Cytosolic Free Calcium Elevation Mediates the Phagosome–Lysosome Fusion during Phagocytosis in Human Neutrophils

M. E. E. Jaconi,* D. P. Lew,* J.-L. Carpentier,‡ K. E. Magnusson,§ M. Sjögren,§ and O. Stendahl§

*Division of Infectious Diseases, Geneva University Hospital, ‡Institute of Histology and Embryology, University Medical Center, Geneva, Switzerland; and §Department of Medical Microbiology, Linköping University, Sweden

Abstract. Cytosolic free calcium ($[Ca^{2+}]_i$) and fusion of secondary granules with the phagosomal membrane (phagosome–lysosome fusion, P-L fusion) were assessed in single adherent human neutrophils during phagocytosis of C3bi-opsonized yeast particles.

Neutrophils were loaded with the fluorescent dye fura2/AM and $[Ca^{2+}]_i$ was assessed by dual excitation microfluorimetry. Discharge of lactoferrin, a secondary granule marker into the phagosome was verified by immunostaining using standard epifluorescence, confocal laser scanning and electron microscopy.

In Ca^{2+}-containing medium, upon contact with a yeast particle, a rapid rise in $[Ca^{2+}]_i$ was observed, followed by one or more Ca^{2+} peaks (maximal value 1,586 nM and median duration 145 s): P-L fusion was detected in 80% of the cells after 5–10 min. In Ca^{2+}-free medium the amplitude, frequency and duration of the $[Ca^{2+}]_i$ transients were decreased (maximal value 368 nM, mostly one single Ca^{2+} peak and median duration 75 s): P-L fusion was decreased to 52%.

Increasing the cytosolic Ca^{2+} buffering capacity by loading the cells with MAPT/AM led to a dose-dependent inhibition both of $[Ca^{2+}]_i$ elevations and P-L fusion. Under conditions where basal $[Ca^{2+}]_i$ was reduced to <20 nM and intracellular Ca^{2+} stores were depleted, P-L fusion was drastically inhibited while the cells ingested yeast particles normally. P-L fusion could be restored in Ca^{2+}-buffered cells containing ingested particles by elevating $[Ca^{2+}]_i$ with the Ca^{2+}-ionophore ionomycin.

The present findings directly indicate that although the ingestion step of phagocytosis is a Ca^{2+}-independent event, $[Ca^{2+}]_i$ transients triggered upon contact with opsonized particles are necessary to control the subsequent fusion of secondary granules with the phagosomal membrane.

THE main function of neutrophils is to phagocytose and kill invading microorganisms. During the ingestion process, various populations of granules fuse rapidly and sequentially with the newly formed phagosome (23). Concomitantly, the various components of the NADPH-oxidase, which are present both in granules and in the cytosol, are inserted into the phagosomal membrane leading to activation of the multifactorial microbicidal systems (28). Thus, phagosome–lysosome (P-L) fusion is a key event for neutrophil microbicidal activity.

Activation of phagocytic receptor triggers a series of biochemical events that link the cell surface with the subsequent cellular responses. It has recently been shown that phagocytosis is associated with the accumulation of inositol, 1,4,5-trisphosphate ($Ins[1,4,5]P_3$) and 1,2-diacylglycerol (DG)[1] (9). In various cellular systems the former induces rapid mobilization of intracellular calcium, whereas the latter is an endogenous activator of protein kinase C (4). In a previous study using populations of quin2-loaded neutrophils in suspension, it was possible to show that during phagocytosis there is a weak elevation of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) preceded by a considerable lag period (17). In contrast, studies using single cell analysis showed rapid and localized elevations of $[Ca^{2+}]_i$ during phagocytosis by macrophages and neutrophils (14, 26). The whole signaling cascade does not appear to be necessary for all the ingestion steps of phagocytosis. In fact, several types of C3bi-opsonized particles could be ingested at exceedingly low $[Ca^{2+}]_i$ ($<10^{-8}$ M), i.e., at concentrations where Ca^{2+}-dependent processes do not occur either in neutrophils or macrophages (7, 17). Under similar experimental conditions there was no enhanced formation of inositol phosphates, but increased accumulation of DG, suggesting that DG, not derived from phosphoinositides, may serve as an important second messenger during the ingestion process (9).

