peptide.

RESULTS

Does global cytosolic free Ca^{2+} concentration in neutrophils change during chemotaxis?

In order to visualise and measure cytosolic free Ca^{2+} changes in neutrophils undergoing chemotaxis, a micropipette containing fMet-Leu-Phe (1 μM) was positioned in a field of fura2-loaded neutrophils. By including fluorescein or fluoresceinated-formylated peptide (formylNle-Leu-Phe-Tyr-Lys-fluorescein derivative), it was established that a local steady state gradient formed around the tip within 2 minutes, by applying a constant pressure (40-50 mbar). The concentration near the tip was at least 100 times greater than at a point 100 μm distant, giving gradients of approximate 0.1 nM/μm (Fig. 1a). After delays of 0.5-2 minutes, neutrophils within the 100 μm field changed shape, moved towards the micropipette tip and clustered around it (Fig. 1b). Visualisation of cytosolic free Ca^{2+} concentration within the neutrophils under these conditions failed to demonstrate any changes in cytosolic free Ca^{2+} concentration in neutrophils undergoing shape change or rapid chemotaxis (Fig. 2). However, the cytosolic free Ca^{2+} concentration in neutrophils within 5-10 μm of the tip rose abruptly to 500-800 nM (Figs 2 and 3). At this point, neutrophils became stationary and often rounded. Apart from the abrupt Ca^{2+} change as neutrophils reached the zone of relatively high peptide concentration, global changes in cytosolic free Ca^{2+} concentration were not detected during chemotaxis in any of the more than 50 individual neutrophils examined.

Was a persistent Ca^{2+} gradient present in migrating neutrophils?

The question arose as to whether a persistent rear-to-front Ca^{2+} gradient was formed in neutrophils during chemotaxis, as found in giant newt eosinophils (Brundage et al., 1991), where the concentration at the rear of the cell was approximately 100 nM higher than the front. Although no difference of this magnitude was detected, a rear-to-front Ca^{2+} gradient of 0.92 nM/μm (Brundage et al., 1991) would produce a difference of only 9.2 nM in a 10 μm cell. Attempts were therefore made to detect differences of this smaller magnitude.

As the detection of a difference in cytosolic free Ca^{2+} concentration between given areas by imaging is essentially statistical, high magnification (×1,000) of the migrating neutrophil was used to provide more sampling points per area of interest. The detection of differences between areas is limited by both the accuracy of pixel to pixel measurement (variance) and the number of pixels covering the areas of interest (see Materials and Methods). Regions of the cell corresponding to the front and rear of the cell were chosen, which occupied sufficient pixel numbers to give a sensitivity of greater than 1 nM/μm (for the variance in the pixel values in those areas). Using these criteria, no significant difference (with 80% power at 0.05 significance level) between the leading edge and the rear was detected (Fig. 4). From the measured standard deviations and pixel numbers, it was calculated that a difference between rear and front as low as 2.7 nM (gradient = 0.17 nM/μm) would have produced significantly different mean pixel values. Similar analysis of five other cells also failed to

