

# Cytosolic free $\text{Ca}^{2+}$ changes and calpain activation are required for $\beta$ integrin–accelerated phagocytosis by human neutrophils

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Phagocytosis of microbes coated with opsonins such as the complement component C3bi is the key activity of neutrophils. However, the mechanism by which opsonins enhance the rate of phagocytosis by these cells is unknown and has been difficult to study, partly because of the problem of observing and quantifying the events associated with phagocytosis. In this study, C3bi-opsonized particles were presented to neutrophils with a micromanipulator, so that the events of binding, pseudopod cup formation, engulfment, and completion of phagocytosis were clearly defined and distinguished from those involved with chemotaxis. Using this approach in combination with simultaneous phase contrast and  $\text{Ca}^{2+}$  imaging, the temporal relationship between changes in cytosolic free  $\text{Ca}^{2+}$  concentration and phagocytosis were correlated. Here we show that whereas small, localized

$\text{Ca}^{2+}$  changes occur at the site of particle attachment and cup formation as a result of store release, rapid engulfment of the particle required a global change in cytosolic free  $\text{Ca}^{2+}$  which resulted from  $\text{Ca}^{2+}$  influx. This latter rise in cytosolic free  $\text{Ca}^{2+}$  concentration also liberated a fraction of  $\beta 2$  integrin receptors which were initially immobile on the neutrophil surface, as demonstrable by both fluorescence recovery after laser bleaching and by visualization of localized  $\beta 2$  integrin labelling. Inhibitors of calpain activation prevented both the  $\text{Ca}^{2+}$ -induced liberation of  $\beta 2$  integrin and the rapid stage of phagocytosis, despite the persistence of the global  $\text{Ca}^{2+}$  signal. Therefore, we propose that  $\text{Ca}^{2+}$  activation of calpain causes  $\beta 2$  integrin liberation, and that this signal plays a key role in the acceleration of  $\beta 2$  integrin–mediated phagocytosis.

## Introduction

Neutrophils are “professional phagocytes” which have a remarkable capacity for phagocytosis. These cells can internalize microscopic particles (0.5–3  $\mu\text{m}$  in diameter) of virtually any surface material. However, the efficiency and speed of phagocytosis is increased by coating the surface of the particles with opsonins such as antibodies or the complement component C3bi. The C3bi-accelerated phagocytosis of infecting microorganisms by neutrophils is probably the main route for the first line of defence by the innate immune system *in vivo*. However, the mechanism for this acceleration of phagocytosis by neutrophils is poorly understood.

However, it is clear that the accelerated phagocytosis is mediated by an interaction with the neutrophil receptor for the complement component C3bi, CD18/CD11b, CR3, or  $\beta 2$  integrin. There is an intricate interplay between this receptor

and  $\text{Ca}^{2+}$  signalling. Although experimental strategies that immobilize  $\beta 2$  integrin on the neutrophil surface trigger changes in cytosolic free  $\text{Ca}^{2+}$  concentration (Jaconi et al., 1991; Ng-Sikorski et al., 1991; Petersen et al., 1993), there is also evidence that changes in cytosolic free  $\text{Ca}^{2+}$  concentration signal outwards to integrins, increasing the effectiveness of the receptor either by increasing its affinity (Li et al., 1998) or clustering ability (Hato et al., 1998; van Kooyk and Figdor, 2000).

One of the problems associated with previous attempts to study C3bi-accelerated phagocytosis in neutrophils has been the difficulty in observing the phagocytic event and of presenting the stimulus to the cell at a defined time and at a defined location on the neutrophil. In some previous studies, phagocytosis has been studied as part of the process of chemotaxis with the cell moving towards the particle before engaging it (Theler et al., 1995). In the study presented here, we avoid the possible crosstalk between chemotactic signalling and phagocytosis by presenting the particle to the neutrophil using micromanipulation. In this way, the events of binding, phagocytosis cup formation, engulfment, and completion of phagocytosis

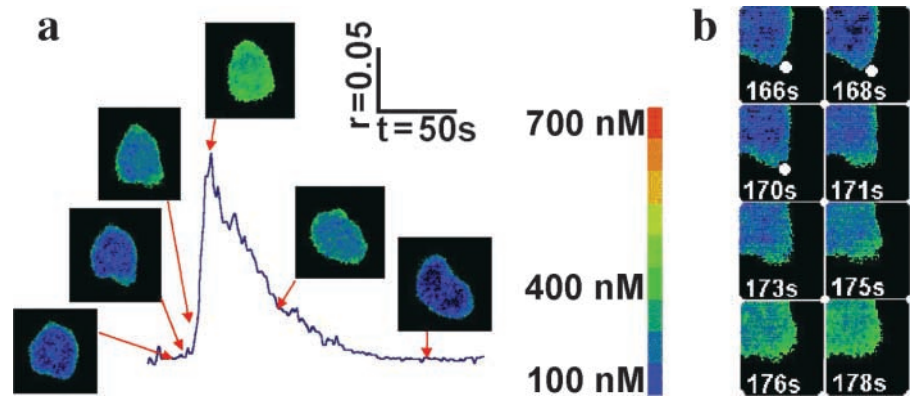
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Figure 1. **Ca<sup>2+</sup> signals accompanying C3bi-mediated phagocytosis.**

(a) The cytosolic free Ca<sup>2+</sup> concentration within an individual neutrophil undergoing phagocytosis is shown. The line graph shows the complete Ca<sup>2+</sup> signal, and the images show the neutrophil shape and Ca<sup>2+</sup> concentration as pseudocolor at the time points indicated by the arrows. The pseudopodia extension and cup formation on the second image is better seen in the sequence in b, where the initial position of the opsonized particle is marked by the filled white circle in the first three images. The formation of the cup and the localized Ca<sup>2+</sup> signal is evident after the third image in the sequence (the filled white circle has not been added to these images so that the localized Ca<sup>2+</sup> events can be clearly seen). In both parts of this figure (and in all subsequent figures), the same pseudocolor look-up-table shown has been used and is shown between parts a and b. This cell was typical of 33 out of 36 cells investigated in which Ca<sup>2+</sup> changed in response to opsonized phagocytosis. In the three remaining cells, complete phagocytosis was observed without any detectable change in cytosolic free Ca<sup>2+</sup> concentration, presumably because phagocytosis proceeded without  $\beta$ 2 engagement. The data sequence shown here is also available online at <http://www.jcb.org/cgi/content/full/jcb.200206089/DC1>.



were clearly defined and distinct from those involved with chemotaxis. The temporal relationship between changes in cytosolic free Ca<sup>2+</sup> concentration and phagocytosis were correlated by simultaneous phase contrast and Ca<sup>2+</sup> imaging. Here we show that small localized Ca<sup>2+</sup> changes occur at the site of particle attachment and cup formation, but that the major effect is a global change in cytosolic free Ca<sup>2+</sup> concentration. It is this latter Ca<sup>2+</sup> signal which accelerates engulfment of the particle. We also show that a global rise in cytosolic free Ca<sup>2+</sup> concentration liberates a fraction of  $\beta$ 2 integrin receptors. As inhibitors of calpain activation prevent the rapid stage of phagocytosis despite the persistence of the global Ca<sup>2+</sup> signal, we propose that Ca<sup>2+</sup>-activated calpain activation plays a key role in the acceleration of integrin-mediated phagocytosis by liberating integrin molecules remote from the initial contact site.

