# Phagosomal oxidative activity during β2 integrin (CR3)-mediated phagocytosis by neutrophils is triggered by a non-restricted $Ca^{2+}$ signal: $Ca^{2+}$ controls time not space

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## Summary

**The temporal and spatial relationship between particle binding to the neutrophil by β2 integrin (CR3), the $Ca^{2+}$ elevation and subsequent oxidase activation has been unclear. This is because of the difficulty in studying the time course of individual phagocytic events in individual neutrophils. Here, we have used a micromanipulation technique to present C3bi-opsonised zymosan particles to the neutrophil under observation. In this way, the moment of particle contact, pseudopod formation and internalisation has been established and cytosolic free $Ca^{2+}$ and oxidation of dichlorodihydrofluorescein (DCDHF)-labelled particles determined simultaneously. Using this approach, we have found that the $Ca^{2+}$ signal, which is triggered by CR3-mediated phagocytosis, can be resolved into two temporally separated components. The first $Ca^{2+}$ signal occurs during β2 integrin engagement as the phagocytic cup forms but does not trigger oxidation of the particle. The second global $Ca^{2+}$ signal, which is triggered about the time of phagosomal closure, causes an abrupt activation of the oxidase. This second $Ca^{2+}$ signal was not restricted to the region of the phagosome yet only triggered the oxidase activation locally in the phagosome, with no evidence of activation at other sites in the neutrophil. This points to a dual control of oxidase activation, with $Ca^{2+}$ controlling the timing of oxidase activation but slower and more localised molecular events, perhaps involving oxidase assembly and phosphatidylinositol 3-phosphate generation, determining the site of oxidase activation.**

Movies available online

Key words: Neutrophils, Phagocytosis, Cytosolic free $Ca^{2+}$, Oxidase activation, β2 integrin

## Introduction

A key event in combating infection is mediated by a programme of activity by circulating neutrophils, which includes neutrophil extravasation, chemotaxis and phagocytosis. It can be argued that the purpose of this series of separate cellular events is to bring the non-mitochondrial oxidase of the neutrophil in close proximity to infecting microbes. This results in the killing of the ingested microbe either directly by oxidase-generated reactive oxygen species, indirectly by oxidants generated subsequently by intraphagosomal myeloperoxidase or by regulation of the intraphagosomal pH or $K^+$ ion concentration to activated proteases (Reeves et al., 2002). Oxidase activation results after the assembly of its components in the phagosomal membrane (Segal, 1996) signalled by binding of phospahtidylinositol 3-phosphate [PtdIns(3)*P*] through PX domains on p40phox and p47phox (Ellson et al., 2001a; Kanai et al., 2001). In vivo, particulate phagocytic stimuli are rapidly opsonised by the complement component C3bi, for which there is a receptor on neutrophils, CR3, the $\alpha_m\beta_2$ integrin (also called CD11b/CD18). Experimentally, engagement of CR3 has been shown to trigger changes in cytosolic free $Ca^{2+}$ (Ng-Sikorski et al., 1991; Jaconi et al., 1991; Petersen et al., 1993). Because, experimentally, an elevation in cytosolic free $Ca^{2+}$ is well known to trigger activation of the oxidase (e.g. Lew et al., 1984; Al-Mohanna and Hallett, 1988), it has been suggested that the CR3-mediated $Ca^{2+}$ signal would trigger oxidase activation during CR3-mediated phagocytosis. However, the temporal and spatial relationship between particle binding to the neutrophil by CR3, the $Ca^{2+}$ elevation and subsequent oxidase activation has been unclear. In part, this is because of the difficulty in studying the time course of individual phagocytic events in individual neutrophils. However, here we have used a micromanipulation technique to present C3bi-opsonised particles to the neutrophil under observation (Dewitt and Hallett, 2002). The moment of contact, pseudopod formation and internalisation has been established with this technique and cytosolic free $Ca^{2+}$ and oxidation of labelled particles determined simultaneously. Using this approach, we have found that the $Ca^{2+}$ signal, which is triggered by CR3-mediated phagocytosis, can be resolved into two temporally separated components. The first $Ca^{2+}$ signal occurs during integrin engagement but does not trigger oxidation of the attached particle. The second global $Ca^{2+}$ signal, which is

triggered about the time of phagosomal closure, causes an abrupt activation of the oxidase. This second $Ca^{2+}$ signal was not restricted to the region of the phagosome, yet only triggered the oxidase activation locally in the phagosome, with no evidence of activation at other sites in the neutrophil. This points to a dual control of oxidase activation, with $Ca^{2+}$ controlling the timing of oxidase activation, but slower and more localised molecular events, perhaps involving oxidase assembly and PtdIns(3)*P* generation, determining the site of oxidase activation.

## Materials and Methods

### Neutrophil isolation

Neutrophils were isolated from the heparinised blood of healthy or myeloperoxidase (MPO)-deficient volunteers as previously described (Hallett et al., 1990). A single MPO-deficient volunteer was recruited with a well-characterised MPO-deficiency (Clark et al., 2002). Informed consent was obtained from all volunteers. Following dextran separation, hypotonic lysis of red cells and centrifugation through Ficoll-Paque, neutrophils were resuspended in Krebs buffer (120 mM NaCl, 4.9 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).

### Preparation of C3bi-opsonised oxidant indicator zymosan

Zymosan particles (10 mg/ml) were opsonised either by incubation with human serum (50% diluted, 30 minutes, 37°C) or with purified human C3bi (1 mg/ml; 30 minutes, 4°C). The particles were then washed by centrifugation and resuspension to remove unfixed C3bi and either labelled immediately or stored at –20°C. Opsonised zymosan labelling was achieved in bicarbonate buffer (0.1 M, pH 8.3, 10 mg/ml) ($NaHCO_3$ and HEPES buffer-titrate). Succimidyl ester of 2′7′dichlorodihydrofluorescein diacetate (1 mg, Molecular Probes, Eugene, Oregon) was dissolved in 100 μl DMSO (anhydrous), and added dropwise to zymosan (1 ml), mixing between each drop. The solution was then drawn into a foil-coated syringe, capped to exclude air and allowed to react overnight at 4°C. This resulted in coupling between the acetylated, oxidant-insensitive probe and the zymosan. In order to de-acetylate the product to generate the oxidant-sensitive dichlorodihydrofluorescein (DCDHF) for use, 100 μl hydroxylamine (105 mg/ml) was added to the suspension, which was then withdrawn into a syringe and capped for a further 3 hours. The DCDHF-labelled zymosan suspension was washed several times by centrifugation and resuspension with BSS (balanced salt solution), aliquotted and stored at –20°C. Fluorescein isothiocyanate (FITC) was conjugated to zymosan by over-night incubation, at 4°C at pH 9.0 as previously described (Morris et al., 2003).

