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Temporal and Spatial Resolution of Ca^{2+} Release and Influx in Human Neutrophils Using a Novel Confocal Laser Scanning Mode

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Confocal laser scanning demonstrated that stimulation of neutrophils with the surface receptor agonist, f-met-leu-phe, resulted in the release of stored Ca^{2+} from a single site. From reconstructions of neutrophils stained with acridine orange, it was shown that the central Ca^{2+} release site was always close to the nucleus, and correlated with a site which stained with $DiOC(6)_3$. Elevated Ca^{2+} was locally restricted to within 1 μm of this site. Release of Ca^{2+} by this pathway was accompanied by the influx of Ca^{2+}, less than 1 second after store release. In each neutrophil, the Ca^{2+} store release component preceded the Ca^{2+} influx, and their spatial separation suggested communication between the Ca^{2+} store and the plasma membrane by a messenger. The $(distance)^2$ of the release site from the plasma membrane was correlated to the time between Ca^{2+} release and influx, consistent with the diffusion of a messenger from the storage site signalling Ca^{2+} influx.

Following extravasation, neutrophils migrate to the site of bacterial invasion, and are stimulated by a variety of mechanisms to phagocytose and destroy pathogens. Bacterial formylated peptides, including formylated Met-Leu-Phe, cause an elevation in cytosolic free Ca^{2+} within the neutrophil which may result in activation of the respiratory burst, chemotaxis [1], degranulation, and upregulation of surface markers, such as the integrins, involved in the inflammatory response. This rise in cytosolic free Ca^{2+} results from both release of Ca^{2+} from intracellular stores, and influx of Ca^{2+} from the extracellular environment.

Until recently, the timing and location of the Ca^{2+} store release site, and the relationship between store release and Ca^{2+} influx, had not been elucidated [2,3]. The neutrophil displays ''classical capacitative Ca^{2+} influx'' [4], whereby Ca^{2+} store release is coupled to influx of Ca^{2+} ions at the plasma membrane [5,6]. Although a soluble factor, similar that reported by Randriamampita and Tsien [7], has been isolated from neutrophils that directly increases Ca^{2+} permeability [8], the role for a diffusible factor remains controversial [9-11]. A key issue for the debate is the relationship of Ca^{2+} release sites to the plasma membrane, a spatial relationship not readily visualised by previous techniques, and its temporal relationship to Ca^{2+} influx [11,12]. Here, we describe a new confocal laser scanning technique which, as well as providing rapid scanning for temporal resolution of Ca^{2+} changes, also has the added advantage of retaining some XZ spatial information, thereby giving both temporal and spatial resolution of the Ca^{2+} store release - influx event. The data presented here demonstrates the temporal and spatial separation of the Ca^{2+} release and influx components of the Ca^{2+} signal in neutrophils, and is suggestive of a diffusible messenger signalling Ca^{2+} influx.

MATERIALS AND METHODS

Neutrophil preparation. Normal neutrophils were isolated by lysis of erythrocytes, and single-step density gradient centrifugation of leukocytes through ficoll-hypaque (Pharmacia; 13). All experiments were carried out in Krebs buffer,

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(120mM NaCl; 4.8mM KCl; 1.2mM KH_2PO_4; 1.2mM $MgSO_4$; 1.3mM $CaCl_2$; 25mM HEPES and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH) at 37 °C.

Neutrophil loading with Fluo3. Freshly prepared neutrophils were loaded with the Ca^{2+} indicator Fluo3 (Molecular Probes, Oregon), by incubation with the acetoxymethyl ester (16.6μg/ml, 20°C, 1hr; 14). The estimated final intracellular concentration of Fluo3 was 50-100μM. The Ca^{2+} concentration in each cell was calculated as previously described [15], using a Kd of Fluo3 of 864nM [16].