## Results

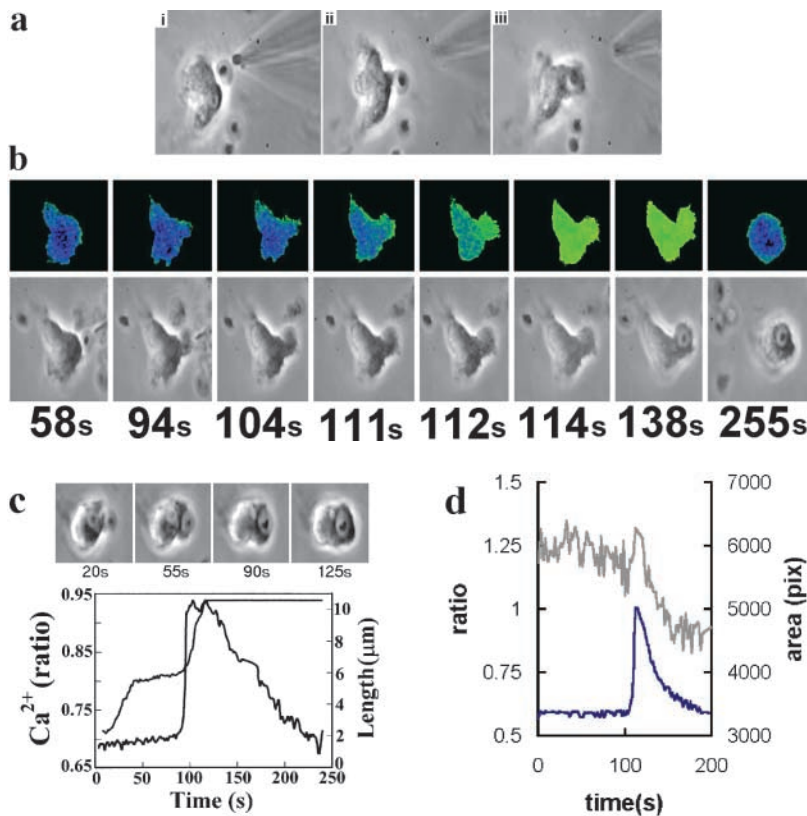
### Ca<sup>2+</sup> changes accompany C3bi-mediated phagocytosis

When permitted to send out random pseudopodia in a field of particles, neutrophils occasionally encounter particles in their near vicinity. When this occurs, four stages of phagocytosis can be distinguished: (a) pseudopod extension; (b) contact with the particle; (c) cup formation; and (d) phagosome closure and pseudopod retraction. With nonopsonized particles, the time to phagosome closure (completion of phagocytosis) was slow (2–4 min) and often did not reach this point. Changes in cytosolic free Ca<sup>2+</sup> were sometimes recorded, but these did not consistently correlate with particular phases of phagocytosis, and Ca<sup>2+</sup> signals were usually seen only after completion of phagocytosis. In contrast, when the particles were C3bi-opsonized, Ca<sup>2+</sup> signals were consistently observed ( $n = 33$ ) during this process as follows: (a) no changes in cytosolic free Ca<sup>2+</sup> concentration occurred during pseudopod extension; (b) local Ca<sup>2+</sup> signals were observed on contact with the particle and during cup formation; and (c) large Ca<sup>2+</sup> changes throughout the neutrophil cytosol occurred immediately before rapid enclosure of the

particle, phagosome closure, and pseudopod retraction (Fig. 1; Video 1, available online at <http://www.jcb.org/cgi/content/full/jcb.200206089/DC1>). In this series of experiments, the neutrophils were free to move and throw out spontaneous pseudopodia, so the possibility existed that the observed Ca<sup>2+</sup> activity was contaminated by cell activities other than phagocytosis. Therefore, an approach was devised which permitted phagocytosis to be triggered on demand and in the absence of spontaneous pseudopodia formation or chemokinesis.

### Ca<sup>2+</sup> changes were triggered by particle contact alone

The method adopted used a micropipette through which slight negative pressure was applied to hold a C3bi-opsonized zymosan particle (2  $\mu$ m in diameter), so that it could be presented to the cell (Fig. 2 a). With this method, the time and location of contact between the particle and the neutrophil was precisely controlled. In particular, chemotactic cytoskeletal changes were not necessary before phagocytosis, as the neutrophil was not required to move towards the particle. Using this approach, contact between the opsonized particle and the neutrophil resulted in the formation of a phagocytic cup and complete phagocytosis. The same sequence of Ca<sup>2+</sup> signalling was observed as with the nondirected phagocytosis, with localized Ca<sup>2+</sup> signals occurring at the contact point during cup formation, and a large global change in cytosolic free Ca<sup>2+</sup> concentration preceding rapid phagosome closure and retraction (Fig. 2 b). As both routes to phagocytosis produced similar Ca<sup>2+</sup> signals, the delivery of the particles by micropipette was preferred as the individual events comprising phagocytosis could be controlled and distinguished. After presentation of the particle by the micropipette and the binding step, the formation of the phagocytic cup was slow and often appeared to temporarily arrest at this stage (Fig. 2 c). However, immediately after the global Ca<sup>2+</sup> signal, there was consistently a rapid spreading of the pseudopodia around the particle (Fig. 2 c). This rapid stage resulted in an abrupt morphological transition toward roundness as the cell engulfed the particle and correlated



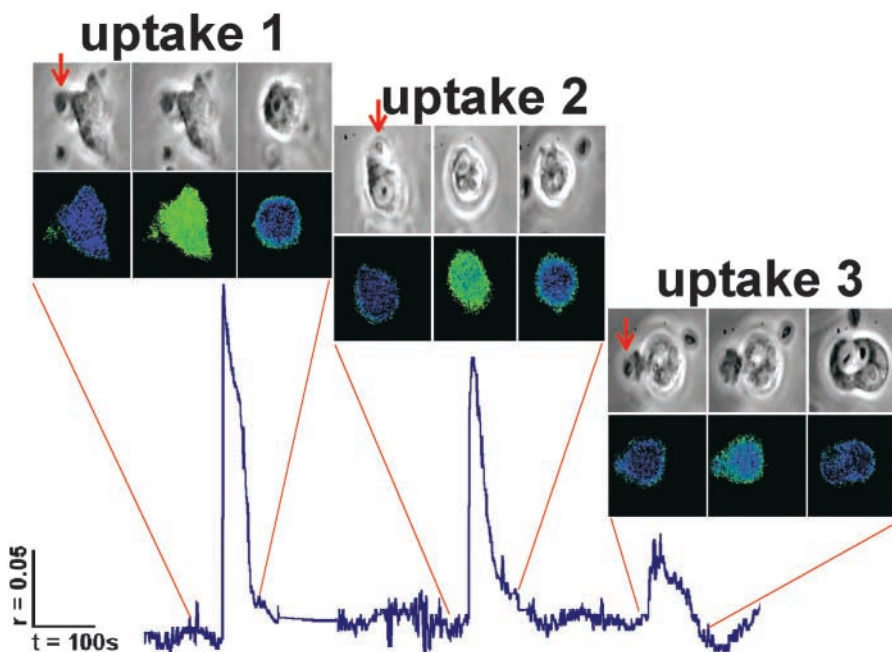
**Figure 2. Ca<sup>2+</sup> signals induced by C3bi-mediated phagocytosis.** (a) The technique of presenting an opsonized particle to an individual neutrophil is shown in the sequence of images where the particle, held by the micropipette, is placed in contact with the neutrophil (i); the micropipette is removed as the particle binds to the neutrophil (ii); and the phagocytic cup forms and phagocytosis proceeds (iii). (b) Selected frames from a sequence of simultaneously acquired Ca<sup>2+</sup> and phase contrast images of a neutrophil presented with a C3bi-opsonized zymosan particle. (Top) Ca<sup>2+</sup> images according to the look-up-table shown in Fig 1. Localized Ca<sup>2+</sup> changes are seen in images at 111 and 112 s, and then the global Ca<sup>2+</sup> change in 114 s. (Bottom) Corresponding phase contrast images, superimposed on to which were high Ca<sup>2+</sup> pixels (with 30% opacity); in order to demonstrate the correlation between cup formation and localized small Ca<sup>2+</sup> changes. The relationship between the triggered Ca<sup>2+</sup> signal and (c) the rate of spreading of the pseudopodia over the surface of the zymosan particle (length of cell:particle contact) and (d) the accompanying whole-cell morphology change (cell area) derived from a semi-automatic adaptive spline tracking program are shown. The Ca<sup>2+</sup> and morphology changes shown here were typical of the majority (70%) of experiments performed. In the remaining cells, the Ca<sup>2+</sup> response had a distinctive double peak (Fig. 5 f).