### Micromanipulated delivery of particles

Neutrophils were allowed to adhere to glass coverslips for 1-2 minutes. Zymosan particles were allowed to sediment among cells and a micropipette (tip diameter 1 μm) was brought into the field of view. A particle was drawn into the mouth of the micropipette by applying slight negative pressure, and was lightly placed in contact with a neutrophil. After adherence between cell and particle was established, the zymosan was released by removing the negative pressure and phagocytosis allowed to proceed (Dewitt and Hallett, 2002).

### Simultaneous cytosolic free $Ca^{2+}$, oxidase and phase contrast imaging

Neutrophils were loaded with the $Ca^{2+}$ indicator, fura-2-AM as previously described (Hallett et al., 1996) or fura2-dextran by micro-injection and allowed to adhere to glass coverslips maintained at 37°C with a temperature-controlled microscope stage heater. Light was transmitted to an inverted microscope (Nikon Eclipse) with an oil immersion 100× objective using a rapid monochromator (Delta RAM, PTI, Surbiton, UK) at three excitation wavelengths – 340 nm, 380 nm (for fura2) and 490 nm (for DCDHF) – delivered sequentially (see below). Phase contrast images were simultaneously taken under far red illumination (690 nm) using an appropriate dichroic mirror and a red-sensitive CCD camera. The fluorescent images were collected using a CCD camera (IC100, Photon Technology International, Surbiton, UK), and 340/380 nm ratio images and 490 nm intensity images were calculated and captured using ImageMaster (PTI, UK) software. The sequence of the excitation wavelengths was 340 nm, 490 nm and 380 nm, thus the two data sets for a single $Ca^{2+}$ ratio image and the DCDHF images were overlapping temporally. Images were acquired through the same emission filter with maximum transmission at 505 nm, with 16-frame averaging, threshold background subtracted and ratio or intensity calculated and pseudocoloured. The sum intensity (SI) (rather than average intensity) of the zymosan particles was used to monitor oxidase activity to avoid errors associated with the periphery of the zymosan particle where the nonoxidised indicator often had subthreshold fluorescence. The SI was therefore calculated as SI=Nø.Iav, where Nø is the number of non-zero pixels and Iav is the average intensity of the pixels.

### Micro-injection of fura2-dextran

The large molecular weight conjugate of fura2, fura2-dextran (molecular mass 10 kDa), was used to measure cytosolic free $Ca^{2+}$ concentration with reduced diffusion of the fura2-$Ca^{2+}$ complex within the cell. The fura2-dextran (Molecular Probes) was micro-injected into neutrophils by the simple lipid assisted micro-injection technique (SLAM) as previously described (Laffafian and Hallett, 1998). The probe was dissolved in intracellular medium (KCl, 150 mM, HEPES, 25 mM, pH 7.0) to give a final concentration of 500 μM and either loaded into a micropipette (tip diameter 0.5 μm), before tip lipid coating, as described previously (Laffafian and Hallett, 1998), or loaded into a pre-lipid-coated micropipette (Cell Engineering, Swansea, UK). On contact between the micropipette and the neutrophil, transfer of fura2-dextran into the cell was monitored by an increase in fluorescence at 360 nm to give intracellular concentrations of fura2-dextran of between 10-50 μM. After successful micro-injection, the neutrophils remained fully functional and able to undergo phagocytosis in response to challenge

## Results

### CR3-mediated phagocytosis produces a complex $Ca^{2+}$ signal

Following presentation of C3bi-opsonised zymosan particles (diameter 2 μm) to adherent neutrophils using a micropipette, binding to the cell and phagocytic cup formation begins before a large change in cytosolic free $Ca^{2+}$ is observed. This sequence has been previously reported (Suzuki et al., 1997; Dewitt and Hallett, 2002) and shown previously to result from $Ca^{2+}$ influx through $Ni^{2+}$-sensitive $Ca^{2+}$ channels and to precede and accelerate phagosome closure (Dewitt and Hallett, 2002). The global $Ca^{2+}$ change often displayed a distinctive double $Ca^{2+}$ peak, or ‘shouldered peak’ (Fig. 1a). Analysis of several $Ca^{2+}$ signals suggested that there were two separate $Ca^{2+}$ signals within each response and that the shape of the peak observed depended on the temporal separation between the two components of the $Ca^{2+}$ signal or the rate of the down slope ($Ca^{2+}$ reuptake). The qualitative shape of the $Ca^{2+}$ signal was constant within an individual neutrophil, with individual

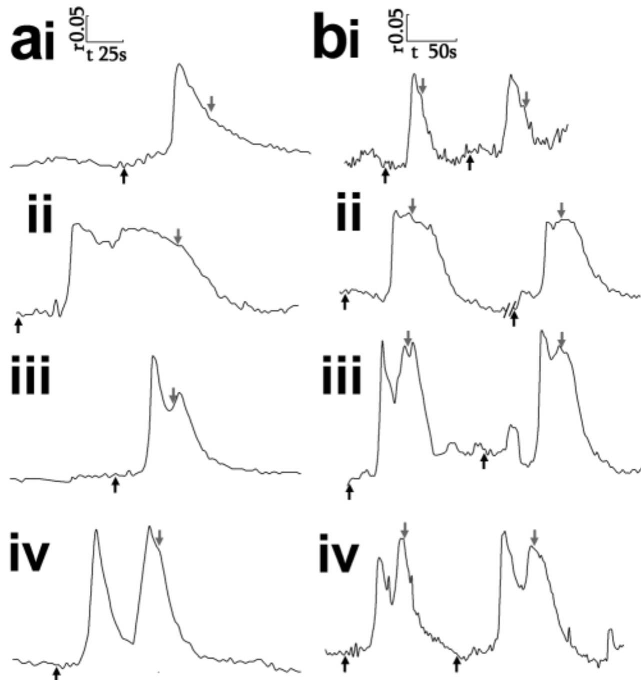


**Fig. 1.** Complexity of phagocytic $Ca^{2+}$ signals. A spectrum of β2 integrin-mediated $Ca^{2+}$ signals was observed. The left-hand column (a) shows examples of these $Ca^{2+}$ signals as (i) single peak, (ii) a single broad peak, (iii) a shouldered peak and (iv) a twin peak. The right-hand column (b) shows $Ca^{2+}$ responses in experiments in which individual neutrophils were challenged with two C3bi-opsonised particles. The character of the second response is similar to the first response by the same cell, whether (i) single peak, (ii) a single broad peak, (iii) a shouldered peak or (iv) a twin peak. In both sets of data, the upward arrow indicates the time of contact between the particle and the cell, and the downward arrow indicates closure of the phagosome.