Thus it has become important to elucidate, at the single cell level, not only the kinetics of the rise in $[Ca^{2+}]_i$ but also whether this $[Ca^{2+}]_i$ change plays any function at all during receptor-mediated phagocytosis. In this investigation, using single cell analysis and manipulation of $[Ca^{2+}]_i$ changes during phagocytosis followed by various immunomicroscopical procedures, we provide evidence that, although the ingestion process may be entirely Ca^{2+} independent, the fu-

1. *Abbreviations used in this paper*: DG, diacylglycerol; P-L fusion, phagosome–lysosome fusion.

sion of secondary granules with the phagosomal membrane is a process that requires receptor-triggered $[Ca^{2+}]_i$ elevations.

Materials and Methods

Isolation of Neutrophils

Neutrophils from healthy donors were isolated from citrate-treated or heparinized blood by Dextran T500 sedimentation, Hypaque-Ficoll gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden) and hypotonic lysis as previously described (18). The cells were finally suspended to 10×10^6/ml in the appropriate buffer.

Reagents

Phorbol myristate acetate (PMA), BSA (fraction V), ionomycin, and FITC were purchased from Sigma Chemical Co. (St. Louis, MO). Fura2/AM was obtained from Molecular Probes, Inc. (Eugene, OR) and MAPT/AM (1,2-bis-5-methyl-amino-phenoxylethane-*N,N,n'*-tetra-acetoxymethyl acetate) from Calbiochem-Behring Corp. AG (La Jolla, CA). Rabbit anti-human lactoferrin serum was a generous gift from Dr. Inge Olsson (University of Lund, Lund, Sweden) and rhodamine-labeled swine anti-rabbit IgG was obtained from Dakopatts (Copenhagen, Denmark).

Loading the Cells with Fura2/AM and MAPT/AM

The cells were suspended to 10×10^6 cells/ml either in Ca^{2+}-medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM NaH_2PO_4, 5 mM $NaHCO_3$, 20 mM Hepes, pH 7.2 supplemented with 0.1% BSA, i.e., $[Ca^{2+}]_o = 10^{-3}$ M, or in Ca^{2+}-free medium, a similar medium with no $CaCl_2$ and supplemented with 1 mM EGTA, i.e., $[Ca^{2+}]_o = 10^{-9}$ M. The cells were warmed to 37°C for 5 min before Fura2/AM was added to a final concentration of 1 μM, and then further incubated for 45 min. When MAPT/AM was used, a final concentration of 10 or 25 μM of the reagent was added 15 min before the incubation with Fura2/AM. The cells were then centrifuged (200 *g*, 5 min) and resuspended in Ca^{2+}- or Ca^{2+}-free medium for the phagocytosis experiments. When cells are loaded in Ca^{2+}-free medium in the presence of MAPT/AM and maintained in Ca^{2+}-free medium the basal level of $[Ca^{2+}]_i$ is decreased severalfold and the cells are depleted of intracellular Ca^{2+} stores (referred to in the text as Ca^{2+}-depleted neutrophils) (6, 17).