strongly with the global Ca<sup>2+</sup> signal (Fig. 2 d). The Ca<sup>2+</sup> signals and phagocytosis could be provoked repetitively within an individual neutrophil. In some neutrophils, the global Ca<sup>2+</sup> change had a distinctive double peak, which was also reproduced in response to subsequent phagocytic challenges (11/36 cells). Up to four particles have been “fed” to a single neutrophil, each phagocytic event being accompanied by a Ca<sup>2+</sup> signal. However, successive Ca<sup>2+</sup> events had a reduced

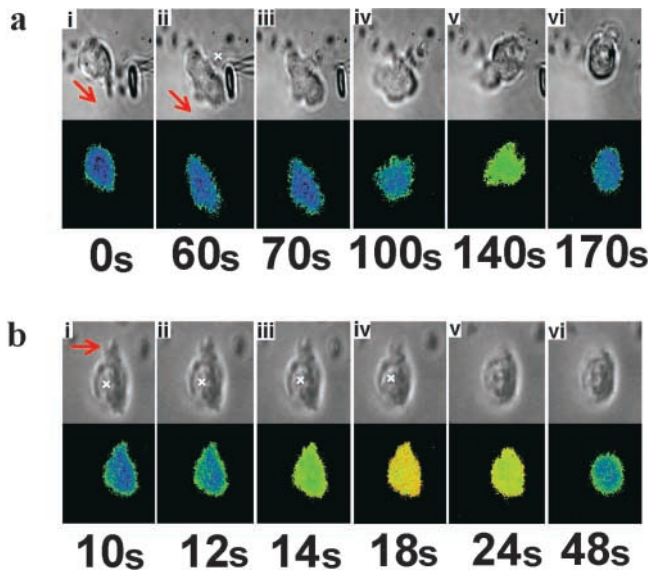
magnitude (Fig. 3) and proceed more slowly (see Fig. 5). This decline in Ca<sup>2+</sup> signal may have resulted from a depletion of available β2 integrin molecules (see Fig. 9) or of other signalling components coupled to the Ca<sup>2+</sup> signal.

### Mechanism of generation of the Ca<sup>2+</sup> signal

It was concluded that the Ca<sup>2+</sup> events accompanying C3bi-accelerated phagocytosis were mediated by β2 integrin en-

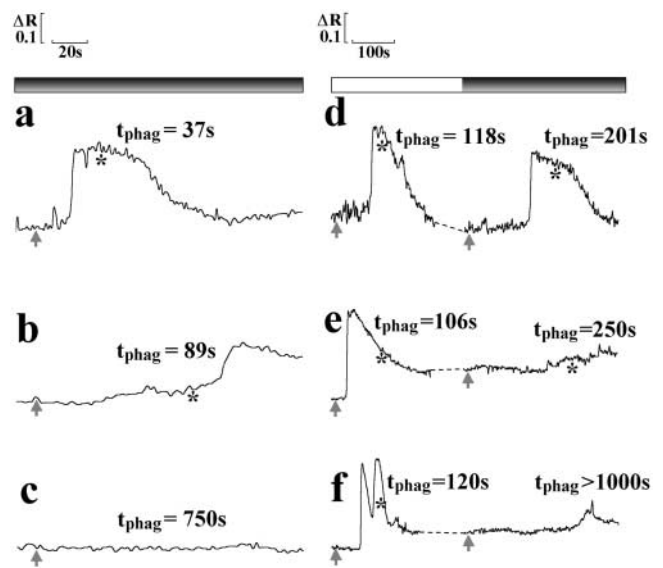


**Figure 3. The Ca<sup>2+</sup> signal decreased with each successive phagocytic event.** The line graph shows the Ca<sup>2+</sup> changes provoked by successive phagocytic events by the same neutrophil. For each event, (labeled “uptake 1–3”), three phase contrast and Ca<sup>2+</sup> images are shown at particle binding, peak Ca<sup>2+</sup> and completion. Each event was allowed to run to completion, Ca<sup>2+</sup> return to resting level and normal motile morphology reestablished (after pseudopod retraction and cell rounding) before the next particle was presented to the cell. The experiment was typical of at least five others.



**Figure 4. Neutrophils signal  $\text{Ca}^{2+}$  and complete C3bi-mediated phagocytosis when presented with particles at sites remote from their leading edge.** Two series of simultaneously acquired phase contrast and  $\text{Ca}^{2+}$  images are shown with the time of acquisition shown. (a) A neutrophil is shown which was undergoing chemokinesis in the direction shown by the red arrow on the phase contrast images. A C3bi-opsonized particle was presented to the cell in ii at the location indicated by the white cross on the phase contrast image. (iii) Binding has occurred. (iv) The phagocytic cup has formed. (v)  $\text{Ca}^{2+}$  response and rapid engulfment phase. (vi) Completion of phagocytosis and restoration of resting cytosolic free  $\text{Ca}^{2+}$  concentration. The complete dataset from which this data is taken is shown as Video 2 (available online at <http://www.jcb.org/cgi/content/full/jcb.200206089/DC1>) with the resultant  $\text{Ca}^{2+}$  measurement. (b) A neutrophil was presented with a particle but removed to leave an abortive phagocytic cup, as indicated by the red arrow on the phase contrast image. The particle was then placed in the position indicated by the white cross. A localized  $\text{Ca}^{2+}$  signal is seen at the point of particle contact (ii) before the global  $\text{Ca}^{2+}$  rises in images (iii) and (iv) and the abortive phagocytic cup retracts in image (v) and the cell assumes a round morphology and resting cytosolic free  $\text{Ca}^{2+}$  concentration (vi).