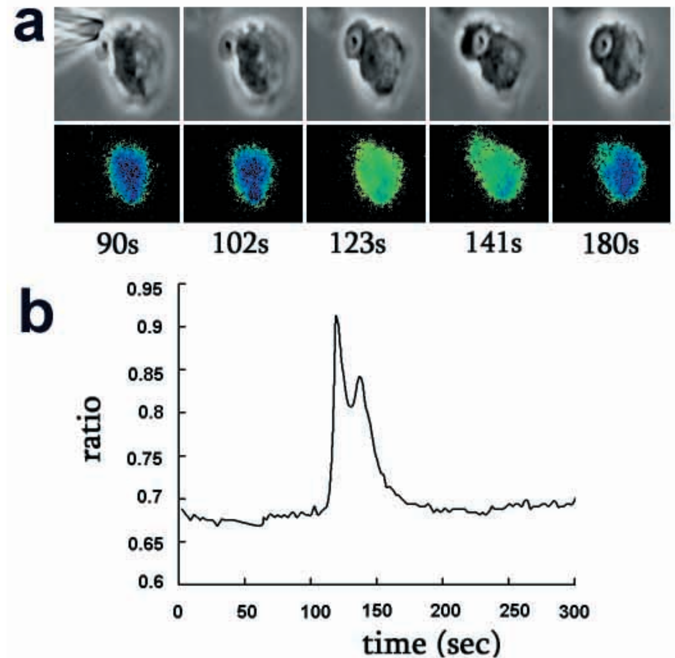


**Fig. 2.** Cytosolic free $Ca^{2+}$ changes during β2 integrin-mediated phagocytosis. The data show a typical experiment in which human neutrophils were presented with a DCDHF-labelled C3bi-opsonised particle for phagocytosis with simultaneous cytosolic free $Ca^{2+}$ measurement. (a) The upper row shows the phase contrast images and the second row shows the accompanying $Ca^{2+}$ images (fura2 ratio image pseudo-coloured to show changes in cytosolic free $Ca^{2+}$ concentration as a change from blue to green), acquired at the times shown below. The images illustrate some of the key features of the phagocytic event. The first image shows the micropipette presenting the particle to the cell; the second, adhesion of the particle to the cell without $Ca^{2+}$ signalling; the third, the formation of the phagocytic cup; the fourth, closure of the phagosome, and the fifth, completion of the event and the return of cytosolic free $Ca^{2+}$ to baseline. (b) The complete time course of the cytosolic free $Ca^{2+}$ data. This experiment was typical of at least 50 others.

neutrophils displaying the same double peak, 'shouldered' or single $Ca^{2+}$ in response to subsequent phagocytic stimuli (Fig. 1b). With nonopsonised zymosan and with neutrophils on which CR3 was blocked by antibody to CD11b/CD18 challenged with C3bi-opsonised zymosan, the characteristic $Ca^{2+}$ signal was absent, although sometimes a rise in $Ca^{2+}$ occurred after completion of phagocytosis (Dewitt and Hallett, 2002).

### Temporal relationship between $Ca^{2+}$ signalling and phagosomal oxidation

DCDHF-labelled zymosan particles triggered the same typical sequence of phagocytic events with particle binding, cup formation and local $Ca^{2+}$ signalling, giving rise to a global $Ca^{2+}$ rise due to influx (and inhibitable by $Ni^{2+}$) and then phagosome closure (Fig. 2). The global $Ca^{2+}$ change was also observed as a single transient increase, with a shoulder or double peak. The DCDHF-labelled zymosan particles were therefore a useful model for studying phagocytosis by neutrophils.

The simple addition of hydrogen peroxide ($H_2O_2$) to a suspension of DCDHF-labelled particles produced an increase in particle-associated fluorescence excited at 490 nm (Fig. 3a). The rate of $H_2O_2$-induced increase in intensity was greatly enhanced by peroxidase activity (Fig. 3c). After phagocytosis, C3bi-opsonised DCDHF-labelled zymosan particles that had been engulfed by adherent neutrophils were significantly brighter than those which had not (Fig. 3b). This was not a general effect of the phagosomal environment on the 'parent molecule', fluorescein, as fluorescein-labelled particles, which were insensitive to oxidation, did not increase in intensity during phagocytosis (4/4 cells) and often decreased in intensity (3/4 cells; Fig. 3d). Thus, the increase in fluorescence intensity of DCDHF-labelled zymosan during phagocytosis was used as a monitor of oxidative activity, particularly in the presence of peroxidase activity.

In order to establish the time sequence of oxidase activation and $Ca^{2+}$ signalling during phagocytosis, phase contrast images, the DCDHF signal from the particle and $Ca^{2+}$ signals from the cell were recorded simultaneously. Using this simultaneous imaging approach, it was immediately evident that the $Ca^{2+}$ signal in the neutrophil began many seconds before the onset of oxidase activation detectable by increased DCDHF fluorescence (42/47 cells). To determine whether the onset