Neutrophil staining with $DiOC_6(3)$ and acridine orange. The membranes associated with Ca^{2+} storage organelles and the nucleus were defined, using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$ Molecular Probes, Oregon), added to neutrophils (0.25μg/ml, for 5 min.). The position of the nuclear lobes was subsequently determined by the addition of acridine orange (5ng/ml, 5 min. Sigma Chemicals, Poole, UK) to the Krebs buffer. The location of each probe was detected by confocal laser scanning microscopy, and reconstructed to provide the relationship of the nucleus to other organelles.

Confocal laser scanning microscopy. Neutrophils were visualised, and images recorded by confocal laser scanning microscopy (Leica). The XY plane scans showed the organelles in relation to each other, and to cytosolic free Ca^{2+} concentration. The XT scan mode was used for rapid-time detection of changes in the cytosolic free Ca^{2+} concentration,

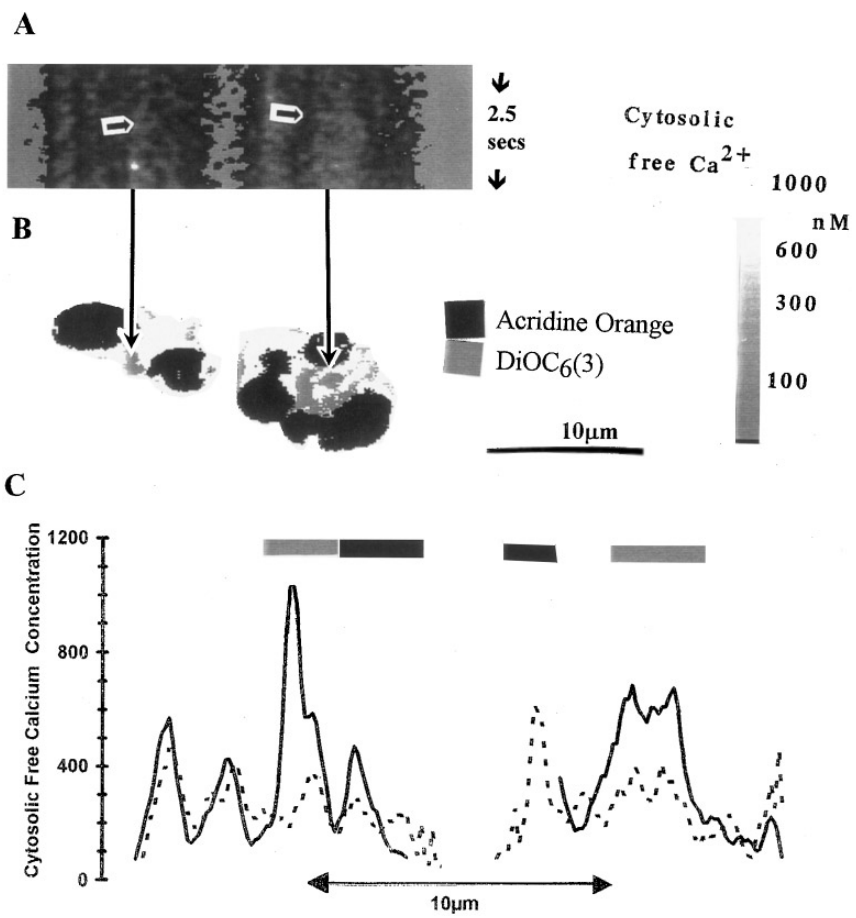

FIG. 1. Ca^{2+} store release in response to the addition of fMLP (100 nM; added at the start of the scan), in the absence of extracellular calcium (1 mM EGTA), (A), detected by confocal XY scanning, shows a central release site (arrowed), located by $DiOC_6(3)$ staining at a position between the nuclear lobes, shown below in (B) The central Ca^{2+} release site correlates to the position of the $DiOC_6(3)$ stained organelle, shown by the arrows. (C) The site of the central $DiOC_6(3)$ staining organelle, shown by the position of the colour bars above the graph, corresponds to the position of Ca^{2+} release 100 ms after fMLP addition (—). The resting cytosolic free Ca^{2+} concentration is also indicated (---). Images showing cytosolic free calcium concentration are pseudo-grey according to the grey scale.