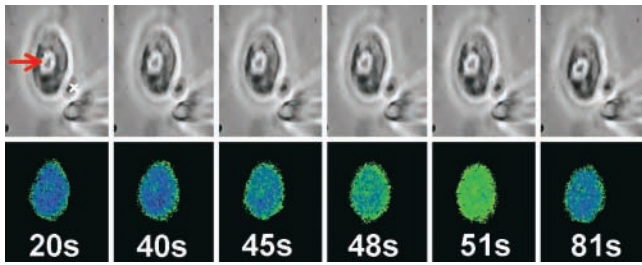
gement because the events were triggered by zymosan opsonized with purified human C3bi. Also, pretreatment of the neutrophils with antibodies to either the  $\alpha_m$  or  $\beta_2$  chain of the integrin prevented both the rapid progression to completion of phagocytosis and the accompanying local and global  $\text{Ca}^{2+}$  signals triggered by either serum and C3bi opsonized particles. In contrast, antibodies to other surface proteins, including CD32 (FCRII), did not inhibit phagocytosis by either serum or purified C3bi-opsonized zymosan, and neutrophils treated with antibodies to  $\beta_2$  integrin were able to take up nonopsonized zymosan. As the leading edge of motile neutrophils and the pseudopodia may be enriched in  $\beta_2$  integrin molecules, contain polymerized actin and activated G proteins (Parent et al., 1998), and possibly have additional  $\text{Ca}^{2+}$  storage sites (Stendahl et al., 1994), it was possible that this morphological organization was crucial for phagocytosis and the accompanying  $\text{Ca}^{2+}$  signalling. To test this, particles were presented to neutrophils at locations remote from either the leading edge of motile neutrophils or the site of pseudopodia formation (Fig. 4, a and b; Video



**Figure 5. Pharmacological inhibition of C3bi-mediated  $\text{Ca}^{2+}$  signaling** The line graphs show  $\text{Ca}^{2+}$  changes in response to C3bi-mediated phagocytosis in the presence of inhibitor or control as indicated by the shaded bar. (a–c) The effect of a single phagocytic challenge in the presence of inhibitor or control. (d–f) The  $\text{Ca}^{2+}$  changes which resulted from two phagocytic challenges, with the inhibitor present only for the second phagocytic event. In all traces, the time to complete phagocytosis ( $t_{\text{phag}}$ ) is shown and the points of particle cell contact and phagosome closure indicated by an arrow and an asterisk respectively. In traces a and d, no inhibitor was present. (b and e) The shaded bars indicate the presence of  $\text{NiCl}_2$  (1 mM). (c and f) The cell was incubated with LY294002 (50  $\mu\text{M}$ , 5 min, 37°C) before the period indicated by the shaded bar. These data are representative of at least nine replicate experiments on neutrophils from different donors.

2, available online at <http://www.jcb.org/cgi/content/full/jcb.200206089/DC1>). Presentation of opsonized particles to motile neutrophils at sites distant from the leading edge were still able to provoke the formation of phagocytic cups, signal local and global  $\text{Ca}^{2+}$  signals, and complete phagocytosis (Fig. 4 a). Similarly, presentation of particles to a site on neutrophils distant from an abortive phagocytic cup (produced by removal of a particle before completion) also resulted in complete phagocytosis and  $\text{Ca}^{2+}$  signalling in neutrophils (Fig. 4 b). There were no consistent sites on the neutrophil surface that failed to trigger  $\text{Ca}^{2+}$  and proceed to phagocytosis. Thus, it was concluded that  $\beta_2$  integrin, local  $\text{Ca}^{2+}$  stores, and all signalling molecules relevant for phagocytosis were available at all sites on the cell surface including sites remote from the leading edge of a motile neutrophil or phagocytic cup.

It has previously been shown that  $\beta_2$ -mediated  $\text{Ca}^{2+}$  signals comprise two components:  $\text{Ca}^{2+}$  store release and  $\text{Ca}^{2+}$  influx (Ng-Sikorski et al., 1991; Petersen et al., 1993; Pettit and Hallett, 1996). The  $\text{Ca}^{2+}$  signal produced by  $\beta_2$  engagement by phagocytic challenge was also composed of these two components, with the global  $\text{Ca}^{2+}$  change resulting from  $\text{Ca}^{2+}$  influx. As extracellular divalent cation ions are required for effective C3bi- $\beta_2$  integrin binding (Li et al., 1998), experiments performed in the absence of extracellular  $\text{Ca}^{2+}$  may not be useful for establishing the role of  $\text{Ca}^{2+}$  influx. In-



**Figure 6. The Ca<sup>2+</sup> signal is not a consequence of cell shape change.** The panel shows simultaneously acquired phase contrast and Ca<sup>2+</sup> images. The neutrophil was shown to be phagocytically competent by presentation of a particle which was internalized by the cell and is indicated in the first image by the red arrow. The cell was then treated with cytochalasin B (5  $\mu$ g/ml) to inhibit actin-dependent cell shape changes, and a second particle presented to the cell. Although the neutrophil was unable to form a phagocytic cup or display any morphological change, the Ca<sup>2+</sup> signal associated with C3bi signaling remained, as can be seen in images 45–51 s.

stead, extracellular Ni<sup>2+</sup> was used as a blocker of Ca<sup>2+</sup> influx, and resulted in blockade of the  $\beta$ 2 integrin-triggered global Ca<sup>2+</sup> signal (8/9 cells). This inhibition was demonstrated by both incubation with the inhibitor before challenge with a single particle (Fig. 5, a–c), and by challenge to an individual neutrophil with two particles, one in the absence and the other in presence of the inhibitor (Fig. 5, d–f). The Ca<sup>2+</sup> signal was also inhibited by pretreatment with the PI-3-kinase inhibitor, LY294002 (9/12 cells). It was concluded that the initial local Ca<sup>2+</sup> changes resulted from the release of Ca<sup>2+</sup> from intracellular stores, whereas the global changes required Ca<sup>2+</sup> influx through Ni<sup>2+</sup>-sensitive Ca<sup>2+</sup> channels and that PI-3-kinase activity was important in its signalling (Fig. 5).

### Role of Ca<sup>2+</sup> signals in phagocytosis

In order to establish the roles of the Ca<sup>2+</sup> signals in phagocytosis, it was important to eliminate the possibility that Ca<sup>2+</sup> signalling was an epiphenomenon caused by events associated with morphology changes. Indeed, it has been shown previously that deformation of the neutrophil surface by blunt micropipettes can induced localized or global Ca<sup>2+</sup> changes (Laffafian and Hallett, 1995). In order to exclude this possibility, neutrophils were pretreated with cytochalasin B (5  $\mu$ g/ml). This treatment totally inhibited neutrophil shape change (6/6 cells), but on contact with opsonised particles, localized and global Ca<sup>2+</sup> signals were still triggered (3/6 cells) despite neither phagocytic cup formation nor any other morphological change. In the example shown (Fig. 6), a phagocytically competent neutrophil (with an internalized zymosan particle from the first challenge clearly visible) was then treated with cytochalasin B before the second challenge which provoked a Ca<sup>2+</sup> signal in the absence of any detectable morphology change. These data were consistent with the Ca<sup>2+</sup> signals being causal for the rapid cell shape change and phagocytosis (rather than being caused by the rapid shape change).

The global Ca<sup>2+</sup> influx signal was required for maximum phagocytic rate. Under conditions in which total Ca<sup>2+</sup> signalling and Ca<sup>2+</sup> influx alone were prevented (LY294002 or Ni<sup>2+</sup>), binding and cup formation occurred, but completion of phagocytosis was slow (3/9 cells) or inhibited entirely (6/9

cells). As the rate of phagocytosis was also reduced by omission of extracellular Ca<sup>2+</sup> (see Fig. 8 a), this led to the conclusion that the global Ca<sup>2+</sup> signal, which resulted from Ca<sup>2+</sup> influx, was the key step in triggering the rapid phase of completion of phagocytosis.

