Early Ca^{2+} signalling events in neutrophils detected by rapid confocal laser scanning

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Characterization of early Ca^{2+} signalling events is crucial for understanding the mechanisms which lead to cell signalling. Rapid confocal laser scanning of Fluo3-loaded neutrophils was used to provide spatially resolved cytosolic free Ca^{2+} measurements from neutrophils stimulated with formyl-Met-Leu-Phe with a time resolution of 12.5 ms. Heterogeneity of the magnitude and timing of the response was observed between individual neutrophils. There was always a delay between contact with the

stimulus and onset of the Ca^{2+} signal, with a minimum delay of 75 ms and a mean delay of 530 ms ($n = 150$). There was no delay in the cytosolic free Ca^{2+} rise induced by ionomycin. The earliest Ca^{2+} event detected following stimulation with formyl-Met-Leu-Phe ($1 \mu\text{M}$) was a localized rise in cytosolic free Ca^{2+} from a location within the cell, pointing to Ca^{2+} store release as the initial event for triggering whole-cell Ca^{2+} changes in these cells.

INTRODUCTION

Receptor activation of cells leads to the generation of the Ca^{2+} signal via a series of events, including G-protein interactions, InsP_3 production and Ca^{2+} -channel opening on intracellular Ca^{2+} stores and at the plasma membrane. Establishing the speed and timing of individual events in the sequence leading to Ca^{2+} signal generation will thus be important for understanding the mechanisms underlying signal transduction. However, the rise in cytosolic free Ca^{2+} concentration following receptor stimulation is rapid, and monitoring the initial rate of the Ca^{2+} rise response with fast time and spatial resolution has been difficult.

In neutrophils, the rise in cytosolic free Ca^{2+} concentration induced by formyl-Met-Leu-Phe acting on chemoattractant receptors has been attributed to both release of Ca^{2+} from intracellular stores and transmembrane Ca^{2+} influx [1,2]. Three possibilities exist to explain this rise in cytosolic Ca^{2+} , namely that receptor occupancy leads (i) to the Ca^{2+} channel opening by direct coupling so that transmembrane Ca^{2+} influx accounts for the initial Ca^{2+} rise, (ii) to the generation of a second messenger (InsP_3) which opens Ca^{2+} channels in both intracellular Ca^{2+} stores and the plasma membrane (so that influx and store release occur together), or (iii) to the generation of a second messenger (InsP_3), which acts only to liberate Ca^{2+} from intracellular Ca^{2+} stores, and another messenger, generated after store release, induces plasma membrane Ca^{2+} channel opening, so that store release occurs before Ca^{2+} influx. These possibilities are difficult to distinguish in neutrophil populations using conventional techniques. Not only would the time resolution of this approach be insufficient, but population measurements can give only 'averaged' responses [3]. Sage et al. [4] have improved the temporal resolution of this approach by using stopped-flow techniques to study the rapid kinetics of the Ca^{2+} response in neutrophils. However, as this technique depends on rapid mixing of populations of cells, only the 'population averaged' rise times can be measured. No account can be taken of the heterogeneity in cell response, particularly in the timing of onset of the response [3]. Furthermore, in these studies, no subcellular spatial

information can be obtained which may assist in determining the routes of Ca^{2+} entry.

Here, we have used a laser scanning technique to measure Ca^{2+} changes in individual neutrophils with a time resolution of 12.5 ms and with one-dimensional spatial information. We demonstrate that there is always a discernible lag, with a minimum of 75 ms, between arrival of formyl-Met-Leu-Phe at the cell surface and the onset of the Ca^{2+} signal. At the sub-second time scale, there is also marked asynchrony in the Ca^{2+} response between individual neutrophils. We also show that this approach can demonstrate the localized release of Ca^{2+} from an intracellular store and that this occurs before Ca^{2+} rises in the remainder of the cytosol.

MATERIALS AND METHODS

Materials

Fluo3-2/AM and pluronic F-127 were purchased from Molecular Probes, OR, U.S.A., ionomycin from Calbiochem, Cambridge, U.K., and formyl-Met-Leu-Phe from Sigma Chemicals, Poole, Dorset, U.K.

Neutrophil isolation

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously [3]. Following dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia) and hypotonic lysis of erythrocytes, 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% BSA, adjusted to pH 7.4 with NaOH).