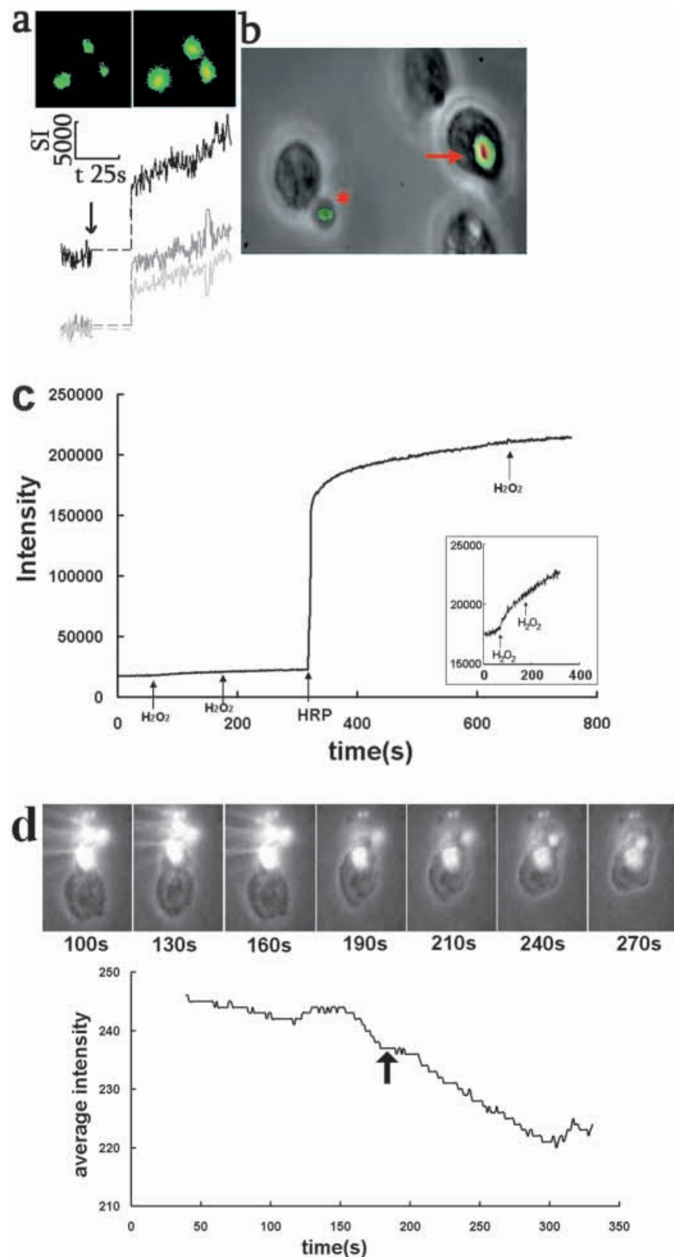


**Fig. 3.** Use of DCDHF as an oxidative indicator during phagocytosis. (a) Three DCDHF-conjugated zymosan particles are shown before and after addition of $H_2O_2$ (0.83 mM). The traces below show the time courses for the increase in fluorescence (SI=summated intensity) for the three particles with the arrow indicating the addition of $H_2O_2$. (b) The fluorescence intensity of internalised (arrowed) and adherent (asterisk) DCDHF-conjugated zymosan particles are compared. The DCDHF intensity image and the phase contrast image have been superimposed for clarity. (c) The dependence of the oxidation response on peroxidase activity is shown. The first two arrows show the addition of $H_2O_2$ (0.83 mM and 1.66 mM, respectively), enlarged in the inset figure. The third arrow shows the addition of horseradish peroxidase (0.25 units/ml), and then a further increase in $H_2O_2$ (2.49 mM). (d) A typical experiment in which FITC-conjugated zymosan particles were internalised is shown. The images show the internalisation of the particles and the graph shows the accompanying intensity change. This result was typical of four other experiments.

correlated with any of the phases of the complex $Ca^{2+}$ signal, neutrophils that displayed distinct double $Ca^{2+}$ peaks were chosen for analysis because the separate $Ca^{2+}$ phases could be more easily distinguished and timed. In the majority of the cells studied (18/25 cells), the onset of DCDHF oxidation (increased fluorescence) was shown to correlate with the second of the two $Ca^{2+}$ peaks (Fig. 4a). It was difficult to time the onset of oxidation accurately in the other cells tested (7/25), either because the particle was moving in and out of focus during phagocytosis or because there was little oxidative increase after phagocytosis. The relationship between oxidation and the later $Ca^{2+}$ signal was not unique to these 'twin peak' cells, as it was also evident in cells with less obvious separation of the two signals, such as those giving a $Ca^{2+}$ shouldered response (Fig. 4b).

The second $Ca^{2+}$ event usually occurred around the same time as phagosomal closure. In the majority of cells, phagosomal closure occurred just after the second peak in the $Ca^{2+}$ signal (21/33); in some cells (8/33) phagosomal closure occurred while the second $Ca^{2+}$ signal was subsiding, but in one cell, phagosomal closure occurred just before the second peak, and in the remainder (3/33), phagosome closure occurred after the return of the second $Ca^{2+}$ peak to base-line. As oxidative activation also occurred near the time of phagosome closure and the second $Ca^{2+}$ peak (see Fig. 4a,b), we considered the possibility that the oxidase was actually activated and peroxidase secreted before phagosome closure, but that the local oxidant and peroxidase concentrations were too low (as a result of diffusional dilution before phagosome closure) to be effective in causing oxidation of DCDHF on the zymosan until the phagosome closed, preventing dilution. However, this explanation seemed unlikely to account for the abrupt increase in oxidation within the phagosome because first, this mechanism would be expected to cause a gradual increase in DCDHF fluorescence, in contrast to the abrupt changes observed (Fig. 4a,b, Figs 5 and 6); second, there was no evidence that the base of the zymosan particle deepest in the phagocytic cup, where the diffusion path to the outside was longest, became oxidised earlier or before phagosome closure (e.g. Fig. 4a,b, Fig. 6); and third, in some cells (e.g. Fig. 4a,b), phagosome closure occurred several seconds before the onset of detectable oxidation, there being no sudden effect of oxidation at the time of phagosomal closure. It was therefore concluded that during phagocytosis, activation of the oxidase and triggering of the release of peroxidase into the phagosome did not occur during the first global change in $Ca^{2+}$ signal but correlated temporally with the second phase of the $Ca^{2+}$ signal.

To establish the role for the phagocytic-induced $Ca^{2+}$ signal in oxidase activation, two pharmacological inhibitors of the $Ca^{2+}$ signal were used. $Ni^{2+}$ (2 mM), which blocked $Ca^{2+}$ influx, totally prevented the global $Ca^{2+}$ signal and slowed phagocytosis (Dewitt and Hallett, 2002). Blocking $Ca^{2+}$ influx in this way also reduced the increase in DCDHF-zymosan fluorescence, the oxidative response being significantly reduced by $Ni^{2+}$ ($P<0.05$, $n=6$, Fig. 5). The phosphatidylinositol 3-kinase [PtdIns(3) kinase] inhibitor, LY294002 (50 μM), also inhibited the $Ca^{2+}$ signal and also slowed phagocytosis (Dewitt and Hallett, 2002). Again, there was a significant decrease in the oxidative response (Fig. 5). It was therefore concluded that during phagocytosis, the global $Ca^{2+}$ signal was obligatory for maximum phagosomal oxidation (during the second $Ca^{2+}$

phase), but it was not sufficient alone to trigger oxidative activation (in the first $Ca^{2+}$ phase).

### Contribution of myeloperoxidase degranulation to oxidation signal

As myeloperoxidase (MPO), which is responsible for the generation of HOCl from $H_2O_2$ in neutrophil phagosomes, is delivered to the phagosome by granule-phagosome fusion, and has a major effect on DCDHF-zymosan oxidation (Fig. 3), it was important to consider the role of this enzyme in the oxidation signal observed. DCDHF-labelled zymosan was therefore delivered to neutrophils in the presence of the potent MPO inhibitor, azide (10 mM $N^{3-}$). Under these conditions, $Ca^{2+}$ signalling was normal, but the fluorescent signal from internalised particles was significantly reduced ($P<0.01$), although it could not be totally inhibited ($n=6$) (Fig. 5). This may have resulted from an inability of azide to cause total peroxidase inhibition, as the residual response could be inhibited further by the $Ca^{2+}$ channel blocker, $Ni^{2+}$ ($P<0.001$, $n=2$, Fig. 5). This reduced the response to that observed in MPO-deficient neutrophils ($P<0.001$, Fig. 5). In these latter cells, the oxidative response was restored to normality by addition of extracellular MPO (5.5 nM, by haem content) or horseradish peroxidase (0.25 units/ml) before phagocytosis. It was therefore concluded that delivery of MPO to the phagosome played an important role in the DCDHF-oxidation signal.