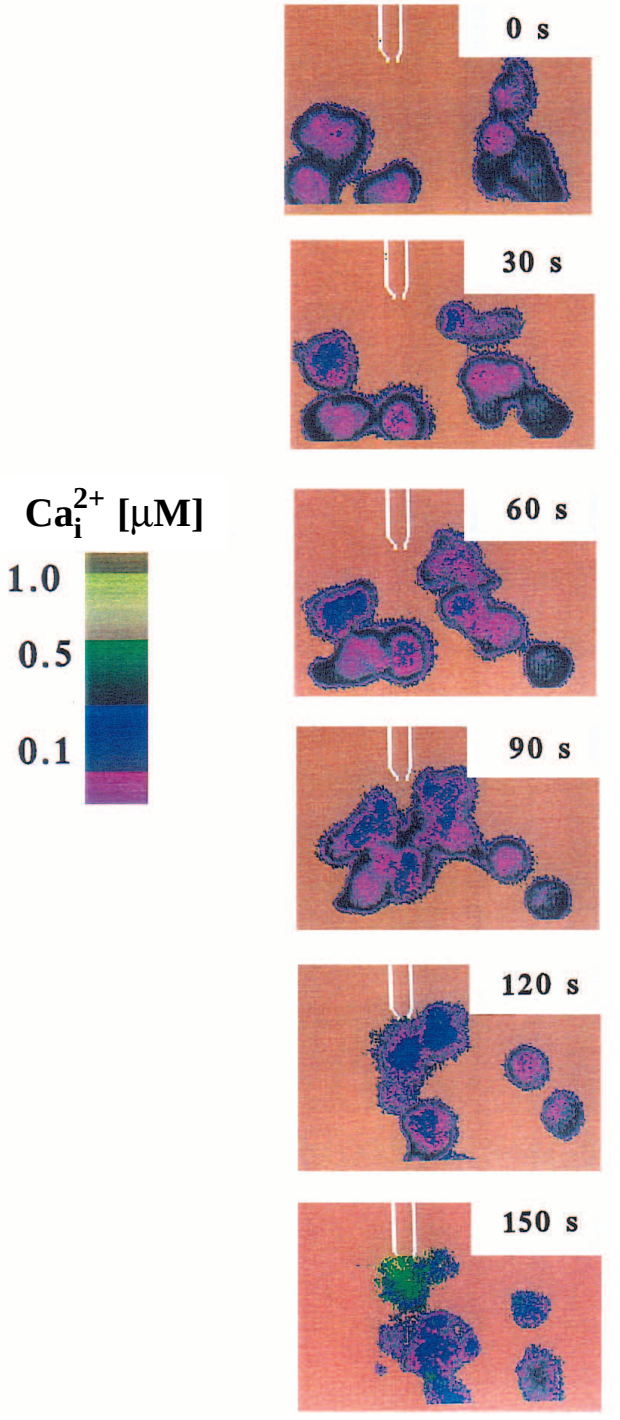

Fig. 2. Cytosolic free Ca^{2+} concentration in neutrophils undergoing chemotaxis. The cytosolic free Ca^{2+} concentration in a group of neutrophils whilst migrating towards a micropipette containing fMet-Leu-Phe (1 μM) is shown, according to the pseudo-colour scale indicated. The position of the micropipette, which contained no fluorescent material and so was not visible, is marked by the white shape. Images shown are for the times indicated, with an acquisition time of 4 seconds. The Ca^{2+} change in neutrophils close to the pipette tip is observed at 150 seconds.

detect statistically significant Ca^{2+} gradients across the cell. The minimum detectable range for such gradients in all six cells was 0.17-0.5 nM/μm (Table 1). It was therefore concluded that although fluctuations in the mean cytosolic free Ca^{2+} concentration in the *three* regions were sometimes apparent, no consistent rear-to-front Ca^{2+} gradient greater than 0.5 nM/μm existed in migrating neutrophils.

Do transient cytosolic free Ca^{2+} increases occur during cell redirecting?

As the formylated peptide gradient developed, the transition of a round stationary neutrophil into a polarized and chemotactic cell occurred without any change in cytosolic free Ca^{2+} concentration (Fig. 3). Also, no global changes in cytosolic free Ca^{2+} concentration or persistent Ca^{2+} gradients were detected during neutrophil chemotaxis. However, transient, localised cytosolic free Ca^{2+} changes were occasionally observed during abrupt neutrophil shape changes (see for example Fig. 3, arrowhead). In order to establish whether this localised change in cytosolic free Ca^{2+} was a cause or a consequence of cell shape change, an empty blunt micropipette was used to produce an abrupt stretching of non-migrating neutrophils, in order to mimic membrane 'stretching', which occurs when cells turn sharply (Fig. 5). Deformation of the neutrophil plasma membrane by advancing the pipette abruptly by 5 μm provoked a transient localised change in cytosolic free Ca^{2+} (Fig. 5) comparable to that seen occasionally during chemotaxis. The possibility thus existed that the occasional change in cytosolic free Ca^{2+} concentration observed during manoeuvring of neutrophils was a result of membrane deformation rather than the reason for the cell to change its direction.