Cytosolic free Ca^{2+} measurement by confocal laser scanning

Human neutrophils were loaded with Fluo3 by incubation with the acetoxymethyl ester and pluronic F-127 (Molecular Probes,

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OR, U.S.A.) as previously described [5,6]. Loaded neutrophils were immobilized on glass coverslips which were then mounted at the base of a warmed perfusion chamber for viewing with an inverted Leica CLSM confocal microscope (Leica Instruments, Milton Keynes, U.K.).

The laser scanning line in the x direction was then positioned 0.2–1 μm above the surface, parallel to the plane of the surface, and re-scanned repetitively to produce an x - t image where one axis represented the spatial dimension and the other time. Lines were accumulated at 80/s, to give a time resolution of 12.5 ms. Cytosolic free Ca^{2+} concentration was calculated using the standard equation with a K_d for Fluo3 of 864 nM [6].

The stimulus was added in a large volume (approx. 200 μl) to adherent cells in the perfusion chamber containing a small volume (approx 10 μl) to produce rapid mixing. The arrival of stimulus at the cells under observation was determined by the inclusion of rhodamine in the stimulus medium, the fluorescence from which rose as a single step within 25 ms. All measurements were performed with cells maintained at 37 $^{\circ}\text{C}$, using a specially constructed heating chamber.

RESULTS

Heterogeneity of Ca^{2+} response in individual neutrophils

In order to establish the timing of the Ca^{2+} response in neutrophils, confocal laser scanning of individual and groups of Fluo3-loaded cells was performed to produce two-dimensional images in which one direction was the spatial dimension through the cells, and the other was time (Figure 1). From these images, following addition of formyl-Met-Leu-Phe (1 μM), an abrupt rise in cytosolic free Ca^{2+} within individual neutrophils was

Figure 1 The laser scanning mode for rapid Ca^{2+} kinetics

The upper part of the Figure shows the relationship between the laser scanning line of the confocal microscope to Fluo3-loaded neutrophils attached to the substrate. The lower portion shows the strings of pixels produced by repetitive scans of the same line, which were accumulated to produce the x - t image. The photon intensity at each pixel was the result of four measurements taken, at 25 μs /pixel, so that the time to scan a single neutrophil was approximately 0.6–2 ms. The time difference between corresponding pixels on adjacent scan lines was 12.5 ms, which sets the time resolution for this system.

Figure 2 Rapid rise in cytosolic free Ca^{2+} concentration following stimulation

The Ca^{2+} changes in three adjacent neutrophils following the addition of formyl-Met-Leu-Phe (1 μM) are shown (a) as an 'x-t' laser scanning image, where the intensity is related to cytosolic free Ca^{2+} concentration and in the three cells is shown against time, and (b) the same data converted into average cytosolic free Ca^{2+} concentration across each cell. On the image the span of 6 s is shown. The exact time of the arrival of the stimulus at the cell is not known in this experiment, because no fluorescent indicator was included with the stimulus solution. Under these conditions accurate quantification of the cytosolic free Ca^{2+} concentration was possible without the interference from the second fluor.

evident (Figure 2a). The peak Ca^{2+} signal rise varied between individual neutrophils from 300 nM to 1200 nM. The maximum up-rate for the Ca^{2+} signal was approximately 1 nM/ms (Figure 2b), with the time to reach the (variable) peak being similar in all cells, peaking by 0.5–1 s after onset of signal generation.

Lag before Ca^{2+} signal

Two notable features of the Ca^{2+} signals from individual neutrophils were the existence and individuality of lag times before the abrupt change in cytosolic free Ca^{2+} concentration was detected (Figures 2a, 2b and 3). In order to determine the time at which the stimulus arrived at the cells, rhodamine was included in the solution containing the stimulus. This demonstrated the existence of a minimum lag, before the generation of the Ca^{2+} signal, of 75 ms (Figure 3b). The lag varied up to several seconds (Figures 2 and 3) and had a mean lag time of 530.5 ± 25.4 ms (median 462.4 ms, $n = 150$ cells). This heterogeneity in response times would lead to an underestimate of the rate of Ca^{2+} rise taken from the population average. The time for the population of cells to reach its measured peak was approx 1.6 s (Figure 3a), whereas the time for individual neutrophils within the population to reach peak Ca^{2+} was 0.5–1 s (Figure 2b).