In the absence of MPO-activity (MPO-deficiency), although the DCDHF signal was reduced and slowed as with $N_3^-$ treatment, a detectable response was observed which commenced at the second phase of the $Ca^{2+}$ signal. As this suggested that the second phase of the $Ca^{2+}$ signal was also important in activating the oxidase (in the absence of peroxidase degranulation), this proposal was tested by measuring oxidation of DCDHF-zymosan during phagocytosis in the presence of extracellular MPO or HRP (0.25 units/ml). This strategy, which would eliminate the dependence of degranulation of peroxidase into the phagosome, had no effect on the timing of the DCDHF response. It was therefore concluded that the oxidase (or delivery of preformed oxidant) was not activated earlier than the second phase of the $Ca^{2+}$ signal and that oxidase activation occurred with the second $Ca^{2+}$ signal.

### Detection of oxidative activity was restricted to the phagosome

As the second $Ca^{2+}$ signal triggered oxidative

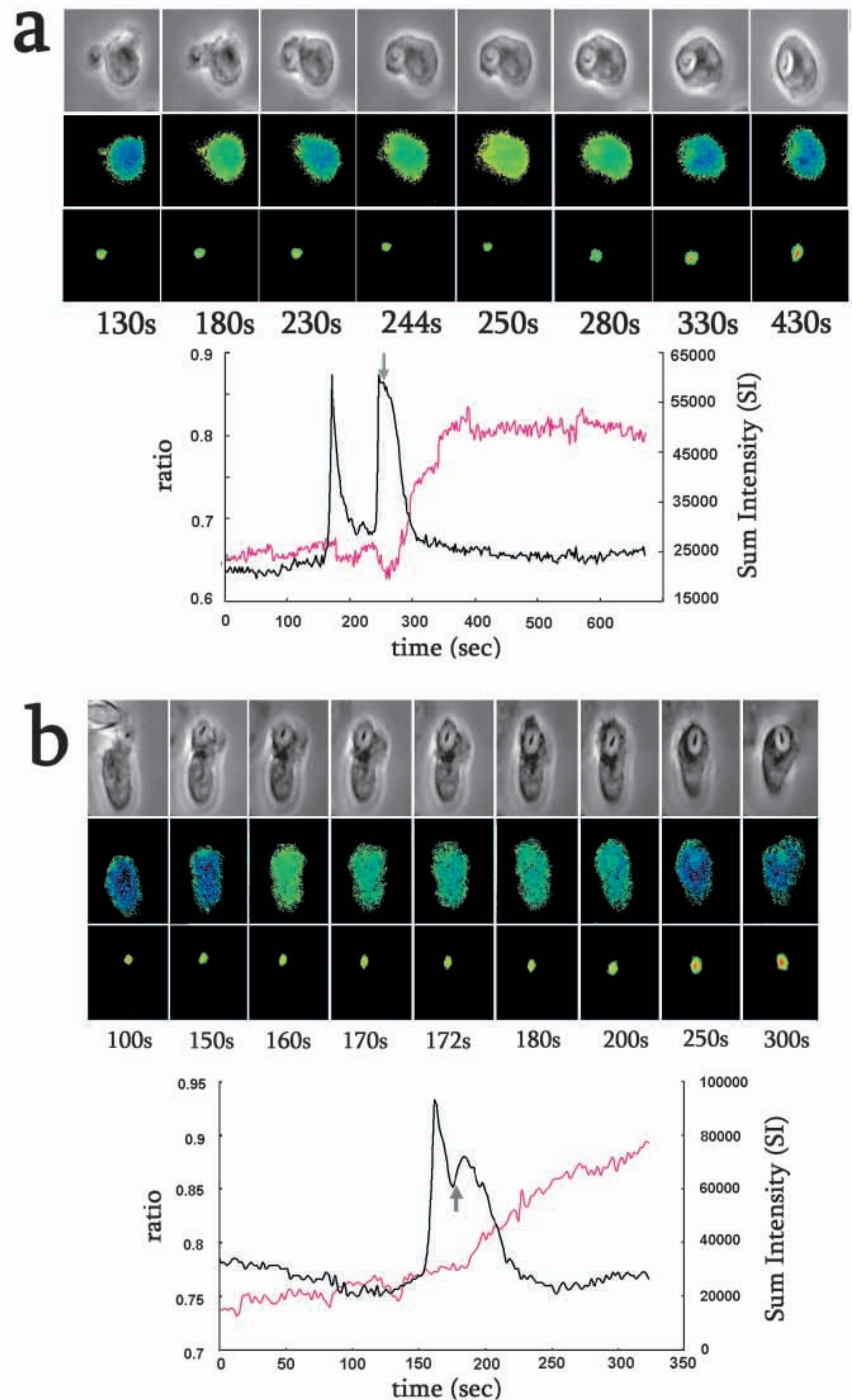


**Fig. 4.** Correlation of oxidative activation with $Ca^{2+}$ and phagocytosis. (a) A typical experiment is shown in which the phagocytic event is shown by phase contrast microscopy (top row); the accompanying cytosolic free $Ca^{2+}$ changes (middle row) and oxidative activity assessed by the DCDHF fluorescent intensity of the internalised zymosan particle (bottom row) are shown. The graph at the bottom shows the complete time course for cytosolic free $Ca^{2+}$ change (black) and DCDHF intensity (SI) with the point of phagosomal closure marked by the arrow. In this example, a cell exhibiting a very marked ‘twin peak’ $Ca^{2+}$ signal is shown. The full data is also shown in Movie 1 (available at jcs.biologists.org/supplemental) in which the oxidative signal from the particle and the $Ca^{2+}$ signal from the neutrophil are superimposed. (b) For comparison, a cell exhibiting a ‘shouldered’ $Ca^{2+}$ signal is also shown. The rows of images are as described above. In both examples, the onset of oxidative activity correlates with the second phase of the $Ca^{2+}$ signal.

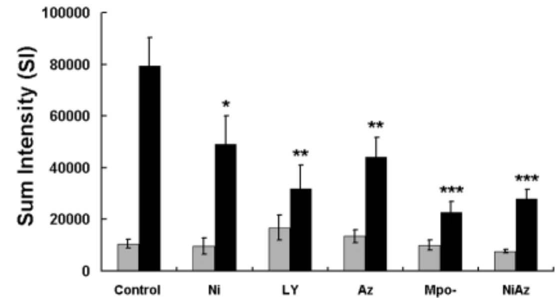


**Fig. 5.** Modulation of the oxidative response. Each pair of histograms shows the mean±s.d. of DCHFC-zymosan fluorescence (sum intensity) before and 200 seconds after phagosome closure in cells which were treated as follows: control, untreated (*n*=15); Ni, in the presence of the $Ca^{2+}$ channel-blocking ion $Ni^{2+}$ (2 mM, *n*=6); LY, pretreated for 15 minutes with the PI(3) kinase inhibitor LY294002 (50 μM, *n*=3); Az, in the presence of the MPO inhibitor, azide (10 mM, *n*=6); MPO–, myeloperoxidase-deficient cells (*n*=2); NiAZ, the presence of both $Ni^{2+}$ (2 mM) and azide (10 mM, *n*=2). The asterisks indicate the level statistical significance for the difference between the control column and the others; where *$P<0.05$, **$P<0.01$, ***$P<0.001$.

