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Invited Review

The microanatomy of calcium stores in human neutrophils: Relationship of structure to function

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Summary. As changes in cytosolic free Ca^{2+} play key roles in coupling responses in neutrophils, it is important to locate and identify Ca^{2+} storage sites within these cells. Here, recent data is presented which highlights the functional link between microanatomical structure and cell signalling function. Fluorescent optical probes for cytosolic free Ca^{2+} have been used, together with organelle specific markers. We present evidence from conventional fluorescence microscopy, together with ratiometric and confocal laser scanning fluorescence microscopy, which pin-points two cellular locations for Ca^{2+} within the neutrophil; one within the nuclear lobes, and the other towards the cell periphery. Knowledge of these two locations provides a clear insight into how signalling in this cell type is regulated and provides a framework for explaining how specific stimuli act to produce specific responses.

Key words: Neutrophils, Calcium stores, Confocal microscopy, Microanatomy, Ca^{2+} signalling

Introduction

Neutrophils are essential white blood cells of the immune system, involved in the inflammatory response occurring during any breach in body defences, such as bacterial attack. In addition to this life-saving function, neutrophils are also the mediators of damaging inflammatory diseases, such as rheumatoid arthritis. A review by Leff and Repine (1993) listed seventeen serious diseases in which neutrophil malfunction plays the major role. Understanding the mechanisms underlying neutrophil activity is thus an important goal for medical research.

Neutrophil extravasation and migration through tissues is the essential first step in any inflammatory response (Marsh et al., 1967). This process is dependent upon neutrophil recognition of adhesion molecules, such as the selectins, and ICAM-1 (Kishimoto and Rothlein, 1994). Neutrophils then respond to a range of chemotactic factors, such as the bacterial peptide, f-met-leu-phe, complement component C5a, and leukotriene B4, which direct their migration towards the site of damage or attack. Here, neutrophils engulf and destroy any pathogens present, using mechanisms which include recognition of opsonised bacteria by specialised receptors in the plasma membrane, such as the Fc and complement receptors. Recognition results in phagocytosis (Brown, 1994), and bacterial killing by release of toxic components from neutrophil granules into the phagosome (Zeya and Spitznagel, 1972). When inflammation has subsided, or a critical number of bacteria have been ingested, the neutrophil begins the apoptotic process, leading to the safe disposal of the spent cell and contents (Haslett, 1992).

Fundamental to each one of these functional processes are changes in neutrophil cytosolic free Ca^{2+} concentration, which are important signals in the mediation between ligand recognition and end response (Pozzan et al, 1983). Thus, the study of molecular processes occurring during neutrophil activity, and especially Ca^{2+} signalling, are central to an understanding of both normal and disease states of inflammation, and to the design of novel therapeutic regimes during inflammation.

Neutrophil microanatomy

The neutrophil is a unique cell, specifically designed for an individual role in the body. It is terminally differentiated, and undertakes a minimal amount of novel protein synthesis during the typical 36-hour life span. The anatomy of this small cell perfectly reflects neutrophil function.

Human neutrophils are 10-15 μm in diameter (Wintrobe, 1981). 20% of the cell volume consists of multi-lobed nucleus, and another 20%, of the dense granules necessary for bacterial killing (Fig. 1). Other typical mammalian cell constituents, such as mitochondria and ER, make up less than 1% of the volume of an average neutrophil (Hallett, 1989). The only

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prominent membranous structure in the neutrophil is a region of vestigial, Golgi-like ER, situated towards the centre of the cell, between the lobes of the nucleus (Fig. 1).

Neutrophil Ca^{2+} signalling

Like all mammalian cells, neutrophils contain a high concentration of calcium (1mM), but the majority is stored in a bound form (Petroski et al., 1963). In the resting neutrophil, the free Ca^{2+} concentration is approximately 100nM, but stimulation can result in the concentration rising to 1 μm (Hallett, 1989). There are two mechanisms for increasing cytosolic free Ca^{2+} in the neutrophil, namely calcium store release, and Ca^{2+} influx, via channels in the plasma membrane (Demareux et al., 1994). The spatial and temporal relationship between these two types of activity, and especially the location and functional roles of calcium store release will be dealt with in greater detail later, but first it is necessary to have a rudimentary understanding of signalling processes in neutrophils.