DISCUSSION

The results presented here show that neutrophils migrate towards a source of fMet-Leu-Phe chemotaxis, on glass, without detectable global elevations or persistent gradients in the cytosolic free Ca^{2+} of greater than 0.5 μM/μm. Occasional

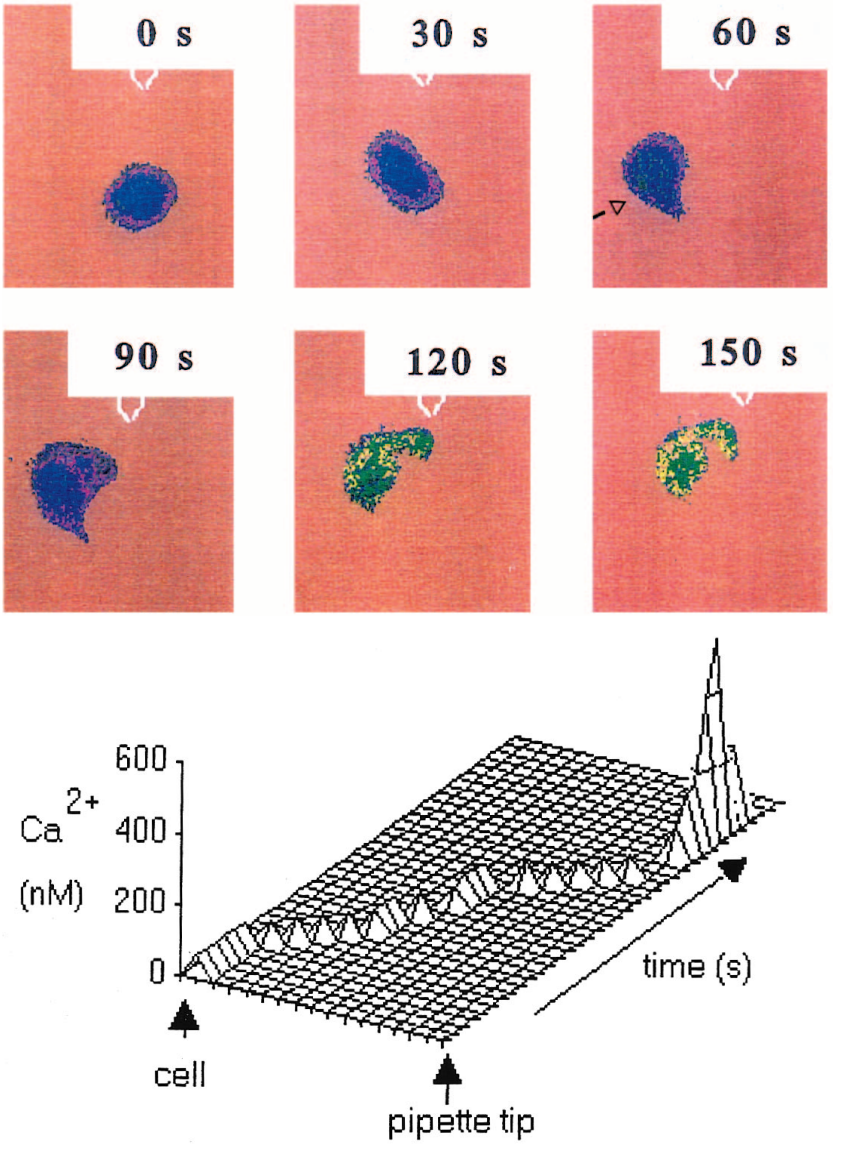

Fig. 3. Cytosolic free Ca^{2+} concentration in an individual neutrophil undergoing chemotaxis. The cytosolic free Ca^{2+} concentration in an individual neutrophil whilst migrating towards a micropipette containing fMet-Leu-Phe (1 μM) is shown, according to the pseudo-colour scale (in Fig. 2). The position of the micropipette, which contained no fluorescent material and so was not visible, is marked by the white shape. Images shown are for the times indicated, with an acquisition time of 4 seconds. On the image for 60 seconds, a small localised Ca^{2+} increase may be seen at the site indicated by the arrowhead. The lower part of the figure also shows the complete the time course, with the cytosolic free Ca^{2+} concentration plotted (*y* axis) at the position of the centre of the cell (*x* axis), for time steps of 4 seconds (*z* axis).