Rapid Monitoring of $[Ca^{2+}]_i$ Changes in Single Neutrophils during Phagocytosis

$[Ca^{2+}]_i$ was measured using the fluorescent dye Fura2 by a dual excitation microfluorimetry technique (Glen Creston Corp,., London, UK), permitting a resolution time of 100 ms (27). The ratio of the fluorescence ($R = F_{340}/F_{380}$) was calibrated to express $[Ca^{2+}]_i$ using the formula proposed previously: $[Ca^{2+}]_i = k_d \cdot \beta \cdot (R-R_{min})/(R_{max}-R)$ (11). Yeast particles (*Saccharomyces cerevisiae*) opsonized with 25% normal human serum, to yield C3bi particles with no detectable IgG (12), were attached to glass coverslips by allowing the yeast particles to sediment at 37°C in a volume of 50 μl at a concentration of 10^6 particles/ml, to have a well-spaced single-particle distribution. The coverslips were then mounted in a temperature-controlled chamber of the inverted microscope. 10^5 neutrophils in a volume of 10 μl added to the chamber, were allowed to adhere to the yeast-coated coverslips, and the neutrophil-yeast interaction was observed continuously in the microscope. At appropriate times, the Ca^{2+}-dependent Fura2 fluorescence was followed in a single neutrophil encountering a yeast particle. Simultaneously with the $[Ca^{2+}]_i$ recording, the number of phagocytosing neutrophils was estimated. After monitoring $[Ca^{2+}]_i$, the cells were fixed to study P-L fusion.

Phagocytosis

To evaluate more precisely the rate of phagocytosis under different calcium conditions, 100 μl of FITC-labeled, C3bi-opsonized yeast particles (2×10^6/ml) were added to neutrophils adherent on glass slides and incubated in a moist chamber. To distinguish between attached and ingested particles, trypan blue (0.2% in PBS) was added before microscopic examination. The dye quenches the extracellular fluorescence but does not reach the phagocytosed fluorescent yeast particles within the phagosome (12, 17).

Phagosome–Lysosome (P-L) Fusion

Fusion between specific granules and the phagocytic vacuole was assayed by studying the release of lactoferrin, a secondary granule marker into the phagosome. Lactoferrin was localized with an antilactoferrin antibody either by indirect immunofluorescence (using standard epifluorescence or confocal laser scanning microscopy) or immunoelectron microscopy.

Immunofluorescence

After incubation for 5–10 min, the cells were fixed in 4% paraformaldehyde in 0.125 M phosphate buffer, pH 7.4, for 45 min, and then thoroughly washed in the phosphate buffer overnight. The cells were then incubated for 60 min with 0.3% Triton X-100 in 0.45 M NaCl and 20 mM phosphate buffer, pH 7.2, supplemented with 200 μg/ml of normal swine IgG, to permeabilize the cells and block nonspecific binding of antibodies. After washing in the same buffer, rabbit antihuman lactoferrin antiserum (diluted 1:40 in the same buffer) was added to the cells. After 60 min, the cells were washed in the same buffer, and finally incubated with 100 μl of rhodamine-labeled swine anti-rabbit IgG at a concentration of 40 μg/ml (in PBS, pH 7.2). After washing in PBS, the slides were mounted with glycerol-PBS, pH 7.2, and examined with epifluorescence microscopy. When a distinct ring of fluorescence was observed around the ingested yeast particle, indicating release of lactoferrin into the phagocytic vacuole, P-L fusion was scored as having taken place. Consequently, cells with no P-L fusion lacked a distinct fluorescent ring around the ingested yeast particles. P-L fusion was expressed as the percentage of phagocytosing cells showing this fluorescence within the phagocytic vacuole. Cells with a fluorescent ring not well defined were scored as doubtful cells. These cells represented between 6 and 7% of the total cells in all experimental conditions tested and were not included in the group of positive cells.

Laser Scanning Confocal Microscopy

To further localize, evaluate, and confirm the fluorescence localization within the neutrophil, a confocal scanning laser microscope (Phoibos 1000; Sarastro AB, Stockholm, Sweden) was used (5). With this technique, thin (0.6 μm) sections of the cell can be observed, eliminating any interfering light outside, below, or above the plane of focus. It thus allows us to rule out differences in fluorescence due solely to differences in thickness of the cytosol or membranes.

Immunoelectron Microscopy

To localize lactoferrin on thin sections, the cells were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.4) for 30 min at 22°C. Cells were then centrifuged at 1,200 rpm for 8 min, resuspended in PBS, centrifuged again, and embedded in Agar, which was cut into small blocks (2–3 mm^3). They were then processed for low temperature embedding in Lowicryl K4M (25).