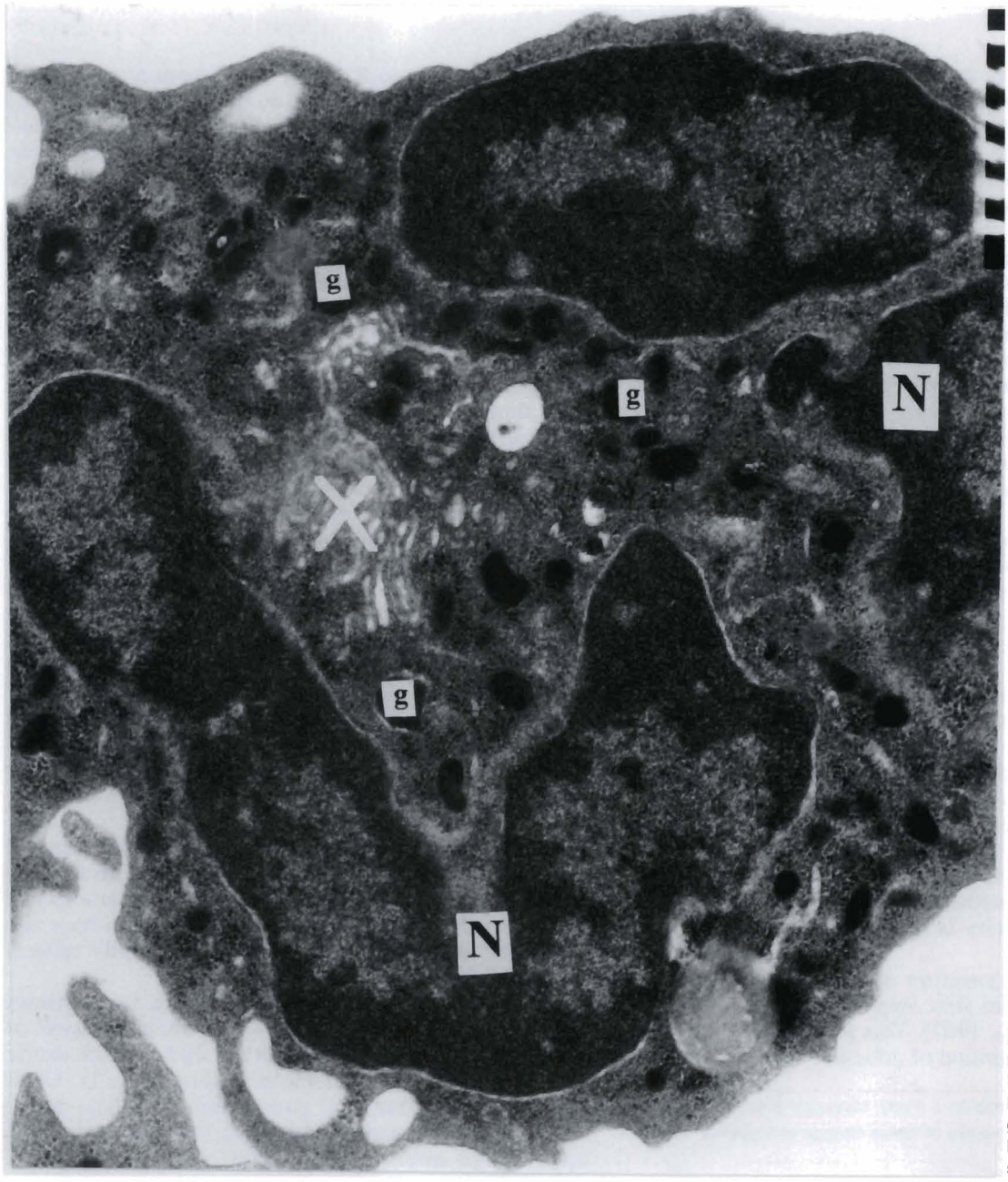


Fig. 1. Electron micrograph of a mature neutrophil, showing the nuclear lobes (N), granules (g), and Golgi-like near-nuclear calcium store (X). x 20,000

(i) Receptor-cytosol signalling

Occupation of neutrophil receptors with their ligands (fMLP, C5a, CD11b/CD18, PAF, Il-8, etc) results in signal transduction across the membrane. In the case of the seven membrane-spanning region chemoattractant receptors, this occurs via heterotrimeric G-proteins coupled to the receptor (Bommakanti et al., 1995). The chemoattractants produce different cellular responses, which depend on their concentration. For example, fMLP at pM-nM concentrations causes priming of the respiratory burst without any detectable change in cytosolic free Ca^{2+}, whilst µm concentrations directly activate the NADPH oxidase, and cause both calcium releases from stores, and influx (Al-Mohanna and Hallett, 1990; Thelen et al., 1993). The mechanisms for other ligands are less well understood, and may, or may not, involve heterotrimeric- or monomeric G-proteins.

The initial interaction between the ligand and receptor results in the activation of one of the range of different types of heterotrimeric G-proteins situated on the inner surface of the membrane - G_{i2}, in the case of fMLP. The G-protein with GDP bound to it is inactive, and conversion to the active form involves dissociation of the ßγ subunits from the α subunit, which contains the nucleotide binding site; GDP is released, and GTP binds to the empty site. The protein is then in the active state, until the GTP is hydrolysed to form GDP again (Fig. 2). Enzymes in the cytosol control the rate of the association-dissociation reaction. Activated G-proteins couple to intracellular effector molecules, and thus cause a cascade of intracellular events, mediated by particular effector molecules coupled to that G-protein.

(ii) Intracellular signalling

One of the enzymes activated by the active form of the heterotrimeric G-proteins is phospholipase C (ß form, PLC; Cockroft and Gomperts, 1985). The function of PLCß is to hydrolyse the plasma membrane lipid, phosphatidylinositol-4,5-bisphosphate. Inositol 1,4,5 trisphosphate (IP_3), is then free to diffuse into the cytoplasm, leaving the lipid diacylglycerol (DAG) in the membrane (Berridge and Irvine, 1989).

The response to fMLP causes IP_3 diffusion to a calcium store by this pathway, leading to occupation of IP_3 receptors in the store membrane. The IP_3 receptor is a calcium channel consisting of four subunits, and IP_3 binding causes channel opening, releasing free calcium ions into the cytosol (Berridge and Irvine, 1984). Phosphorylation of IP_3 generates IP_4, and although its actions are not well established, it has been suggested that it may open Ca^{2+} channels on the plasma membrane (Meldolesi and Pozzan, 1987). The inositol phosphate signalling pathway was virtually unknown twenty years ago (Michell, 1995). The generation of an increase in cytosolic free calcium concentration is summarised in Figure 3.

Calcium is cleared from the cytosol by the Ca^{2+}-ATPase pumps, both in the store membranes, and the plasma membrane (Lagast et al., 1984). Rapid clearing of the calcium ions allows the neutrophil to prepare for a second wave of calcium activity, which occurs in some situations such as integrin mediated signals (Pettit and Hallett, 1996a,b), and during chemotaxis. There is little evidence to suggest that calcium-induced calcium release (CICR) pathways operate in neutrophils, nor do neutrophils posses caffeine, or ryanodine-sensitive receptors, typical of other mammalian cells such as adrenal medullary cells (Cheek et al., 1990).

The effects of an increase in cytosolic free calcium on the neutrophil are very varied. Calcium ions activate protein kinase C (PKCγ), via its translocation to the plasma membrane, and association with DAG (Dang et al., 1995). Protein kinase C is responsible for the phosphorylation of proteins important in neutrophil