Table 1. The distribution of cytosolic free Ca^{2+} concentration in neutrophils during chemotaxis determined in the leading segement (front) and rear segment (tail)

	Cytosolic free Ca^{2+} concentration (nM)			
	Front	Body	Tail	δ_o (nM)
Cell 1	118±32 (1069)	115±29 (1853)	116±38 (605)	>2.7
Cell 2	110±40 (855)	111±3 (947)	109±35 (629)	>6.3
Cell3	121.5±34 (1012)	122±29 (1544)	120±38 (709)	>5.35
Cell 4	109±33 (988)	109±28 (1619)	110±38 (624)	>6.25
Cell 5	142±38 (1121)	141±35 (1423)	142±46 (521)	>7.98
Cell 6	103±32 (508)	102±27 (1241)	102±35 (432)	>6.15

The values shown are the mean ± standard deviation (number of pixels in the area). The value δ_o is the actual difference that would have been statistically detected with 80% power.

transient localised changes were observed during cell manoeuvring, which could similarly be provoked by deformation of the neutrophil membrane. These data question the relevance of Ca^{2+} gradients, which are present during chemotaxis of giant non-mammalian cells, to human neutrophil chemotaxis.

An early study on individual neutrophils reported that chemotaxis was associated with elevated Ca^{2+} at the front of the neutrophil (Sawyer et al., 1985). These data from non-ratiometric imaging of quin2 were probably artefactual. In recent studies, using ratiometric imaging of fura2-loaded neutrophils, both local and global changes in cytosolic free Ca^{2+} concentration were detected (Marks and Maxfield, 1990a,b). However, the possibility existed that Ca^{2+} signalling was the result of neutrophil integrin engagement (Ng-Sikorski et al., 1991; Petersen et al., 1993) rather than chemotaxis (Marks et al., 1991). In larger migrating cells, such as amoeba or newt eosinophils, the cytosolic free Ca^{2+} concentration becomes elevated at the rear (Taylor et al., 1980; Brundage et al., 1991, 1993; Gilbert et al., 1994), the Ca^{2+} gradient from rear to front in migrating newt eosinophils being 0.92 nM/μm (Brundage et al., 1991). In some recent elegant studies by Fay and colleagues (Gilbert et al., 1994) a model, based on spatial separation between diacylglycerol causing Ca^{2+} pump activation and InsP_3 releasing Ca^{2+}, has been proposed to explain the mechanism for these gradients (Gilbert et al., 1994). If the spatial elements and diffusion constants for this model in large newt eosinophils apply to human neutrophils, and so give rise to a similar Ca^{2+} gradient, then the expected difference in Ca^{2+} concentration between the front and the rear of the human neutrophil (diam., 10 μm) would be only 9.2 nM. The data presented here do not support the hypothesis that a persistent Ca^{2+} gradient of this