activity but was not restricted within the cytosol, the possibility that oxidase and peroxidase release were also triggered at non-phagosomal sites was investigated. Neutrophils were chosen that had oxidant-detecting zymosan particles close by or touching, which would act as a sentinel reporting extracellular oxidation during phagocytosis. In these experiments, no oxidants were detected at extracellular sites around the cell (47/47) even if the sentinel zymosan particles were near the open mouth of the phagosome (e.g. Fig. 6a, particle 3). Sentinel particles near or touching the outer face of the phagocytic cup (Fig. 6a, particle 2) also failed to become oxidised. Thus, it appeared that the second phase $Ca^{2+}$ rise triggered oxidase assembly and granule fusion restricted to the inner face of the forming phagosome. However, the concentration of oxidants near extracellular zymosan particles (i.e. not within phagosomes) was expected to be less as a result of dilution and

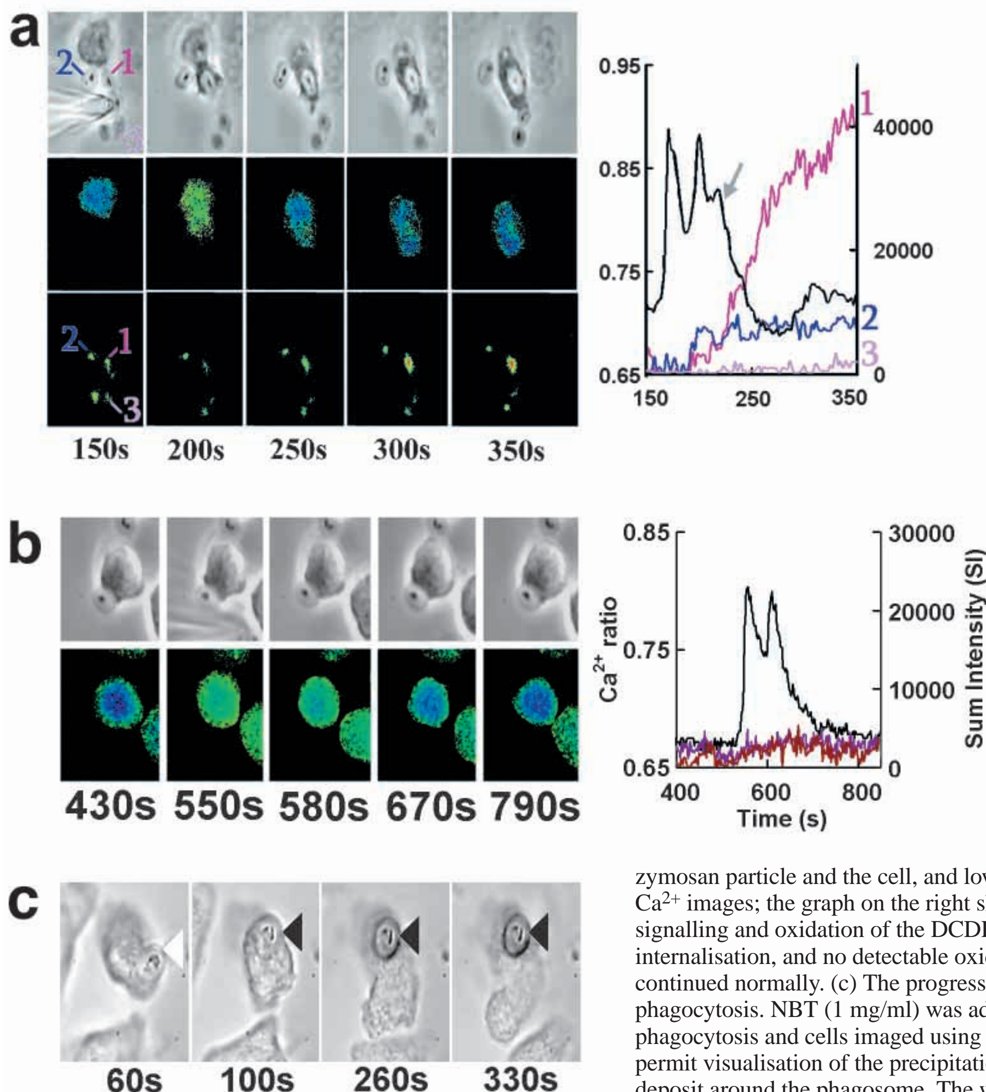


**Fig. 6.** Extracellular oxidants measured by sentinel particles and NBT. (a) The data shows a typical experiment in which an opsonised zymosan particle (labelled '1') is presented to a neutrophil for phagocytosis together with a nonphagocytosed sentinel particle (labelled '2') and another particle near the mouth of the open phagosome (labelled '3'). The upper row shows the phase contrast images, the middle row, the $Ca^{2+}$ images, pseudocoloured as before and the lower row shows the intensity of DCDHF on the particles in the field. The graph on the right shows the complete data for the $Ca^{2+}$ signal (twin peaked) with the arrow marking the time of phagosome closure, and the intensity for the three particles. As before, the intensity of the particle undergoing phagocytosis increased abruptly at the time of the second phase of the $Ca^{2+}$ signal, whereas the other particles fail to respond. (b) The result from a typical experiment in which completion of phagocytosis was prevented by pretreatment of the neutrophils with cytochalasin B (5 μg/ml). The upper series of images show the contact between the zymosan particle and the cell, and lower series, the corresponding cytosolic $Ca^{2+}$ images; the graph on the right shows the complete data for $Ca^{2+}$ signalling and oxidation of the DCDHF-zymosan. Despite inhibition of internalisation, and no detectable oxidation of the zymosan, $Ca^{2+}$ signalling continued normally. (c) The progressive precipitation of formazan during phagocytosis. NBT (1 mg/ml) was added to the cells before the onset of phagocytosis and cells imaged using bright field with weak phase contrast to permit visualisation of the precipitation of formazan, which is seen as black deposit around the phagosome. The white arrow in the first image indicates the exposed portion of the C3bi-opsonised zymosan particle after contact but before complete engulfment. The black arrows indicate the point of phagosome closure, which occurred at 100 seconds and the subsequent images show that the deposition of formazan continued for at least 230 seconds. This experiment was typical of at least three others.