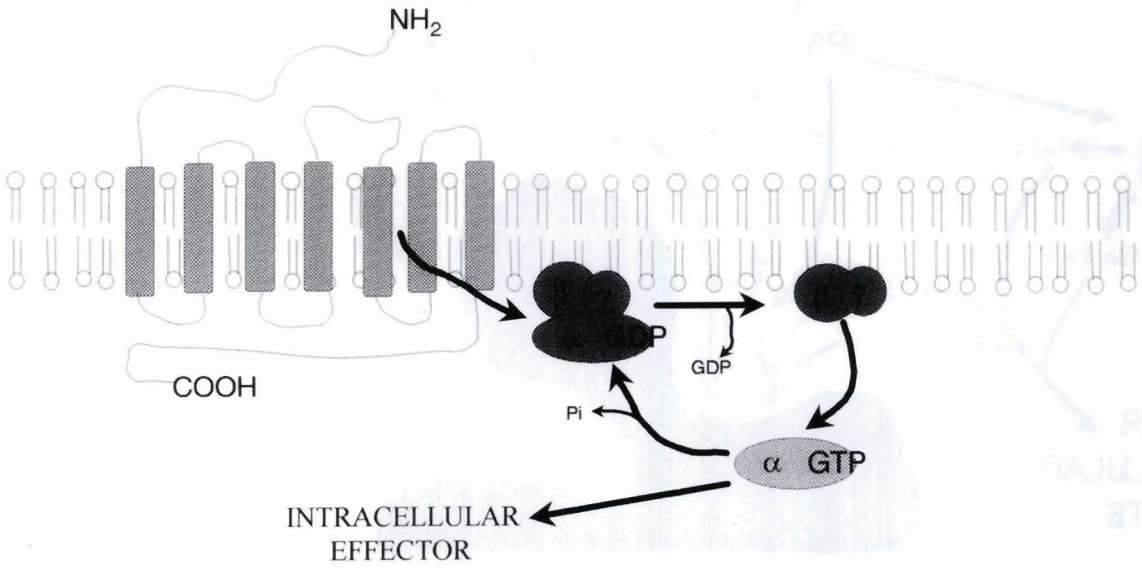


Fig. 2. The interaction between heterotrimeric G-proteins and the chemotactic receptor, with the generation of an intracellular signal. The fMLP-type receptor (seven transmembrane domains), coupled to the G-protein (αßγ), causes the exchange of guanidine diphosphate (GDP), and guanidine triphosphate (GTP) to activate the G-protein and cause dissociation.

activities, such as NADPH oxidase components (respiratory burst), and vimentin (cytoskeleton). Calcium is involved in regulating the assembly of F-actin, and pseudopod formation, which occurs during chemotaxis (Fernandez-Segura et al., 1995), phagocytosis, and adhesion (Westlin et al., 1992). An increase in cytosolic free calcium is associated with phagocytic events, but not necessarily essential for them to occur (Theler et al., 1995). Release of calcium ions during phagocytosis is mediated by the occupation of the Fc γ receptors. Phagocytic events may also result in the movement of calcium stores to the periphagosomal region of the cell (Sawyer et al., 1985; Stendahl et al., 1994; Theler et al., 1995). Cytosolic free calcium increases during non-specific adherence (Jaconi et al., 1988), and can be correlated to neutrophil spreading (Kruskal et al., 1986; Hendry and Maxfield, 1993; Theler et al., 1995). The effect of cytosolic free calcium concentration on actin filament assembly and disassembly are mediated by gelsolin (Howard et al., 1990), and profilin (Goldschmidt-Clermont, 1990). The range of actions of calcium and IP3 are reviewed by Albritton et al. (1992).

(iii) Other signalling mechanisms.

The active form of the heterotrimeric G-proteins can also be coupled to other effector molecules, and may cause the formation of a number of other intercellular, or possibly intracellular messengers, in addition to calcium. Phospholipase A2 hydrolyses phosphatidylcholine and phosphatidylethanolamine to produce arachidonic acid. This, with DAG and calcium ions, activates protein kinase C, and is a precursor for the formation of leukotriene B4 (LTB4), secreted as a chemoattractant to recruit more neutrophils (Ford-Hutchinson et al., 1980). Phospholipase D hydrolyses phosphatidylcholine, to produce DAG and choline. DAG can be enzymatically converted to phosphatidic acid, which may be a second messenger for oxidase activation (Agwu et al., 1991; Dana et al., 1994).

Another activity of G-proteins in neutrophils, which remains unclear, is the modulation of the cytosolic cyclic-AMP concentration (Tsu et al., 1995). In other cells responding to hormonal stimulation, different receptors coupled to G-proteins can either activate, or inhibit the adenylate cyclase enzyme, which in its active form, causes an increase in cAMP levels. The cAMP then either activates, or inhibits, protein kinases, causing different intracellular effects. One of the functions of calcium-calmodulin is to activate the phosphodiesterase enzyme, which decreases the cAMP concentration. The presence of cAMP has been demonstrated in neutrophils, but little is known about its functions.

The importance of the Ca^{2+} store in neutrophils

The use of increasingly effective fluorescent microscopic techniques, and the wide variety of calcium indicators, buffers and chelators, has resulted in exciting new developments in calcium signalling research in