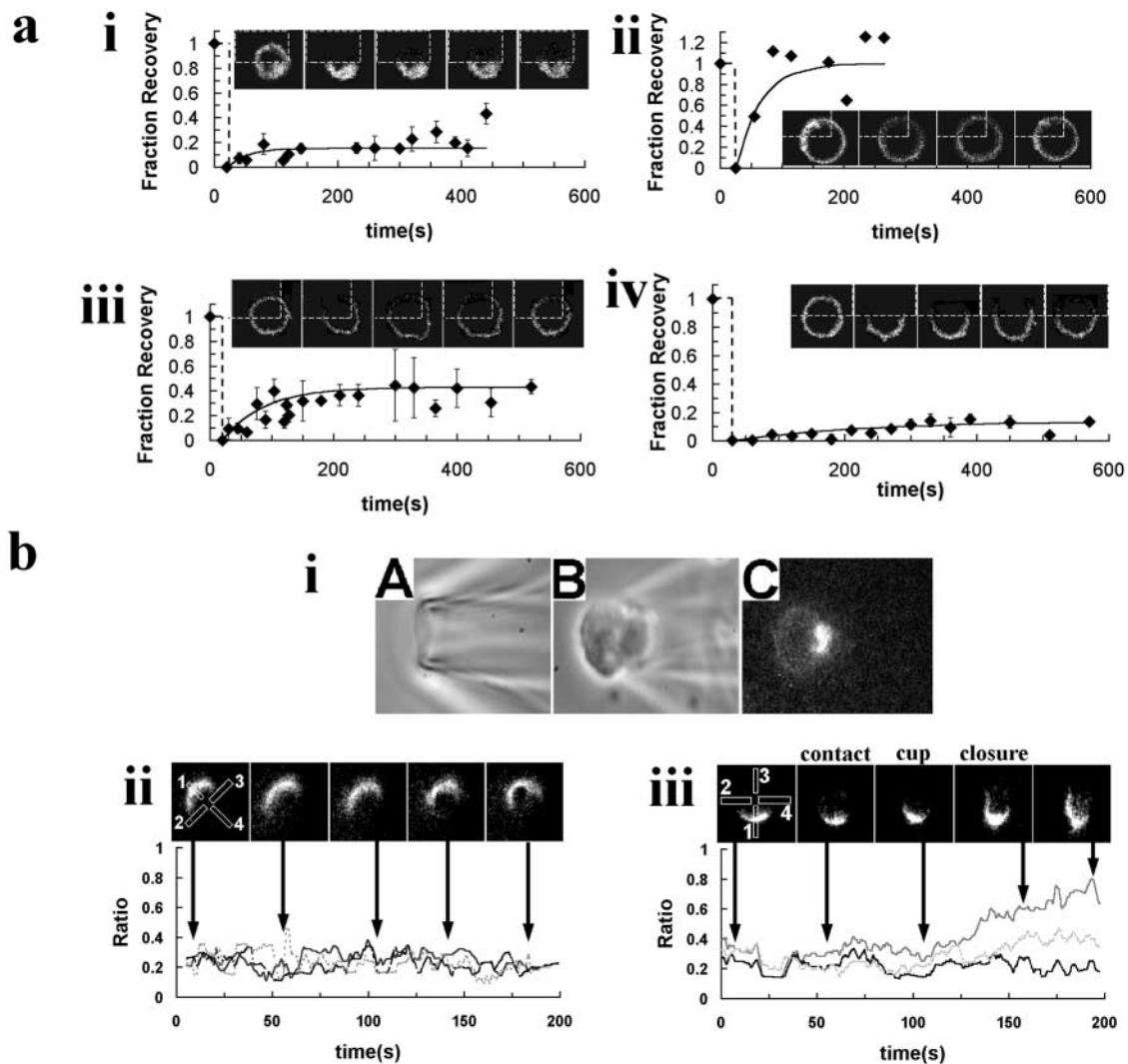
### Ca<sup>2+</sup> increased mobility of integrin receptors

Calpain has previously been suggested to have a role in un-tethering integrin molecules from their cytoskeletal anchors (Stewart et al., 1998; Leitinger et al., 2000; Hogg and Leitinger, 2001). Therefore, the possibility existed that the global Ca<sup>2+</sup> signal observed in neutrophils undergoing phagocytosis freed integrins from sites distant from the contact point to participate in the internalization process. In order to test this, the mobility of  $\alpha$ m $\beta$ 2 (fluorescent antibody-labelled CD18/CD11b) was determined by laser FRAP. In resting neutrophils,  $\beta$ 2 integrin molecules were poorly mobile (Fig. 7 a, i) with only 15.5% ( $\pm$ 5%,  $n = 4$ ) of the fluorescence molecules free to diffuse. However, after activation of calpain with high cytosolic free Ca<sup>2+</sup> concentrations (>5  $\mu$ M average cytosolic concentration), or moderately elevated (0.8–1  $\mu$ M), the fraction of mobile integrin molecules increased to 100 and 43% ( $\pm$ 16%,  $n = 7$ ), respectively (Fig. 7 a, ii and iii). Elevated cytosolic free Ca<sup>2+</sup> significantly increased the fraction of mobile molecules ( $P < 0.001$ ), but did not significantly affect the rate of recovery. The increase in the mobile fraction induced by raising cytosolic free Ca<sup>2+</sup> concentration was prevented by the calpain inhibitor (PD 150606), with the mobility of  $\beta$ 2 integrin molecules being reduced to below that of the resting neutrophils (Fig. 7 a, iv). As the rate of fluorescent recovery after elevation of cytosolic free Ca<sup>2+</sup> was similar under these conditions, but the fractional recovery was altered, this suggested that once liberated,  $\beta$ 2 integrin molecules moved at a similar rate as would be expected for simple diffusion. These data were consistent with elevated cytosolic free Ca<sup>2+</sup> releasing tethered integrin molecules for diffusion by a calpain-dependent step.

In order to determine whether such  $\beta$ 2 integrin un-tethering occurred during phagocytosis, part of the neutrophil surface was labelled with fluorescent antibody using a wide mouthed micropipette containing the antibody (Fig. 7 b, i). As demonstrated by FRAP, the labelled  $\beta$ 2 integrin molecules were essentially immobilised and did not diffuse around the cell periphery (Fig. 7 b, ii). However, after challenge with a C3bi-opsonized particle, these labelled  $\beta$ 2 integrin molecules became again free to diffuse (Fig. 7 b, iii).

### Effect of calpain inhibitors on the phagocytic sequence

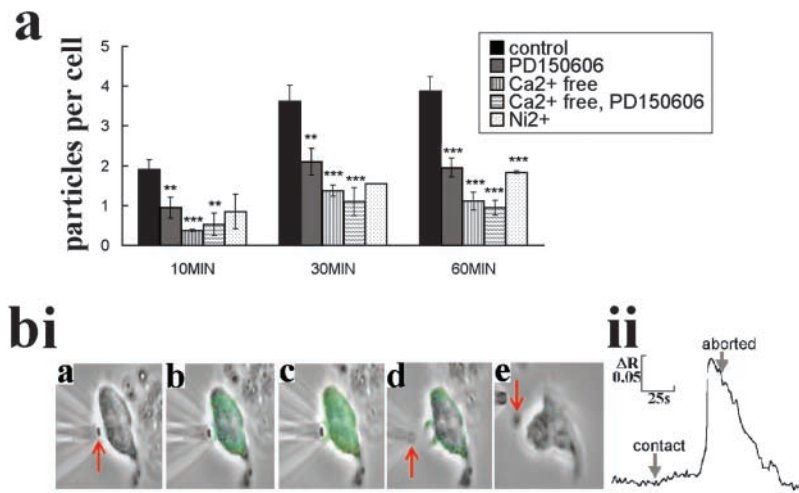
In order to probe whether the calpain-sensitive step was involved in the rapid completion of phagocytosis, pharmacological inhibitors of calpain were employed. An inhibitor of the Ca<sup>2+</sup> binding site on calpain (PD 150606; Lin et al., 1997) inhibited the rapid (opsonin-dependent) uptake of particles and reduced the number of particles which were internalized (Fig. 8 a). The effect was specific for the Ca<sup>2+</sup> influx route, as PD150606 did not inhibit phagocytosis in the absence of extracellular Ca<sup>2+</sup>. Inhibitors of the protease activity of calpain (calpeptin; Tsujinaka et al., 1988) and ALLN had similar inhibitory effects on phagocytosis, reduc-