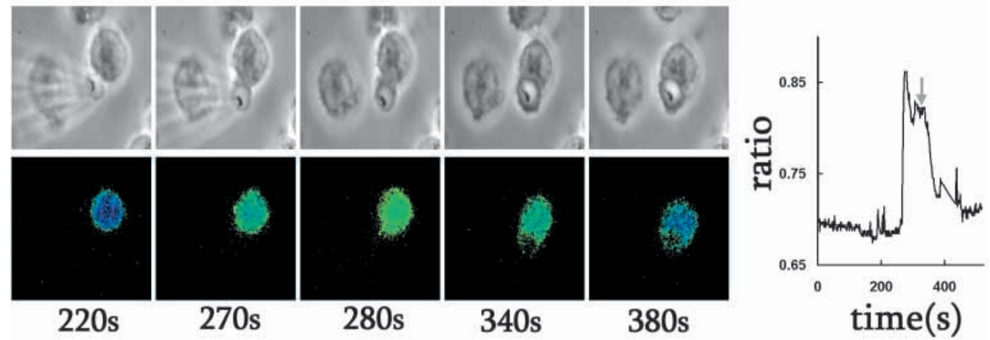


**Fig. 7.** Local oxidase activation and $Ca^{2+}$ signal reported by fura2-dextran conjugate. The data shows a typical experiment in which neutrophils were micro-injected with fura2-dextran conjugate. In the example shown, a single neutrophil has been micro-injected and hence only that cell appears in the $Ca^{2+}$ image (other neutrophils in the field having no fluorescent signal). The micro-injected cell was challenged with an opsonised particle (at 220 seconds) as before and the phagocytic cup (270 seconds), phagosome closure (340 seconds) and completion of the $Ca^{2+}$ signal (380 seconds) is shown in the upper set of phase contrast images. The lower set of images show the corresponding fura2-dextran signal and the graph on the right shows the complete $Ca^{2+}$ data, with the point of phagosome closure marked by the downward arrow.

so detection of oxidation of DCDHF might have been preferential within the phagosome. This was confirmed in neutrophils in which pseudopodia formation and internalisation of particles was prevented by pretreatment with cytochalasin B (5 μg/ml). Although this treatment did not inhibit the $Ca^{2+}$ signal (Dewitt and Hallett, 2002), no oxidation of the extracellular particle was detected (Fig. 6b). Cytochalasin B does not inhibit but enhances oxidase-mediated oxygen consumption (Al-Mohanna and Hallett, 1987) and causes nondirected MPO release, which was consistent with extracellular dilution of oxidants preventing the detection of extracellular oxidation by this approach. This conclusion was also supported by the failure of phorbol myristate acetate (PMA), a non-phagocytic stimulus of the oxidase, but not of MPO release, to oxidise extracellular DCDHF-zymosan. A second indicator of oxidase activity, independent of MPO, was therefore used. Nitroblue teterazolium (NBT) is reduced by $O_2^-$ (and may also accept electrons directly from the oxidase) to form insoluble formazan. As the precipitate cannot diffuse, it provides spatial information of oxidase activity. This indicator reported strong oxidation within the phagosome, beginning on closure of the phagosome ($n$=3), but produced no evidence of oxidase activity at adjacent plasma membrane sites (Fig. 6c). There was evidence of oxidation of NBT within the cytosol near the phagosome, perhaps as a result of diffusion of oxidants from localised oxidase activity within the phagosome (Fig. 6c) (see also Sullivan, 2003). There was, however, no clear formazan precipitation at membranes other than within the phagosome (Fig. 6c).

Whether or not activation of the oxidase was restricted absolutely to the inner phagosomal membrane, formazan precipitation clearly showed it was localised at least to that part of the cell undergoing phagocytosis. As the activation of the oxidase appeared to be spatially restricted, yet $Ca^{2+}$ signalling was unrestricted throughout the cytosol, we examined the possibility that the apparent global distribution of elevated $Ca^{2+}$ was generated artefactually as a result of the rapid diffusion of the fura2-$Ca^{2+}$ complex away from a more restricted region of the cytosol. This possibility was considered because the initial small localised $Ca^{2+}$ event, which occurs on initial contact between an opsonised particle and a neutrophil, is best observed when the geometry of the phagocytic cup includes a narrowing before the main body of the cell (Dewitt and Hallett, 2002). This would be explained if the narrowing at the base of some phagocytic cups imposed a 'diffusion resistance' to the fura2-$Ca^{2+}$ complex and so more accurately reported the site of elevated $Ca^{2+}$. The possibility that the oxidase-triggering second $Ca^{2+}$ signal was also restricted to a zone around the phagosome was therefore tested by using a larger molecular weight dextran conjugate of fura2 (molecular mass 10 kDa) as a more slowly diffusible $Ca^{2+}$ reporter. After micro-injection with fura2-dextran (50-100 μM), the phagocytic event was identical to that seen in noninjected neutrophils or those micro-injected with an irrelevant molecule (lucifer yellow). Furthermore, apart from the initial $Ca^{2+}$ event, which was observed as localised in 1/3 phagocytotic events, fura2-dextran also reported synchronised global $Ca^{2+}$ signals (3/3), with no evidence of a time delay between the periphagosomal region and elsewhere (Fig. 7). Also, fast confocal imaging (33 mseconds resolution) failed to reveal a wave of $Ca^{2+}$ originating from the phagosome (data not shown). There was thus no evidence that the global $Ca^{2+}$ changes originated from a localised source of $Ca^{2+}$ in the phagocytic region or that the cytosolic free $Ca^{2+}$ concentration was higher there (in $Ca^{2+}$ ratio images, the DCDHF signal contaminates the $Ca^{2+}$ image as it gets brighter after phagocytosis and give an artefactually higher ratio value at that location). It was therefore concluded that the $Ca^{2+}$ signal that triggered the oxidation response was the result of influx of $Ca^{2+}$ across the plasma membrane around the whole cell perimeter. However, as this elevated $Ca^{2+}$ alone was insufficient to trigger oxidase activity in non-phagosomal locations in the cell, it was concluded that other key factors required for activation of the oxidase and peroxidase release by $Ca^{2+}$ were limited to the phagosomal membrane.

## Discussion

The work presented here has shown that the $Ca^{2+}$ signals that accompany phagocytosis are composed of at least two components but that only the second phase is associated with oxidative activity in the phagosome, the earlier $Ca^{2+}$ changes failing to trigger oxidase activation. It is therefore proposed that elevated cytosolic free $Ca^{2+}$ concentration alone is insufficient to activate the oxidase and that other obligatory but slower events, which are complete by the time of the second phase of the $Ca^{2+}$ signal, are required. It is probable that the slower events include the assembly of oxidase components (Segal, 1996) and PI(3)P generation (Ellson et al., 2001a; Kanai et al., 2001) in the

phagosomal membrane. The data here also shows that while the $Ca^{2+}$ signal controls the timing of oxidase activation, it plays no role in restricting the activity to the phagosome.