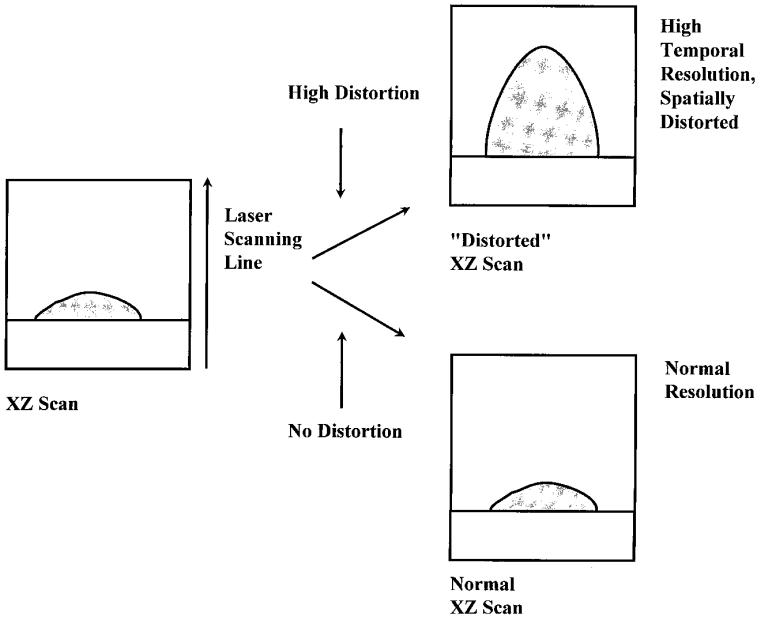

FIG. 2. Cartoon showing the origin of the spatial distortion in the ''distorted'' XZ scan.

and the novel ''distorted'' XZ scan also allowed the distance of the release site from the plasma membrane in the XZ plane to be determined. We estimate that detection of Ca^{2+} store release by this method would be restricted to those cells in which influx occurred later than 200ms after store release.

RESULTS AND DISCUSSION

A Single, Central Ca^{2+} Release Site in Neutrophils

In order to visualise the location of Ca^{2+} release in response to fMLP, extracellular Ca^{2+} was chelated by the addition of EGTA (1mM), and the rapid-time confocal XT scan was used. Addition of fMLP to neutrophils resulted in Ca^{2+} store release from a central location in the cell (Figure 1A). Staining the same neutrophils with $DiOC_6(3)$ and acridine orange enabled the relationship between the release sites, $DiOC_6(3)$ staining organelles, and nuclear lobes to be determined (Figure 1B). The position of the $DiOC_6(3)$ staining, and the Ca^{2+} store release were correlated (Figure 1C), suggesting that an organelle at that location was responsible for Ca^{2+} release. Having identified the Ca^{2+} release site, and its relationship to surrounding organelles, in the absence of Ca^{2+} influx, it was necessary to devise a method for monitoring both Ca^{2+} release and influx in the presence of extracellular Ca^{2+}.

Characterisation of the ''Distorted'' XZ Scan

In order to resolve Ca^{2+} store release from Ca^{2+} influx in neutrophils stimulated with fMLP, with both temporal and spatial information, a ''distorted'' XZ scan was developed and characterised (Figure 2). In this novel scan mode, adherent neutrophils were visualised by the XZ scan. Each line in the X direction took 17msec to scan, with the average neutrophil in the scan line for approximately 9msec. Differences in the time of scanning between each side of the neutrophil in the X direction was minimised by averaging the intensity over two lines scanned with an interval of 8.6msec. There was therefore negligible temporal and spatial distortion in the X direction. However, in order to resolve Ca^{2+} release from influx, as the laser scanning line moved towards the upper surface of the cell in the Z direction, a spatial