The lag before the generation of the Ca^{2+} signal could not be attributed to the time of arrival of the stimulus at individual cells, since the fluorescence of rhodamine, included with the stimulus, rose abruptly and before the Ca^{2+} change (Figure 3b). Also, the cytosolic free Ca^{2+} in neutrophils treated with ionomycin rose without any minimum detectable lag (Figure 3c), indicating that the 75 ms minimum lag with formyl-Met-Leu-Phe was intrinsic

Figure 3 The distribution of Ca^{2+} lag times in response to formyl-Met-Leu-Phe and ionomycin

(a) The cumulative distribution for the fraction of neutrophils responding to formyl-Met-Leu-Phe (fmlp) is shown. The frequency distribution for the delays between the arrival of (b) fmlp ($1 \mu\text{M}$) and (c) ionomycin ($4 \mu\text{M}$) and the onset of Ca^{2+} signal is shown for 150 and 16 neutrophils respectively. The inset Figures in (b) and (c) show typical 'x-t' laser scanning images for stimulation with fmlp or ionomycin, where the intensity is related to cytosolic free Ca^{2+} concentration displayed against time. The time of arrival of fmlp ($1 \mu\text{M}$) or ionomycin ($4 \mu\text{M}$) was determined by the inclusion of rhodamine with the stimulus, and is indicated on each image and as time zero on the graphs.

to stimulation by this route, rather than a consequence of the detection system.

The individual variation in lag times before the generation of the Ca^{2+} signal could not be attributed to differences in the timing of arrival of the stimulus at individual cells, since the fluorescence of rhodamine, included with the stimulus, rose uniformly across the plane of view without significant difference at individual cells (Figure 3b). Also it was theoretically unlikely that adjacent cells, within a few microns of each other, could experience significantly different extracellular concentrations of stimulus persisting for many hundreds of milliseconds (Figure 2). The possibility that the Fluo3 within the individual neutrophils had different properties or subcellular locations which accounted for the differences in lags was also excluded as the Ca^{2+} ionophore, ionomycin ($4 \mu\text{M}$), which provoked a slower and less abrupt

Figure 4 The release of stored Ca^{2+} in the presence and absence of extracellular Ca^{2+}

The three-dimensional plots show the dimension across the cell as the x axis, the change in cytosolic free Ca^{2+} concentration as the y axis and time receding into the page (as the z axis). (a) The Ca^{2+} response from a single neutrophil stimulated with formyl-Met-Leu-Phe ($1 \mu\text{M}$) in the absence of extracellular Ca^{2+} (1 mM EGTA), and the inset Figure showing, in detail, the left-hand side of the same cell where slow diffusion towards the cell edge was apparent. (b) The inset Figure shows an 'x-t' laser scanning image, where the intensity is related to cytosolic free Ca^{2+} , with the arrow indicating the initial rise in Ca^{2+} before the rest of the cytosol. The larger Figure shows the same data as a three-dimensional plot. The same initial Ca^{2+} responding point is indicated.

Ca^{2+} rise, did so without the same wide distribution of lag times (Figure 3c). Unlike the formyl-Met-Leu-Phe, there was little variation in the lag times between individual neutrophils. The lag and the heterogeneity of lag times with formyl-Met-Leu-Phe thus cannot be accounted for simply, and may point to an important feature of stimulus- Ca^{2+} -signal-generation coupling.

Ca^{2+} store release was detected first

In order to determine the relative timing of release of Ca^{2+} from stores within the neutrophil, the subcellular distribution of the rise in Ca^{2+} was analysed across the one-dimensional spatial line. In the absence of extracellular Ca^{2+} , when an elevation of cytosolic free Ca^{2+} concentration must originate from within the cell, the addition of formyl-Met-Leu-Phe failed to elicit a detectable Ca^{2+} response in most cells examined. However, in approx. one-twentieth of cells examined, a localized rise in cytosolic free Ca^{2+} concentration was observed (Figure 4a). The possibility existed that, in these cells, the laser scanning line had fortuitously passed near a Ca^{2+} storage site. The 'cloud' of elevated Ca^{2+} showed little lateral diffusion (Figure 4a). In the