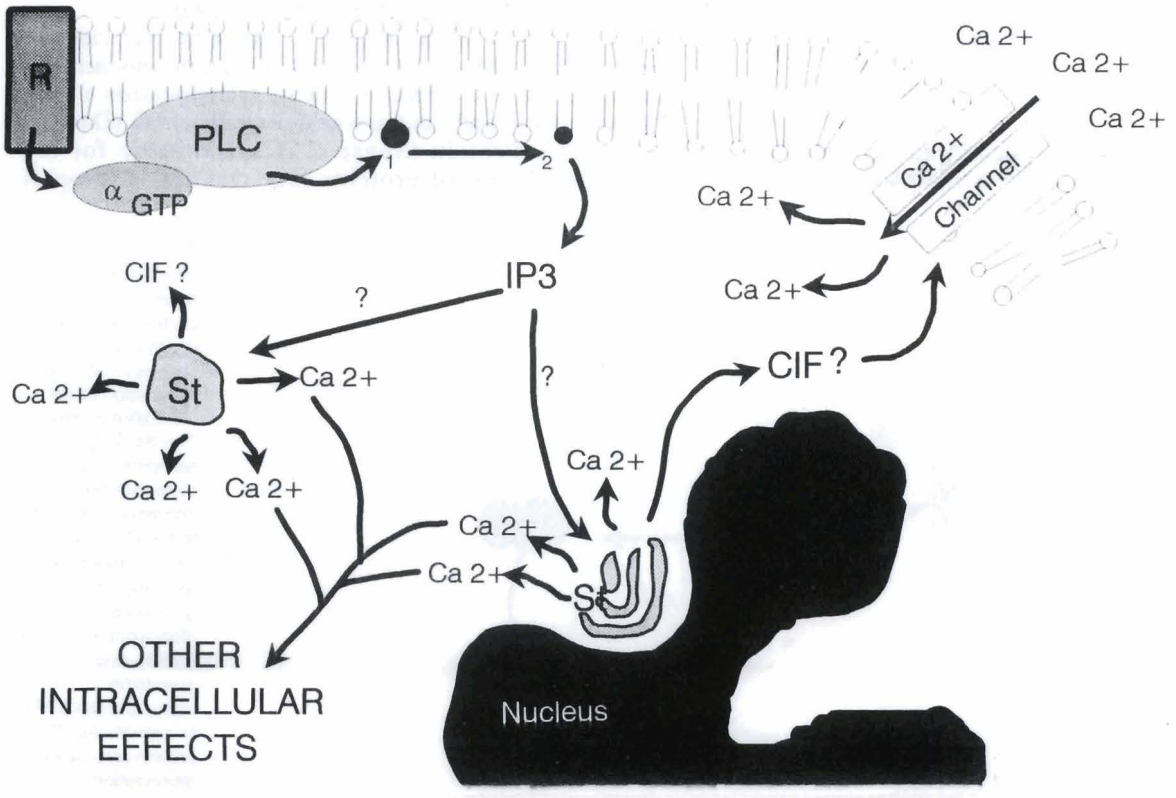


Fig. 3. Intracellular calcium signalling. Generation of inositol trisphospate (IP_3) by activated phospholipase Cγ (PKC) causes calcium. ion release (Ca^{2+}) from intracellular stores (St). PKC is activated by the active form of the G-protein, generated by the receptor interaction (R). 1: phosphatidyl-inositol-4,5-bisphosphate; 2: diacyl-glycerol; CIF: calcium influx factor; α: activated G-protein.

human neutrophils. Recent research (Parekh et al., 1993; Randriamampita and Tsein, 1993; Davies and Hallett, 1995a) has suggested that "calcium influx factor" (CIF) may be liberated from stores at the same time as calcium itself. CIF is thought to diffuse to the plasma membrane, and cause opening of calcium channels there, allowing extracellular calcium ions to flood the cytosol, and replenish the emptied stores. There has been lively debate concerning the truth, and relevance, of these observations (Berridge, 1996; Hallett et al., 1996).

Closely tied to the CIF debate, has been the observation by several workers of discrete locations within the neutrophil from which, calcium is released after cell stimulation by a variety of pathways (Burgoyne et al., 1989; Stendahl et al., 1994). The two stimulation pathways that have formed the basis of these observations are mediated by the two main receptor types on the neutrophil surface. The chemoattractant receptors have been the classical model in neutrophil signalling. Almost all of our present knowledge of neutrophil signalling processes is derived from the study of this type of receptor, and its agonists. Recently, however, a second type of receptor has been more widely studied. This class of receptor includes the functionally important Fc receptors, and the $ß_2$ (CR3, Mac1) integrin family of adhesion/complement receptors. The fundamental difference between these types of receptor are that a one-to-one occupation of the second type does not result in signal transduction. Ligand recognition must be followed by some form of physical connection - "clustering" - before signal transduction is initiated. Experimentally, this has usually been achieved by crosslinking, using a secondary antibody.

Recently, crosslinking of the FcγRII (CD32) on the surface of neutrophils, by immune complexes or by secondary antibody has been reported to increase cytosolic free Ca^{2+} (Davies et al., 1994; Davies and Hallett, 1995b). Similarly, the $ß_2$ integrin family were originally thought to be adhesion receptors, with no role in signal transduction or neutrophil activation. However, several workers have reported that crosslinking the integrin causes cytosolic free calcium concentration to increase (Ng-Sikorski et al., 1991; Petersen et al., 1993). Recently, novel techniques have pinpointed the intracellular origin of calcium store release mediated by the engagement, or engagement and clustering, of the types of receptor discussed above (Pettit and Hallett, 1996a,b). Here, we present recent findings concerning the location, and functional roles, of different calcium stores in human neutrophils.

Calcium store location

Functionally, calcium stores can be defined as membrane bound organelles containing calcium at a higher concentration than the cytosol. This is achieved by both an inwardly-facing Ca^{2+}-ATPase, which "pumps" calcium into the store, and also a calcium binding protein within the organelle, which increases the capacity of the organelle. Attempts to locate the site of calcium storage in neutrophils have been of two types; (i) electron microscopy, or (ii) fluorescently labelled probes. At the EM level, organelles called calciosomes have been identified, which contain the calcium binding protein, calsequestrin. These small organelles are widespread throughout the cell (Volpe et al., 1988). A fluorescent probe, chlortetracycline, which binds calcium may be used to locate calcium stores (Caswell, 1979; Naccache et al., 1979a,b). Although it cannot give a quantitative measure of calcium concentration, chlortetracycline is accumulated in calcium storage sites, because the calcium bound form of the molecule does not freely diffuse across membranes. This feature enables the precise localisation of high Ca^{2+} regions in neutrophils (Fig. 4).

We have identified two regions of high calcium concentration in neutrophils, one towards the centre of the cell, the other under the plasma membrane, on the cell periphery (Fig. 4). The central store may correspond to the region of vestigial, Golgi-like ER, between the lobes of the nucleus (Fig. 1), and the peripheral store, to the one released during integrin engagement and clustering (Pettit and Hallett, 1996a,b), and Fc receptor crosslinking (Davies, et al., 1994; Davies and Hallett, 1995b, 1996).

The distinct locations of these two storage sites may indicate different functional roles in neutrophil signalling and activation. In order to establish the relationship between structure and function, several different fluorescent microscopic techniques have been employed.

The single, juxta-nuclear Ca^{2+} store

The neutrophil response to fMLP involves the signal transduction pathway mediating the calcium activity described in the introduction, with IP_3 generating the

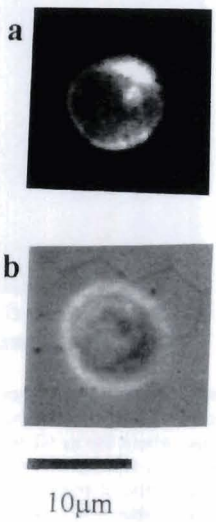


Fig. 4. Calcium storage sites detected by the accumulation of chlortetracycline. **a.** The fluorescent image, recorded by excitation at 380nm and emission greater than 410nm, and captured on an intensified CCD camera. **b.** The same cell with bright field illumination.

store release signal. This release is followed almost instantaneously by calcium influx, so that the two activities cannot normally be resolved. However, the removal of calcium ions from the extracellular medium by a chelator, such as EGTA, or prevention of calcium influx by the channel blocker, SKF96365, allows the visualisation of store release by preventing the calcium influx (Davies, et al., 1991, 1992; Pettit and Hallett, 1995). Under these conditions, ratiometric imaging of neutrophils loaded with Fura-2 has shown that cytosolic free Ca^{2+} rises initially from a single focus within the neutrophil (Fig. 5).