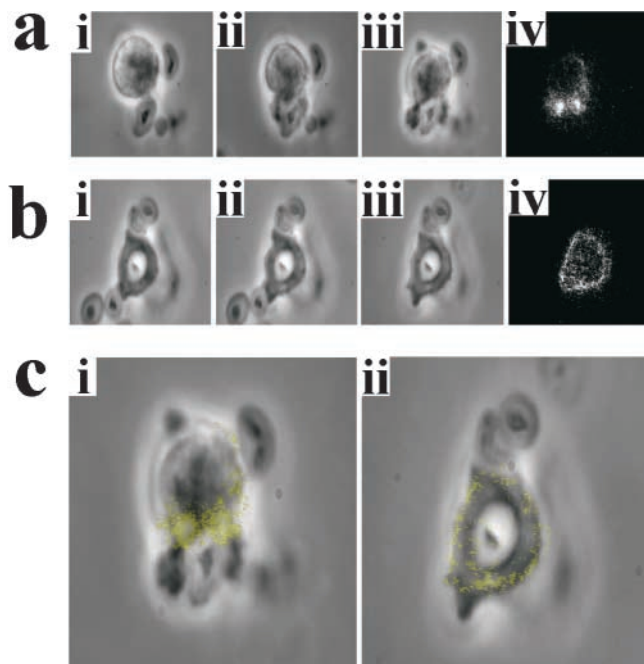
**Figure 7. Changes in the mobility of  $\beta 2$  integrin with  $\text{Ca}^{2+}$  and phagocytosis.** (a) Neutrophils were labeled with phycoerythrin-labeled anti-CD11b antibody for confocal imaging. A confocal plane was chosen which approximately bisected the cell and the mobility of  $\beta 2$  integrin determined after localized photobleaching. (i–iv) The insert shows the image of a representative cell before localized laser bleaching, with the area to be photobleached marked. The subsequent images show the immediate effect of laser bleaching, followed by three images showing the recovery and equilibrium state. The graphs show the recovery of fluorescence in the bleached area over time as a fraction of the unbleached area. The mean and standard deviations are shown ( $n = 4-7$ ), for data from neutrophils with (i) resting  $\text{Ca}^{2+}$  ( $\sim 0.1 \mu\text{M}$ ); (ii) high  $\text{Ca}^{2+}$  ( $> 5 \mu\text{M}$ ) elevated by ionomycin ( $4 \mu\text{M}$  plus  $13 \text{ mM}$  extracellular  $\text{Ca}^{2+}$ ); (iii) elevated cytosolic  $\text{Ca}^{2+}$ ,  $\sim 0.8-1 \mu\text{M}$  induced by ionomycin ( $4 \mu\text{M}$ ); and (iv) elevated cytosolic  $\text{Ca}^{2+}$  ( $\sim 0.8-1 \mu\text{M}$  as in iii) after preincubated with an inhibitor of calpain activation, PD150606 ( $50 \mu\text{M}$ , 15 min). (b) Localized labeling of  $\beta 2$  integrin was achieved by use of wide-mouthed micropipettes (tip diameter,  $5-10 \mu\text{m}$ ), loaded with stock fluorescent (RPE) antibody to CD11b receptor, shown in i A. The micropipette was moved into contact with a cell and slight negative pressure applied to form a seal between tip and cell i B. After 10–30 s, zero pressure was restored and the tip gently taken away, leaving the region of membrane in contact with the pipette contents, fluorescently labeled i C. (ii and iii) A series of images from part-labeled neutrophils at the time indicated by the arrows. The graphs show the ratio of fluorescence in the unlabeled regions (marked 2–4 on the images) with the labeled region (marked 1). The data for a resting neutrophil is shown in ii. (iii) Data is shown from a neutrophil in which a C3bi-opsonized zymosan particle was brought into contact with the cell (marked “contact”), a phagocytic cup formed (marked “cup”), and phagocytosis continued toward closure. These data were typical of at least three similar experiments.

ing both the rate and capacity for phagocytosis. Treatment of individual neutrophils with PD150606 or calpeptin also reduced the speed of phagocytosis in some neutrophils (4/9 with calpeptin) and prevented phagocytosis entirely in the remainder (3/3 cells with PD 150606; 5/9 cells with calpeptin). In all cases, the intact  $\text{Ca}^{2+}$  signalling sequence was evident on presentation of the opsonized particle (Fig. 8 b). After inhibition of calpain, binding of the particle and formation of the phagocytic cup could still occur, but pro-

gression to complete phagocytosis was prevented (Fig. 8 b, i). The consequent abortive phagocytic cups were deficient in antibody-accessible  $\beta 2$  integrin (Fig. 9, b and c), despite being uniformly distributed on the rest of the cell surface. In contrast, calpain-competent cells had an abundance of antibody-detectable  $\beta 2$  integrin at the phagocytic cup (Fig. 9 a). Together, these data pointed to a crucial role for  $\text{Ca}^{2+}$  activation of calpain activity as an important step in liberating  $\beta 2$  integrin for facilitation of phagocytosis.



\* is  $P < 0.05$ , \*\* is  $P < 0.01$ , and \*\*\* is  $P < 0.001$  compared with untreated control. (b) A typical experiment is shown in which a neutrophil treated with the calpain inhibitor calpeptin (100 mg/ml, 15 min, 37°C) was presented with an opsonized zymosan particle. (i) The series of images show the neutrophil morphology changes with high cytosolic free Ca<sup>2+</sup> pixels overlain. (a) Presentation of the particle, indicated by red arrow, provokes a loose binding (b) and phagocytic cup forms (c), but fails to progress so that on release of the particle from the micropipette, the particle is not bound to the cell (d, red arrow). The cytosolic free Ca<sup>2+</sup> signal is triggered and the cytoplasm of the neutrophil moves toward the region of the abortive cup (e), but the particle has not been internalized and is visible outside the cell (red arrow). (ii) The complete Ca<sup>2+</sup> signal is shown with the points at which contact was made and the phagocytic event aborted indicated by the arrows.