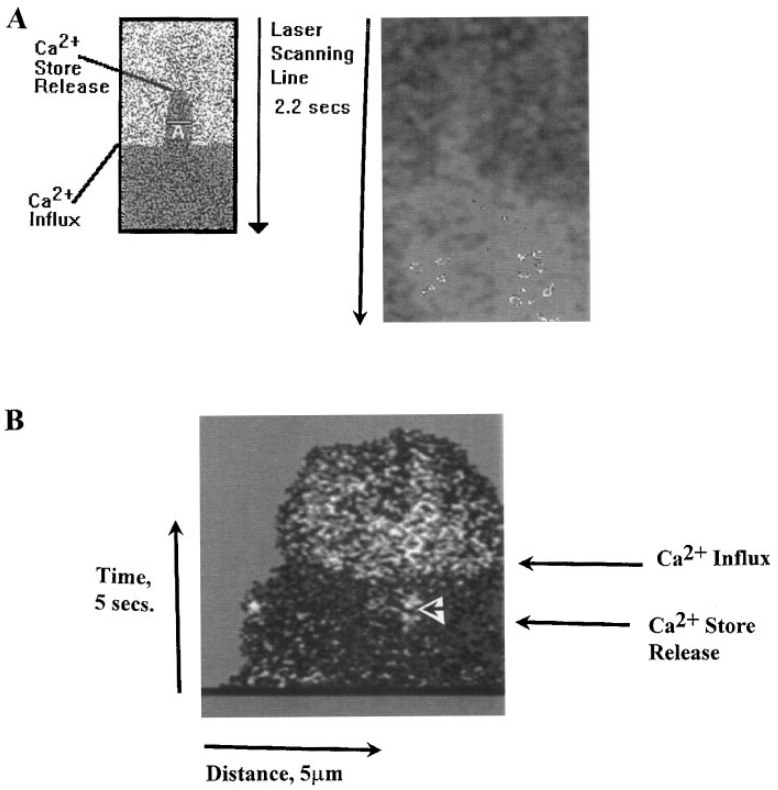

FIG. 3. Neutrophil response to 100nM fMLP in the presence of extracellular Ca^{2+}. (A) Section of a neutrophil, as explained by the schematic diagram. Use of the ''distorted'' XZ scan has allowed resolution of the Ca^{2+} store release (''A'') and influx. (B) An entire neutrophil scanned in the ''distorted'' XZ scan mode, showing Ca^{2+} store release (arrowed) and influx. The grey scale converting colour to cytosolic free Ca^{2+} concentration is shown in Figure 1.

distortion was introduced. In this way, the neutrophil was scanned with an increased time resolution, whilst spatial resolution in the XZ direction was retained to a greater or lesser degree. In neutrophils with sufficient temporal resolution, and in which the time between Ca^{2+} store release and Ca^{2+} influx was sufficiently prolonged, different phases of Ca^{2+} activity were distinguishable within the cell, and the spatial relationship in the XZ plane preserved.

Relationship of Central Ca^{2+} Store Release to Ca^{2+} Influx

In order to visualise the relationship between Ca^{2+} store release and Ca^{2+} influx, experiments were conducted in the presence of extracellular Ca^{2+}. The Ca^{2+} store release, which reached an average of 0.78μm ($\pm$0.41μm; n=13) in diameter, always preceded the Ca^{2+} influx, which was detected an average of 840msec ($\pm$470msec; n=12) after the first detected store release. Because the nature of the scanning mode used favoured detection of cells in which influx was unusually slow, this number represents an over-estimate of the time between store release and influx. The influx phase was rapid, reaching uniformity across the cell within 200msec, and there was no exclusion by the nucleus, suggesting that this membrane presented no significant barrier to Ca^{2+} diffusion under these conditions (Figure 3). The time taken for the concentration of Ca^{2+} from the influx phase to reach uniformity correlated to the square of the distance across which diffusion took place (r=0.63; n=7). This relationship would be expected for a