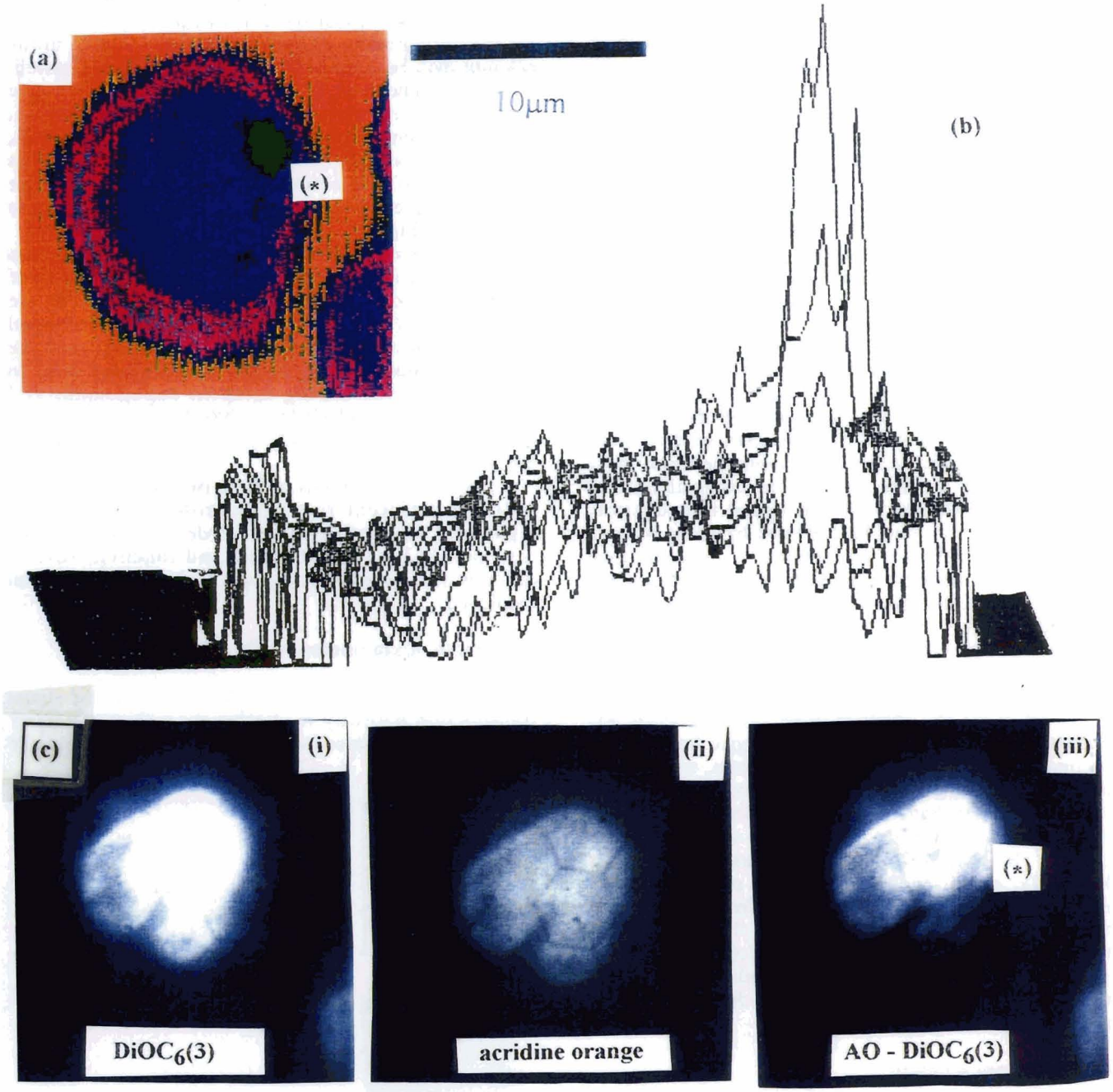


Fig. 5. The juxta-nuclear Ca^{2+} release site in neutrophil. The figure shows the localised release of Ca^{2+} from the store induced by f-met-leu-phe (1μM) in the absence of extracellular Ca^{2+} (**a**) as a ratiometric image of a fura2 loaded neutrophil, where blue and green represents resting and high cytosolic free Ca^{2+} respectively, and (**b**) as a pseudo-3D map where the xy plane are the dimensions of the cell and the z axis is proportional to the cytosolic free Ca^{2+} concentration. In the lower panel, the same cell is stained with (i) acridine orange, as a marker for the position of the nucleus, (ii) $DiOC_6(3)$ as a marker for membranous organelles, and (iii) by subtraction of image (i) from image (ii) the non-nuclear membranous structures. In the final image (iii), a "hot spot" of membrane staining (*) is observed at a position which corresponds to the position of elevated Ca^{2+} (*) in the Ca^{2+} image (a).

Recently, rapid time scan confocal microscopy of neutrophils loaded with the calcium indicator, Fluo3 (Merritt et al, 1991; Pettit and Hallett, 1996b) has provided further details of the location of the release site, and has permitted its visualisation, even in the presence of extracellular calcium. This demonstration was only possible because the time resolution of the laser scanning technique was sufficiently fast to resolve store release from influx, with both events occurring within approximately 200ms. This was also demonstrable by conventional imaging of unusually large myeloid cells, where the timing between store release and influx was tens of seconds (Roberts, et al., 1995). The use of EGTA, and the calcium channel blocker, nickel, confirmed that the activity was indeed calcium store release (Pettit and Hallett, 1995). In addition, this approach has enabled the visualisation of Ca^{2+} diffusion from the store after release. Ca^{2+} release from the site does not diffuse much beyond the nuclear lobes. Before calcium had diffused to the membrane from the centrally located store, influx had already begun. This result was consistent with the presence of another, more rapidly diffusible messenger, such as CIF (Pettit and Hallett, 1995).