**Figure 9. Distribution of  $\beta 2$  integrin in phagocytic cup in calpain-competent and inhibited neutrophils.** The distribution of  $\beta 2$  integrin in neutrophils undergoing phagocytosis was determined by pulsing the cell with fluorescent anti-CD18 as the phagocytic cup formed. It was not possible to prelabel  $\beta 2$  integrin as this inhibited phagocytosis by this route, so labeling was performed after engagement of  $\beta 2$  integrin, cup formation and at the time of Ca<sup>2+</sup> signaling. (a) A typical untreated neutrophil in which the progression of phagocytosis is shown from (i) binding, (ii) initiation of cup formation, and (iii) cup formation and the point of Ca<sup>2+</sup> signaling, at which time fluorescent antibody was pulsed, resulting in the fluorescent image (iv). The position of labeled  $\beta 2$ -integrin is shown superimposed on the cell image (ci) to demonstrate  $\beta 2$  integrin near the phagocytic cup available for facilitating phagocytosis. Panel (b) shows a similar typical experiment in a neutrophil treated with the calpain inhibitor

### Figure 8. Calpain inhibition reduces phagocytosis without interfering with Ca<sup>2+</sup> signaling.

(a) Populations of neutrophils were incubated at 37°C with C3bi-opsonized zymosan particles for 10, 30, and 60 min, before being fixed, stained, and phagocytosis quantified. The histograms show the mean and standard errors for the number of particles/cell internalized by at least 100 neutrophils from separate donors ( $n$  value). Data is shown for untreated neutrophils (control,  $n = 11$ ), those treated with the calpain inhibitor PD150606 (50  $\mu$ M, 15 min,  $n = 7$ ), untreated neutrophils in the absence of extracellular Ca<sup>2+</sup> (1 mM EGTA; Ca<sup>2+</sup> free,  $n = 4$ ), those treated with the calpain inhibitor PD150606 (50  $\mu$ M, 15 min) and then phagocytosis performed in the absence of extracellular Ca<sup>2+</sup> (Ca<sup>2+</sup> free, PD150606,  $n = 4$ ), and untreated neutrophils in the presence of extracellular Ca<sup>2+</sup> and Ni<sup>2+</sup> (1 mM; Ni<sup>2+</sup>,  $n = 2$ ).

The asterisks indicate significant difference where

## Discussion

In this paper, we have shown that phagocytosis of C3bi-opsonized particles by neutrophils is accelerated by a global increase in cytosolic free Ca<sup>2+</sup> concentration which follows localized integrin engagement at the point of contact between the particle and the cell. The global Ca<sup>2+</sup> change resulted from Ca<sup>2+</sup> influx and was responsible for increased mobility of  $\beta 2$  integrin molecules distant from the contact site. The mechanism for this latter stage was dependent on calpain activation via its Ca<sup>2+</sup> binding sites. As inhibition of calpain also inhibited C3bi-accelerated phagocytosis, we propose the following model to explain how C3bi opsonization facilitates phagocytosis. The initial contact between the C3bi-opsonized particle and the neutrophil causes crosslinking of local  $\beta 2$  integrin molecules which results in localized Ca<sup>2+</sup> release from storage sites near the plasma membrane and formation of the phagocytic cup. The rate of further  $\beta 2$  integrin binding to the particle is limited by the supply of  $\beta 2$  integrin molecules which are tethered to cytoskeletal components. However, depletion of localized Ca<sup>2+</sup> stores generates a diffusible signal which opens Ca<sup>2+</sup> channels in the plasma membrane distant from the initial contact site and Ca<sup>2+</sup> influx occurs across the entire neutrophil surface. This Ca<sup>2+</sup> influx activates calpain which releases dis-

PD150606 (50  $\mu$ M, 15 min). The images show (i) the neutrophil chosen with an internalized particle, as evidence that it was phagocytically competent before treatment with the inhibitor (ii) binding of two particles and abortive cup formation after inhibition of calpain and (iii) cup formation of the upper particle (and detachment of the lower) and the point of Ca<sup>2+</sup> signaling, at which time fluorescent antibody was pulsed, resulting in the fluorescent image (iv). Although labeled  $\beta 2$ -integrin was visible over most of the neutrophil surface, the superimposed images (c, ii) reveals that unlike in the uninhibited cells, no  $\beta 2$  integrin was available in either of the two abortive phagocytic cups. These experiments were typical of at least three others.

tant  $\beta 2$  integrin molecules from their tethers and permits their diffusion to the contact site to complete the phagocytic event.

It is well established that the  $\beta 2$  integrin is coupled to cytosolic free  $\text{Ca}^{2+}$  signalling when it is immobilized or crosslinked. Thus, antibody crosslinking with anti- $\beta 2$  antibodies (Ng-Sikorski et al., 1991) or permitting neutrophils to sediment onto anti- $\beta 2$  antibody coated surfaces triggers (Pettit and Hallett, 1996) release of  $\text{Ca}^{2+}$  from internal stores and influx of  $\text{Ca}^{2+}$  from the extracellular environment. Using confocal z sectioning, it was shown that the release of intracellular  $\text{Ca}^{2+}$  occurred only at the points of cell contact with the anti- $\beta 2$  integrin (Pettit and Hallett, 1996), whereas  $\text{Ca}^{2+}$  influx occurred from all surfaces. It was also observed that the global  $\text{Ca}^{2+}$  signal was also followed by rapid neutrophil spreading onto the integrin-engaging surface. As  $\text{Ca}^{2+}$  signals often precede neutrophil spreading (Kruskal et al., 1986; Marks and Maxfield, 1990), the possibility that global  $\text{Ca}^{2+}$  signals caused neutrophil spreading was investigated by releasing caged  $\text{Ca}^{2+}$  in neutrophils which had newly sedimented onto a surface but had yet to spread. These experiments showed that there was a linkage between globally elevated  $\text{Ca}^{2+}$  and neutrophil spreading but only onto  $\beta 2$  integrin engaging surfaces (Pettit and Hallett, 1998). The data we present here now provides the explanation for those experiments, as neutrophil spreading onto  $\beta 2$ -integrin engaging surfaces and C3bi-mediated phagocytosis are essentially equivalent cell events. The increased mobility of  $\beta 2$  integrin molecules may also underlie the changes in receptor affinity and avidity which occur on cell activation (Hato et al., 1998; Li et al., 1998; van Kooyk and Figdor, 2000). Thus, the positive feedback provided by the loop of integrin engagement signalling  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  permitting further integrin engagement may be the motor for phagocytic acceleration. The second phase of integrin binding may be responsible for the second  $\text{Ca}^{2+}$  peak observed in some neutrophils. It is possible that the two  $\text{Ca}^{2+}$  events that are visible in these cells are present in all cells, but that they manifest as a single  $\text{Ca}^{2+}$  peak (Fig. 5 e), a  $\text{Ca}^{2+}$  peak with shoulder (Fig. 5 a), or double  $\text{Ca}^{2+}$  peak (Fig. 5 f) depending on the kinetics of cytosolic free  $\text{Ca}^{2+}$  reuptake. A recent mathematical model suggests that such differences may arise in individual cells as a result of differences in the amounts of their  $\text{Ca}^{2+}$  storage proteins (Baker et al., 2002).