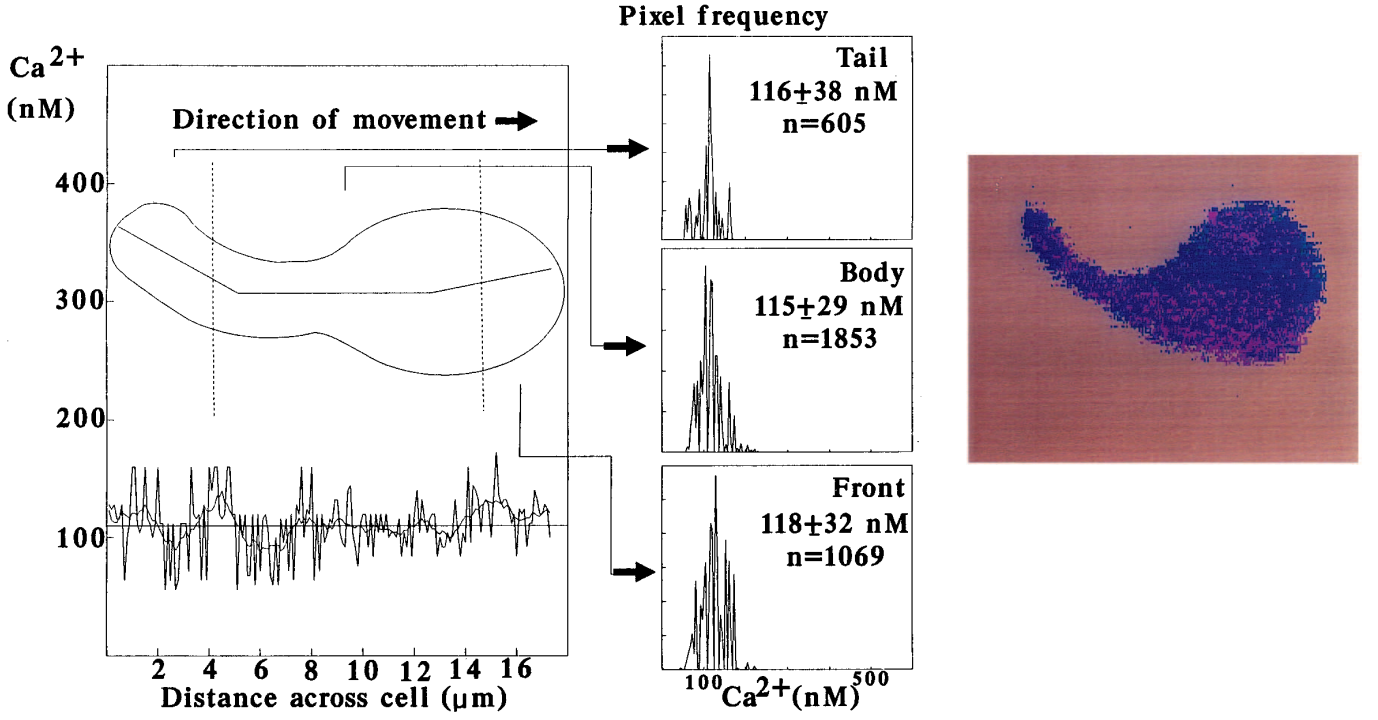

Fig. 4. Analysis of Ca^{2+} within a neutrophil during chemotaxis. The image (right) shows the cytosolic free Ca^{2+} concentration in an individual neutrophil during chemotaxis (acquisition time 2 seconds, magnification ×1,000), coloured according to the pseudo-colour scale (Fig. 2). The Ca^{2+} values for a single line of pixels through the cell from rear to front (together with smoothed data) is shown for the line indicated on the cell outline (left). Histograms of pixel frequency for sample areas from the three regions indicated are shown (middle). The values on the histograms show the mean Ca^{2+} for that area with standard deviation and total number of pixels. The standard errors for the mean Ca^{2+} values in the tail, body and front were 1.5 nM, 0.67 nM and 0.98 nM, respectively. A similar analysis failed to detect statistically significant Ca^{2+} gradients across five other neutrophils.