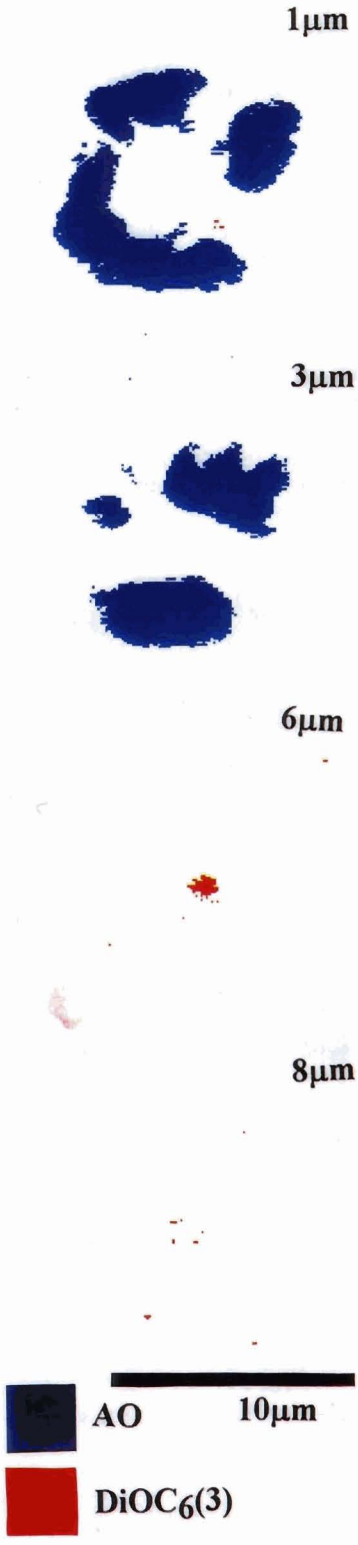


Fig. 6. Relationship of nuclear lobes to central organelle. Confocal XY slices at 1 μm steps, from top to bottom (1, 3, 6 and 8 μm shown) were taken through a neutrophil loaded initially with $DiOC_6(3)$ (0.25 μg/ml) to display membranous organelles, and acridine orange (5ng/ml, 5 mins) to display the nuclear lobes. The relationship between the two structures was determined by the superimposition of the two sets of confocal slices, showing in this case, the DiOC6(3) staining above the nuclear lobes.

The question arises as to what micro-anatomical structure is responsible for the Ca^{2+} release at this site. By using the fluorescent membrane-staining dye $DiOC_6(3)$, which stains endoplasmic reticulum membrane (Terasaki et al., 1984), and acridine orange, to stain the nuclear lobes, the relationship of the release site to these structures has been established. By conventional fluorescence microscopy, the release site was associated with a $DiOC_6(3)$ staining organelle near the nuclear lobes (Fig. 5). The three-dimensional relationship of the Ca^{2+} release site to these microanatomical components, has now been established using confocal optical sectioning. Following the detection of calcium activity in neutrophils, they were stained with $DiOC_6(3)$, and acridine orange in turn. The z-stepper function of the confocal microscope allowed serial optical sections to be taken through the cell (Fig. 6), which could be superimposed and reconstructed in three dimensions. Rotation of the resultant image showed the relative positions of the store and the nucleus from several different angles (Fig. 7). The data can also be viewed as a "wide-eye" random dot image (Fig. 8). This central location also explains the delay in sensing calcium at the plasma membrane, using the membrane probe, FFP-18 (Davies and Hallett, 1996).

From these observations, we concluded that the calcium store released by the addition of fMLP was the central, chlortetracycline- and $DiOC_6(3)$-stained membranous organelle, nestled in the space between the nuclear lobes, or, in a few cases (Fig. 6), just above or below the plane of the centre of the nuclear lobes. In the vast majority of cases, the distance from the store site to the plasma membrane precluded any physical link between the two from being responsible for the initiation of calcium influx.

The peripheral Ca^{2+} stores

The peripheral calcium stores detected by chlortetracycline staining can be released by engagement and

clustering of both Fc, and $ß_2$ integrin receptors on the neutrophil surface. The signal transduction pathways involved in the release of calcium from the peripheral stores remain to be elucidated. The role of G-proteins and IP_3 are unclear.

Crosslinking of the FcγRII (CD32) on the surface of neutrophils, by immune complexes or by secondary antibody results in a biphasic increase in cytosolic free Ca^{2+}. The first phase of this activity involves IP_3-independent calcium store release. Like the fMLP response, the second phase is calcium influx, but this stage is temporally isolated from, and can occur in the absence of, initial store release (Davies, et al., 1994; Davies and Hallett, 1995b). Unlike the situation with fMLP, the membrane indicator FFP-18 which detects changes in cytosolic free calcium immediately inside the plasma membrane, suggests that the Ca^{2+} release sites triggered by FcγRII crosslinking are located close to the membrane (Davies and Hallett, 1996).

Calcium ions are also released from peripheral stores during $ß_2$ integrin clustering. Again, the store release and influx are sufficiently temporally dissociated to allow visualisation of the store release in the presence of extracellular calcium (Pettit and Hallett, 1995). Initial integrin clustering causes a calcium transient at the specific site of engagement (Fig. 9).

This calcium store was also visualised, in three-dimensional relationship to the nuclear lobes, using $DiOC_6(3)$, and acridine orange, and detected by the confocal z-stepper, or by the "sideways" XZ scan (Fig. 10). In several cases, both the peripheral, and central stores could be visualised in the same cell, using this method.

Physical and functional roles

There are fundamental differences in the effects on the neutrophil of calcium release from these two sites. Calcium release from the juxta-nuclear store is followed almost instantaneously by calcium influx, such that the two activities cannot usually be resolved. However, in the case of peripheral store release, the calcium influx is delayed by up to 100 seconds, so that the two phases of activity are easily resolved. In addition, store release is essential for influx in the case of integrin clustering (Pettit and Hallett, 1996a,b), but influx continues when store release is prevented before FcγRII crosslinking (Davies and Hallett, 1995a,b). This data would suggest