presence of extracellular Ca^{2+} , in most cells cytosolic free Ca^{2+} concentration rose uniformly throughout the cell without any major intracellular heterogeneity. However, occasionally (in approx. one-twentieth of cells examined), cytosolic free Ca^{2+} was observed to rise at a focus within the cell before Ca^{2+} in the rest of the cytosol rose (Figure 4b). The rise in Ca^{2+} in the cytosol lagged behind this initiation point, but within 1 s was uniform across the cell (Figure 4). Again, in these cells the possibility existed that the laser scanning line had fortuitously passed near a Ca^{2+} storage site and that release of Ca^{2+} from that site was detected before the general cytosolic rise. There was no evidence for a wave of Ca^{2+} emanating from the initiation site and in no cell was more than one such Ca^{2+} initiation point found.

DISCUSSION

The data in this paper have demonstrated a time lag between the arrival of the agonist at the cell surface and onset of Ca^{2+} signal generation, and provided the minimum time for this event (75 ms). Also, for the first time in neutrophils, it was shown that release of Ca^{2+} from an intracellular site was the earliest detectable Ca^{2+} event following physiological stimulation.

Sage et al. [4] have previously used stopped-flow fluorimetry to examine the rapid kinetics of neutrophil activation and also reported a Ca^{2+} response lag, somewhat longer than those reported here (approx. 1 s). Presumably, the population averaging nature of the stopped-flow method limited detection of the fastest responding cells and so produced the larger delays in that study. The delay before Ca^{2+} signal generation may provide an explanation for the time lags before Ca^{2+} -dependent responses, such as exocytosis [7] and oxidase activation [8], which are detected in individual neutrophils. Several events may account for the delay before the Ca^{2+} rise, including the time required for receptor binding, the generation of InsP_3 , diffusion of InsP_3 to the Ca^{2+} release sites and diffusion of a calcium influx factor from the store to the plasma membrane. From the association rate constant for formylated peptide receptor binding, $k_{\text{on}} = 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ [9], and the fractional receptor occupancy for Ca^{2+} signal generation, $< 0.1\%$ [10], it can be shown that the binding time would be less than 0.5 ms and would thus not significantly contribute towards the observed lag times. The time for Ca^{2+} release from an intracellular Ca^{2+} store located within the cell would depend upon the rate of generation of InsP_3 and its diffusion time to the store. With a diffusion constant of $283 \mu\text{m}^2/\text{s}$ [11], it can be estimated that InsP_3 would diffuse from the neutrophil periphery to its centre ($5 \mu\text{m}$) in approx. 44 ms. If a diffusible calcium influx factor of similar size (and diffusion constant) were liberated by the store [12], there would be sufficient time for signalling to and from such a storage site during the minimum lag time (75 ms).

The variation in lag times of individual cells after addition of stimulus is not trivial in origin, as it was specific for the receptor-operated route (Figure 3). It was unlikely that differences in rate

of receptor binding to individual cells could contribute to the variation, since lag times of greater than 1 s would require a binding rate less than $1/2000$ the mean rate. Theoretical models, based on InsP_3 - Ca^{2+} reinforcement coupling, however, have been proposed with the property that small differences in receptor occupancy would generate large differences in latency before cytosolic free Ca^{2+} signal generation [13]. The possibility also exists that during signal transduction there are additional slower steps between receptor occupancy and the elevation of cytosolic free Ca^{2+} concentration which give rise to individual cell variation.

The initial Ca^{2+} rise from a single focus reported here (Figure 4) is similar to the single cloud of elevated Ca^{2+} previously observed by ratiometric Fura2 imaging [14]. The infrequency with which this event was observed (approx. 5%) would be accounted for by the cells lying randomly with respect to the laser scanning line and the area of the cytosol in which Ca^{2+} was elevated being restricted (Figure 4a). As a wave of elevated Ca^{2+} did not spread through the cell under these conditions, it is unlikely that Ca^{2+} -induced Ca^{2+} release exists in neutrophils, and the possibility exists that emptying of the Ca^{2+} store plays another role. As the release of stored Ca^{2+} preceded Ca^{2+} influx (Figure 4b), the possibility existed that store emptying signals Ca^{2+} influx. Although it was not clear from our data whether this occurred via a diffusible factor, such as that recently reported [12,15], a similar factor extracted from neutrophils has been shown to cause Ca^{2+} influx in these cells [16].

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