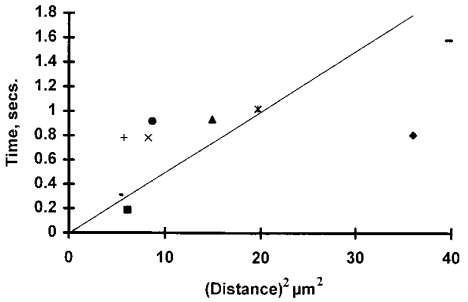

FIG. 4. Graph showing the relationship between the square of the distance from the Ca^{2+} release site to the plasma membrane, and the time taken to signal Ca^{2+} influx. The points are experimental data from individual neutrophils, and the line shows the relationship for a molecule with a diffusion constant of 20 μm^2/sec.

diffusible ion, and hence gives a measure of the precision of this method of correlation between different neutrophils with varying Ca^{2+} buffering capacities.

The Characteristics of a Diffusible Factor Signalling Ca^{2+} Influx

The diameter of the released Ca^{2+} ''cloud'', was a measure of how advanced the response was at the time at which the laser scanning line intercepted it. This was poorly correlated to the time between Ca^{2+} release and Ca^{2+} influx ($r=0.37$; $n=12$; data not shown), indicating that there was no systematic error in the timing of the release event in relation to the Ca^{2+} influx. However, the square of the distance of the Ca^{2+} store release site from the furthest plasma membrane in the X direction, (4.28μm $\pm$2.25μm; $n=10$), was proportional to the time between Ca^{2+} release and influx ($r=0.7$; $n=10$; Figure 4). A diffusible factor, travelling an average distance of 4.28μm to the plasma membrane to signal influx within the time detected for each neutrophil, would have a diffusion constant of approximately 20μm^2/sec, giving an approximate molecular weight of 15kDa [17].

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REFERENCES

1. Maher, J., Martell, J. V., Brantley, B. A., Cox, E. B., Neidel, J. E., and Rosse, W. F. (1984) *Blood* **64,** 221–228.
2. Pettit, E. J., and Hallett, M. B. (1995) *Biochem. J.* **310,** 445–448.
3. Pettit, E. J., and Hallett, M. B. (1996) *Histology and Histopathology,* in press.
4. Putney, J. W., Jr. (1990) *Cell Calcium* **11,** 611–624.
5. Montero, M., Alvarez, J., and Garcia-Sancho, J. (1991) *Biochem. J.* **277,** 73–79.
6. Scharff, O., and Foder, B. (1993) *Physiol. Rev.* **73,** 547–582.
7. Randriamampita, C., and Tsein, R. Y. (1993) *Nature* **364,** 809–814.
8. Davies, E. V., and Hallett, M. B., (1995) *Biochem. Biophys. Res. Commun.* **206,** 348–354.
9. Berridge, M. J. (1995) *Biochem. J.* **312,** 1–11.
10. Clapham, D. E. (1995) *Nature* **375,** 634–635.
11. Hallett, M. B., Pettit, E. J., and Davies, E. V. (1996) *Biochem. J.* **314,** 1054–1055.
12. Berridge, M. J. (1996) *Biochem. J.* **314,** 1055–1055.
13. English, D., and Anderson, B. R. (1974) *J. Immunol. Met.* **5,** 249–252.
14. Minta, A., Kao, J. P. Y., and Tsien, R. Y. (1989) *J. Biol. Chem.* **264**(14), 8171–8178.
15. Pettit, E. J., and Hallett, M. B. (1996) *in* Ion Measurements in Cells: Microscopic Measurement and Biological Activities (Morgan, A. J., Sigee, D., Sumner, A. T., and Warley, A., Eds.), Portland Press, in press.
16. Merritt, J. E., McCarthy, S. A., Davies, M. P. A., and Moores, K. E. (1991) *Biochem. J.* **269,** 513–519.
17. Luby-Phelps, K., Lanni, F., and Taylor, D. L. (1984) *J. Cell Biol.* **101,** 1245–1256.