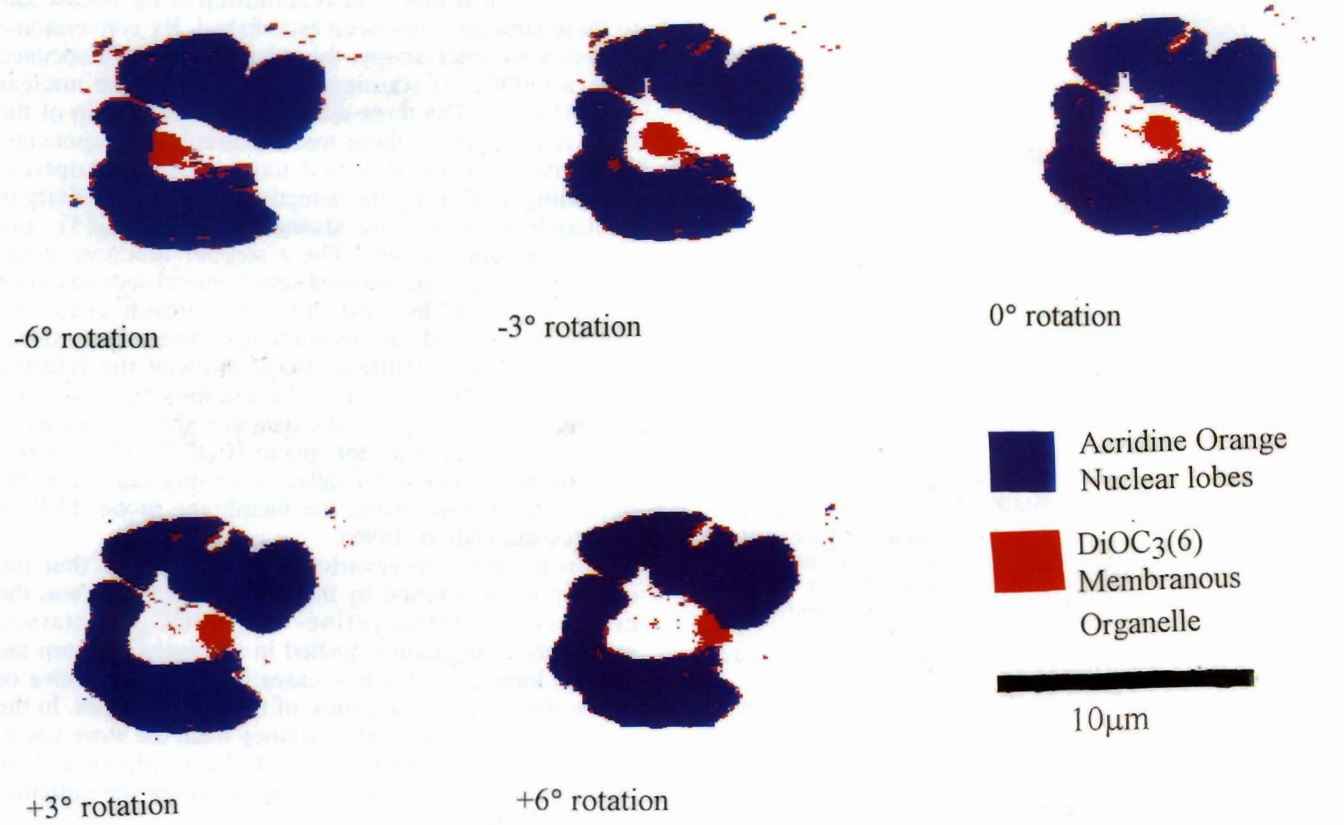
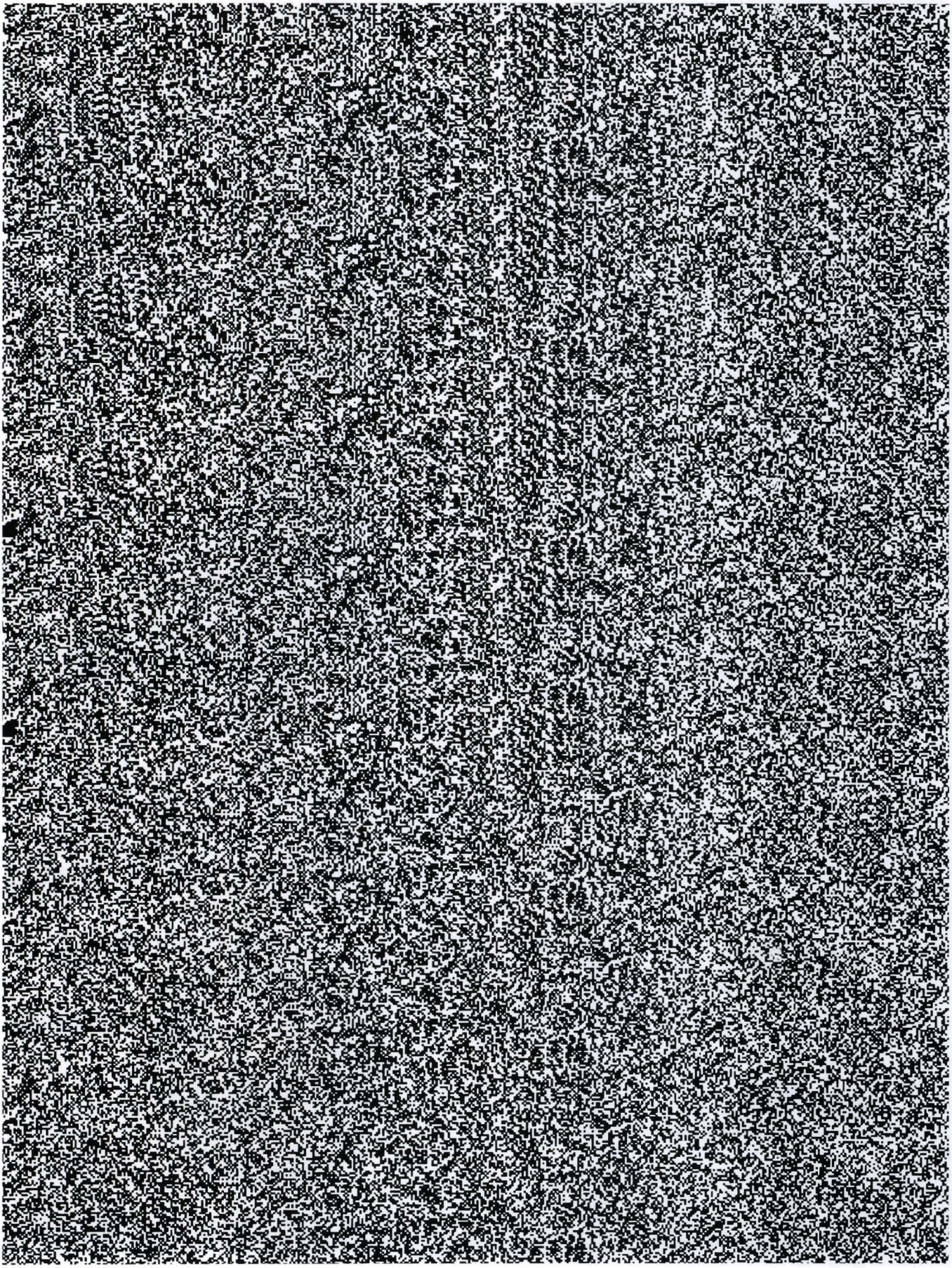


Fig. 7. The data from figure 6 is displayed, from below, as a three-dimensional reconstruction, and the same data rotated by +/- 3°, to show the relationship between the calcium store and nuclear lobes.