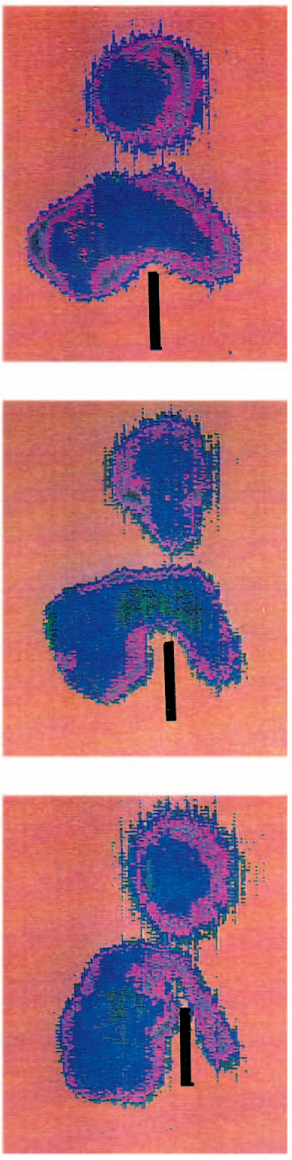

Fig. 5. Cytosolic free Ca^{2+} concentration in an individual neutrophil undergoing membrane deformation by a blunt micropipette is shown, according to the pseudo-colour scale (Fig. 2). The position of the micropipette, which contained no fluorescent material and so was not visible, is marked by the black bar. The top image shows the neutrophil with the blunt pipette near the cell; the middle image shows the image taken 4 seconds after advancing the pipette 5 μm, when a localised cytosolic free Ca^{2+} change was evoked; and the lower image shows the result of advancing the pipette a further 5 μm (4 second acquisition time).

magnitude exists in migrating human neutrophils, but they suggest that if any gradient exists it must be less than 0.5 nM/μm. A similar Ca^{2+} gradient in kinetoplasts (diam., 2 μm), small motile neutrophil fragments (Keller and Bessis, 1975; Malawista and Chevance, 1982), would generate a rear-to-front Ca^{2+} difference of only 1 nM (1% of the resting level).

Although the biochemical events accompanying chemotaxis are not resolved, the movement of neutrophils is associated with an increase in the polymerization of actin (Sheterline and Rickards, 1989). This biochemical event can be dissociated from the rise in cytosolic free Ca^{2+} when measured in neutrophil populations (Al-Mohanna and Hallett, 1990; Downey et al., 1990; Howard et al., 1990). The possibility exists that high-affinity receptors for fMet-Leu-Phe are coupled to events other than a rise in cytosolic free Ca^{2+} (Meshulam et al., 1986). For example, lower concentrations of fMet-Leu-Phe (Huang et al., 1990) or other chemotactic agents (Lloyds et al., 1995) signal tyrosine phosphorylation.

In some previous studies with human neutrophils and in migrating newt eosinophils, globally elevated Ca^{2+} levels during sharp turns have been reported (Marks and Maxfield, 1990a,b; Brundage et al., 1991, 1993; Gilbert et al., 1994). In the study reported here, small changes in cytosolic free Ca^{2+} concentration were also sometimes observed during neutrophil manoeuvring (Fig. 3). These changes in cytosolic free Ca^{2+} concentration are unlikely to trigger the manoeuvring, since procedures that inhibit changes in cytosolic free Ca^{2+} concentration do not prevent cell manoeuvring or chemotaxis (Meshulam et al., 1986; Zigmond et al., 1988; Perez et al., 1989). As membrane deformation alone was sufficient to elicit similar cytosolic free Ca^{2+} concentration changes (Fig. 5), the possibility exists that, rather than triggering cell shape change, these changes were a consequence of the shape change. The mechanism of Ca^{2+} change in neutrophils in response to membrane deformation may be related to the stretch-activated channels observed in some other cells, such as endothelial cells (Lansman et al., 1983; Goligorsky, 1988) and epithelial cells (Boitano et al., 1992). In macrophages, membrane deformation induces hyperpolarization (Reis et al., 1979) as a result of Ca^{2+} influx and activation of Ca^{2+}-activated K^+ channels. Alternatively, experimentally 'prodding' the cell could have elicited temporary and local membrane 'damage' that generated the Ca^{2+} signal. Whatever the mechanism for the membrane deformation-induced Ca^{2+} signal, the possibility exists that such signalling occurs during neutrophil migration and manoeuvring.

The data presented here thus question whether cytosolic free Ca^{2+} plays a role in signalling or controlling chemotaxis in human neutrophils.

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