This study raises some important questions. The first set of questions concern the mechanism underlying the complexity of the phagocytic $Ca^{2+}$ signal. We have recently shown that the initial β2 integrin-mediated $Ca^{2+}$ signal causes an increase in mobility of β2 integrin molecules distant from the contact site, which results in an acceleration of phagocytosis (Dewitt and Hallett, 2002). The second phase of β2 integrin binding may be responsible for the second $Ca^{2+}$ peak observed in some neutrophils. It is probable that the same events exist in all neutrophils, but appeared as a single $Ca^{2+}$ peak, a $Ca^{2+}$ peak with shoulder or a double $Ca^{2+}$ peak, depending on the some critical values for key kinetic parameters of integrin mobilisation or $Ca^{2+}$ homeostasis. A recent mathematical model suggests that splitting of $Ca^{2+}$ signals may arise in individual cells as a result of differences in the number of their $Ca^{2+}$ storage proteins (Baker et al., 2002) or in $Ca^{2+}$ reuptake rates. The question also arises about the mechanism by which the global $Ca^{2+}$ signal is generated. Clearly, with the technique used here, the stimulus delivered to the cell was very localised, yet no evidence for localisation of the 'global' $Ca^{2+}$ signal was found (Fig. 4a,b, Fig. 7). As the global $Ca^{2+}$ signal was driven by $Ca^{2+}$ influx, three possibilities exist: first, a factor was released into the medium that diffused extracellularly to trigger remote $Ca^{2+}$ influx; second, a factor was released into the cytosol that diffused intracellularly to trigger remote $Ca^{2+}$ influx; or third, a factor was released into the plasma membrane that diffused around the cell membrane to trigger remote $Ca^{2+}$ influx. There is some evidence in support of either of the latter two possibilities, as a water-soluble factor ($Ca^{2+}$ influx factor) has been described (Randriamampita and Tsein, 1993) and can be isolated from neutrophils (Davies and Hallett, 1995), and inhibitors of PI(3) kinase inhibit $Ca^{2+}$ signals triggered by β2 integrin (Dewitt and Hallett, 2002) and phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)$P_3$] itself can induce $Ca^{2+}$ signals in neutrophils. For any of the three mechanisms, the $Ca^{2+}$ concentration may be elevated nearer the generation site of the '$Ca^{2+}$ influx signal' as it diffuses down a concentration gradient. In an earlier study, a wave of $Ca^{2+}$ (17 μm/s) originating near the phagosome (Schwab et al., 1992) was indeed reported. However, we and others (Theler et al., 1995) have not been able to show such a $Ca^{2+}$ wave.

The $Ca^{2+}$ influx signal was responsible for abrupt oxidation of internalised zymosan (Fig. 5). This may be attributed to the abrupt delivery to the phagosome of MPO by granule-phagosome fusion, the abrupt activation of the oxidase in the phagosome membrane, the delivery of long-lived oxidants preformed in a granule (that perhaps also contain the MPO) or a combination of these. It has previously been shown that high $Ca^{2+}$ levels are required for MPO-granule-phagosome fusion, with $K_{50}$ values estimated by quin2 buffering as 2.6 μM (Lew et al., 1986; Jaconi et al., 1990) or by internal perfusion at near 100 μM (Nüsse et al., 1998). As these levels of $Ca^{2+}$ are higher than the bulk $Ca^{2+}$ level (approximately 700 nM), either β2 integrin engagement increased the efficacy of $Ca^{2+}$ for degranulation or $Ca^{2+}$ just under the phagosomal membrane reached much higher levels. Although two early papers reported a long-lived localised $Ca^{2+}$ signal around the phagosome (Sawyer et al., 1985; Murata et al., 1987), this could not be shown here. However, $Ca^{2+}$ influx in neutrophils has been shown to cause high submembrane changes in $Ca^{2+}$, to give a $Ca^{2+}$ concentration of at least 50 μM (Davies and Hallett, 1998), which has been suggested to mediate subplasma membrane calpain activation ($K_d$ 30 μM) (Dewitt and Hallett, 2002). As the concentration of $Ca^{2+}$ within the phagosome decreases after phagocytosis (Lundqvist-Gustafsson et al., 2000), the possibility exists that $Ca^{2+}$ channel opening in the phagosomal membrane may elevate $Ca^{2+}$ concentrations to very high levels immediately around the phagosome. However, it is clear that this $Ca^{2+}$ signal alone is insufficient to activate the oxidase as its activity was not triggered by the initial $Ca^{2+}$ change. The possibility exists that between the first and second $Ca^{2+}$ event, the oxidase was assembled in a form awaiting the $Ca^{2+}$ signal for full activation. It is known that components of the oxidase p40phox and p47phox must be assembled (Segal, 1996) and that PtdIns(3)*P* binding and generation occurs (Ellson et al., 2001a; Kanai et al., 2001). If PtdIns(3)*P* is localised to the phagosomal membrane in neutrophils, as it is in RAW 264.7 cells (Ellson et al., 2001b), the effect of the global $Ca^{2+}$ signal would only be evident locally within the phagosome. Although the oxidase complex may be partially active without the $Ca^{2+}$ signal, and thus account for the inability to completely inhibit oxidative activity by blocking the $Ca^{2+}$ signal (Fig. 5), the $Ca^{2+}$ signal was associated with a massive increase in oxidative activity in the phagosome. As it is well documented that activators and inhibitors of protein kinase C (PKC) activity also activate and inhibit oxidase activation (e.g. Cooke and Hallett, 1985), and PKC associates with the oxidase component p40phox (Reeves et al., 1999) the possibility exists that the action of $Ca^{2+}$ on the oxidase may be mediated by PKC activity.

An issue important for pathology raised in this study is whether leakage of oxidants into the extracellular medium can occur from the activated oxidase. No leakage was detected by zymosan particles near by or even at the mouth of the phagosome. However, this may have arisen artefactually as a result of dilution of oxidants in the extracellular medium (an event which cannot occur within the enclosed phagosome). In some cells, efflux of oxidants from the forming phagosome may thus remain a possibility, because although there was a good correlation between the second phase on the $Ca^{2+}$ signal and the activation of oxidative activity, this was not tightly coupled to the time of phagosomal closure. This means that in some cells, the oxidase was activated before complete closure of the phagosome and that, theoretically, extracellular oxidation would be possible.

The work here has therefore provided a role for the second of the two $Ca^{2+}$ signal phases triggered during β2 integrin-mediated phagocytosis – namely, in activation of the oxidase and triggering granule-phagosome fusion. Previously, we have shown that the first phase was required for the liberation of immobile β2 integrin to cause acceleration of phagocytosis. However, it still remains to be established whether the first localised $Ca^{2+}$ signal fulfils any role and how the $Ca^{2+}$ signal interacts with other events before a full explanation of the roles of the complex $Ca^{2+}$ signalling of phagocytosis by neutrophils can be given.

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