Fig. 8. A 'wide eye' random dot 3D image, generated from the data in figure 6. The eyes must be de-focused to view the 3D structure of the confocal slices. When this is achieved, the two dots at the top of the image merge to form three.



that there are different pathways for store release, and different relationships between store release and influx in each case. One difference between the two signal transduction mechanisms is that massive tyrosine phosphorylation activity occurs in response to integrin (Burridge, et al., 1992; Fuortes et al., 1994), or FcγRII clustering, but not in response to fMLP. Localised phosphorylation events could sensitise peripheral store IP_3 receptors, which in the absence of phosphorylation remain insensitive, leaving the central store to respond in this case (Jayraman, 1996). The site of the central store, between the nuclear lobes, is also the location of other interesting organelles in the neutrophil, such as the Golgi-like region, and the microtubule organising centre (MTOC). Although most workers quote the Golgi region as the calcium store, the MTOC has also been identified as playing a role in calcium release in other cells (Brundage et al., 1993). It will be possible to resolve which of these two structures is responsible for the calcium release, by using electron microscopic immuno-localisation of molecules such as IP_3 receptor and Ca^{2+} ATPase. This will provide the link between the structure, and its function in calcium signalling.

Conclusion

The evidence for a "two-stores, two-pathways" signalling arrangement in human neutrophils is gradually accumulating. There is release of calcium from a single, centrally located store upon fMLP addition, and recent work by several groups has documented the presence of peripheral stores (Fig. 11). Many questions now remain to be answered in the search for full understanding of the physical and functional roles of calcium stores in human neutrophils. The localisation of calreticulin, calcium ATPase and IP_3 receptors in relationship to calcium release will be an important step, as will further investigation of the role of tyrosine phosphorylation, and the use of caged IP_3 to release stores in different parts of the cell. Understanding these associations will also shed light on the continuing CIF debate, as well as mechanisms of phagocytosis mediated by antibody Fc fragments, and complement component C3bi.

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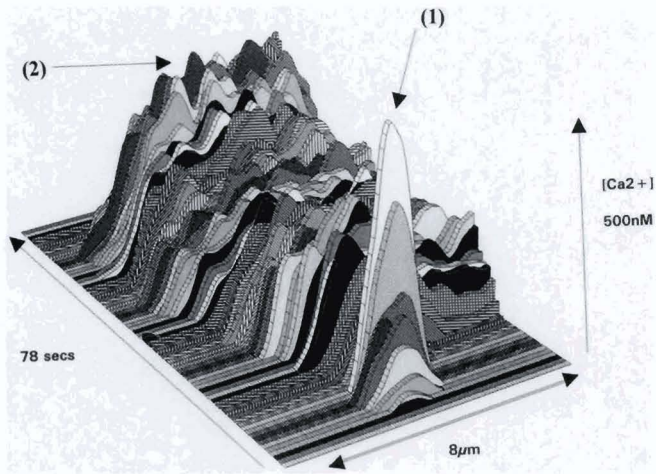

Fig. 9. Initial store release, followed by calcium influx, in a neutrophil undergoing engagement and clustering of CD11b/CD18 integrins. Data from the XT (rapid time) scan mode of the confocal microscope. In order to capture the store release event, the laser scanning line was positioned no more than 1 μm above the integrin engaging substrate. The data can be displayed as a three-dimensional graph, showing the increase in cytosolic free calcium (y axis), neutrophil diameter in contact with the integrins (x axis), and time (z axis). Store release (1) and calcium influx (2) are arrowed.

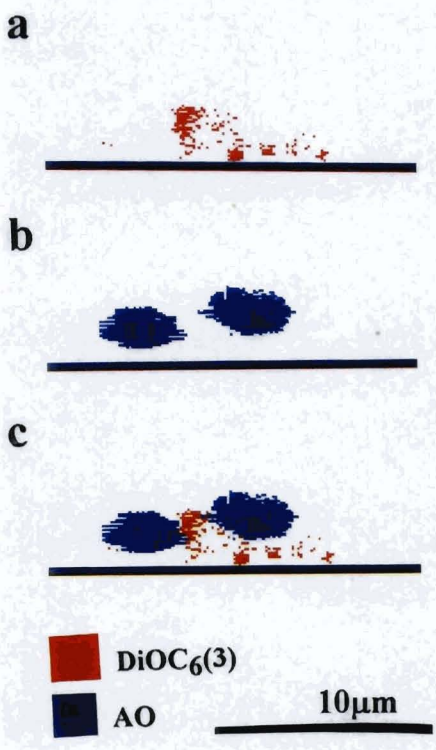

Fig. 10. Confocal slices in the XZ direction were taken through a neutrophil loaded initially with **(a)** $DiOC_6(3)$ (0.25μg ml) to display membranous organelles, and **(b)** acridine orange (5ng/ml, 5 mins) to display the nuclear lobes. The relationship between the two structures was determined by the superimposition of the two sets of confocal slices **(c)**.

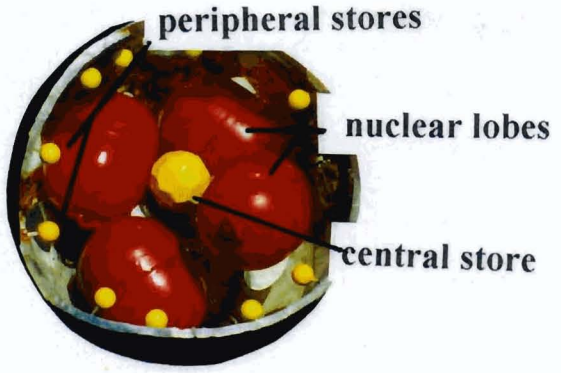

Fig. 11. Three dimensional model of a human neutrophil, showing the juxta-nuclear and peripheral stores, and their relationship to the nuclear lobes.

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