However, some important questions remain unresolved. The first relates to the mechanism by which calpain liberates  $\beta 2$  integrin molecules in neutrophils. It is not clear which is the crucial calpain substrate in neutrophils and whether this is the linkage between  $\beta 2$  integrin and the cytoskeleton. However, talin, a known cytoskeletal linker, is a strong candidate as it is established that an elevation of cytosolic free  $\text{Ca}^{2+}$  concentration in neutrophils results in the cleavage of this molecule (Sampath et al., 1998). Another question relates to the specificity of the  $\text{Ca}^{2+}$  signal for calpain activation. It is clear that neutrophils are able to complete multiple tasks, such as phagocytosis, oxidase activation, and exocytosis and that changes in cytosolic free  $\text{Ca}^{2+}$  have been implicated in each of them (Scharff and Foder, 1993). Although it has not yet been possible to establish how specific outcomes can arise from  $\text{Ca}^{2+}$  signalling, for calpain activation, the explanation may be clearer. The two forms of calpain ( $\mu$  and  $m$ ) differ in their affinity for  $\text{Ca}^{2+}$ , with  $\mu$ -calpain having an apparent dissociation constant

for  $\text{Ca}^{2+}$  of  $\sim 25 \mu\text{M}$  (Michetti et al., 1997). As bulk cytosolic free  $\text{Ca}^{2+}$  never reaches this level under physiological conditions, calpain is essentially held in an inactive form. However, during  $\text{Ca}^{2+}$  influx, the concentration of free  $\text{Ca}^{2+}$  just beneath the plasma membrane rises to at least  $30 \mu\text{M}$  (Davies and Hallett, 1999). Thus,  $\mu$ -calpain would be activated specifically just beneath the plasma membrane at the strategic site required for talin cleavage. Other questions which arise include whether similar mechanisms accompany IgG-mediated phagocytosis by neutrophils. Although it has been suggested that the mechanisms of phagocytosis by the two routes may differ with respect to pseudopod protrusion (May and Machesky, 2001), with the micropipette method used here, presentation of either C3bi or IgG-opsonized zymosan particles resulted in similar pseudopod extension. A final question concerns the role of the initial  $\text{Ca}^{2+}$  release event. Although it may be a necessary prelude for  $\text{Ca}^{2+}$  influx, the possibility exists that it may also be important for local oxidase activation or phagosome-lysosome fusion. In a recent paper (Müller-Taubenberger et al., 2001), it has been shown in *Dictyostelium* lacking the  $\text{Ca}^{2+}$  storage proteins calreticulin and calnexin, that phagocytosis was impaired as a result of a defect in actin polymerization. These authors suggested that the  $\text{Ca}^{2+}$  storage of these cells was directly linked to actin regulation. If a similar situation exists in neutrophils, then the initial local  $\text{Ca}^{2+}$  signal may “seed” actin polymerization for phagocytic cup formation. Clearly, further work will be required to establish the answers to these and other questions. However, the data presented here lays the foundation for understanding the link between C3bi engagement,  $\text{Ca}^{2+}$  signals and phagocytosis.

## Materials and methods

### Neutrophil isolation

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously (Hallett et al., 1990). After dextran sedimentation, centrifugation through Ficoll-Paque (Amersham Biosciences) and hypotonic lysis of red cells, neutrophils were washed and resuspended in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 25 mM Hepes and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH).

### Simultaneous cytosolic free $\text{Ca}^{2+}$ and phagocytosis quantitation and imaging

Neutrophils were loaded with fura-2 from the acetoxy-methyl ester for ratio imaging measurements as previously described (Hallett et al., 1990). Excitation wavelengths (340 and 380 nm) selected using a rapid monochromator (Delta RAM; PTI), which was connected to a Nikon Eclipse inverted microscope. The cells were maintained at  $37^\circ\text{C}$  using a temperature controlled microscope stage heater. The images at each excitation wavelength were collected using an intensified CCD camera (IC100; PTI) and the ratio image calculated using Image Master software (PTI). All images were captured using an oil immersion  $100\times$  objective. Phase contrast images were taken under far red illumination simultaneously with acquisition of  $\text{Ca}^{2+}$  images. This was achieved using an appropriate dichroic mirror and a second red-sensitive CCD camera. Fura-2 ratio images were collected from adherent neutrophils while a suspension of C3bi-opsonized zymosan particles was added. 340/380nm ratio fluorescence images were acquired with 16-frame averaging and threshold background subtraction at a rate of at least 0.6 frames/second. The ratiometric ( $\text{Ca}^{2+}$ ) images were pseudocolored according to the scale shown in Fig. 1, and the average ratio value of the pixels was calculated and plotted over the time course. The morphological changes (e.g., cell area, roundness, and perimeter contact between particle and cell) were determined using purpose-written software (A. Hoppe, University of Glamorgan, Wales, UK) and Optimas. The stimulus was added to the cells under view while recording continuously. All experiments were repeated on separate occasions with neutrophils isolated from different donors.



### C3bi opsonization and presentation of zymosan

Zymosan particles (10 mg/ml) were opsonized either by incubation with human serum (50% diluted, 30 min, 37°C) or with purified human C3bi (1 mg/ml; 30 min, 4°C). The particles were then washed by centrifugation and resuspension to remove unfixed C3bi and used immediately or stored at -20°C. Human neutrophils were allowed to adhere to glass coverslips for 1 to 2 min before presentation of C3bi-opsonized zymosan particles (~2 μm in diameter). Zymosan particles were allowed to sediment among the cells. A micropipette (tip diameter, 1–1.5 μm; WPI), with slight negative pressure applied, was used to pick up and hold a single zymosan particle. This was brought to the target neutrophil and contact between the neutrophil and the particle made. When adhesion between the opsonized particle and the neutrophil had occurred, and the particle was released from the micropipette and phagocytosis was allowed to proceed (Fig. 2 a).

### Mobility of integrins

The β chain (CD18) and α chain (CD11b) of the β2 integrin were labelled with fluorescently labelled antibody (phycoerythrin or fluorescein) by incubation of the cells with excess antibody. A confocal plane was chosen which approximately bisected the cell to provide an image of the cell equator at low laser power. The voltage on the photomultiplier tube was set to maximum and line averaging was used. The portion of the cell to be photobleached was subjected to high power laser scanning for 10–30 s. Confocal images were then acquired as the fluorescence within the bleached area recovered. The ratio of the intensities of the bleached to nonbleached regions of the cell (*I*<sub>r</sub>) were measured, at time intervals (3–30 s) over the following 2 min. The rate of recovery after photobleaching (*I*<sub>r</sub>) and the fraction of mobile molecules (*Mf*) was calculated as  $I_r = Mf(1 - e^{-kt})$ , where  $k = 1/\ln_2 t/2$  and *t* = time after bleaching.

In experiments in which the location of β2 integrin was determined during phagocytosis, it was not possible to preincubate the cells with antibody to either CD18 or CD11b, as this inhibited accelerated phagocytosis. Therefore, experiments were performed in two ways. The first approach was to label part of the neutrophil surface remote from the phagocytic event. This was achieved by using a wide micropipette (~5 μm; WPI) containing the fluorescent antibody. A neutrophil was drawn into the mouth of the micropipette with slight negative pressure and held for ~20 s, before it was expelled by restoring zero intrapipette pressure. This ejected the partially β2 integrin-labelled neutrophil without expelling antibody into the surrounding medium. The second approach was to label β2 integrin after initiating phagocytosis in neutrophils by adding a pulse of excess fluorescently labelled antibody to the cell at the point of phagocytic cup formation. This permitted CD18/CD11b labelling and washing of nonadherent antibody to be achieved within ~30 s of the onset of the Ca<sup>2+</sup> signal.

### Source of reagents

Fura-2-AM was purchased from Molecular Probes. Purified human C3bi, ALLN, and PD150606 were purchased from Calbiochem, LY294002 from Sigma-Aldrich, and antibody to CD18 and CD11b from Dako. All other standard chemicals were purchased from Sigma-Aldrich.

### Online supplemental material

Video clips of the full data from which Figs. 1 and 4 were derived are available online at <http://www.jcb.org/cgi/content/full/jcb.200206089/DC1>. These video clips show the cytosolic free Ca<sup>2+</sup> concentration changes which occur on contact between the C3bi-coated particle and the neutrophil, either spontaneously (Video 1) or on presentation with a micropipette (Video 2). In the Video 2, the phase contrast image is also shown together with the Ca<sup>2+</sup> measurement, so that the correlation between Ca<sup>2+</sup> elevation and the accelerated phase of phagocytosis can be clearly seen.

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