Thin sections of Lowicryl K4M-embedded cells were collected on nickel grids and immunolabeled by an antilactoferrin antibody and an anti-IgG-gold complex (10 nm) (Janssen Life Science Products, Beerse, Belgium) as previously described (24). The grids were then rinsed with distilled water between each incubation step. Immunolabeled sections were double-stained with uranyl acetate and lead citrate before examination on the electron microscope.

Two separate experiments were performed. In each experimental condition, 80–90 pictures (45–50 per experiment) of phagosomes were randomly photographed. Pictures were taken at an initial magnification of 19,000.

The number of gold particles per squared micron of the phagosomal space encompassing the yeast and delimited externally by the phagosome-limiting membrane was evaluated on positive prints at a final magnification of 57,000. The intensity of the labeling was assessed as the number of gold particles per squared micron of this phagosomal space and recorded with an electronic pen on a graphic tablet (type 4983; Tektronics, Inc., Beaverton, OR) connected with a microprocessor system (IBM PC-AT) that was programmed to calculate the number of particles per squared micron.

Exocytosis Experiments

To assess release into the extracellular medium vitamin B12-binding protein (secondary granules), β-glucuronidase (primary granules) and lactate dehydrogenase (LDH) (cytoplasmic content) were assayed in the supernatants and calculated as a percentage of total protein released from an aliquot of the same cell suspension treated with 0.1% Triton X-100 for 5 min at 37°C

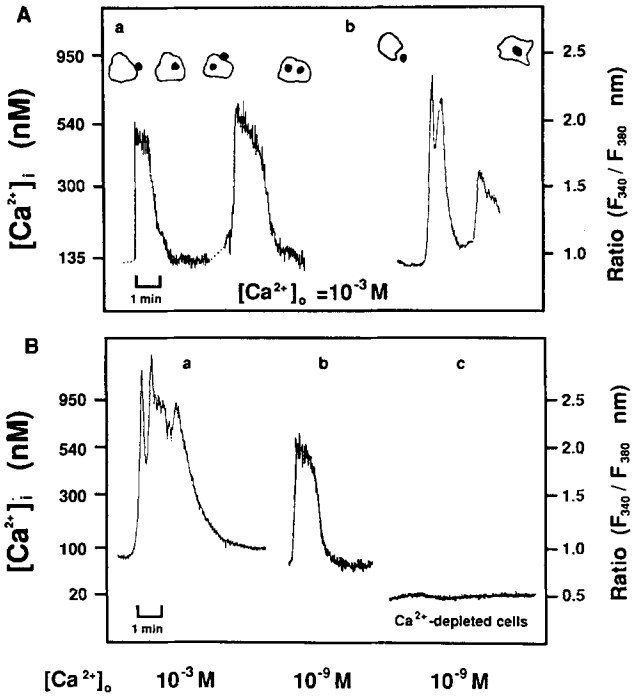

Figure 1. Monitoring of $[Ca^{2+}]_i$ in single adherent neutrophils during surface phagocytosis. (*A*) Continuous monitoring of $[Ca^{2+}]_i$ in single neutrophils during phagocytosis of serum-opsonized yeast particles. (*Top*) Cell shape observed during the recording by rapid switch to phase-contrast microscopy during monitoring of the Ca^{2+} signal. (*B*) Typical recording of a single cell measurement of $[Ca^{2+}]_i$ in fura2-loaded human neutrophils during surface phagocytosis of serum-opsonized yeast particles. (*a*) Normal cells in Ca^{2+} medium; (*b*) normal cells in Ca^{2+}-free medium; (*c*) Ca^{2+}-depleted cells with 25 μM MAPT/AM in Ca^{2+}-free medium. The extracellular concentration of Ca^{2+} ($[Ca^{2+}]_o$) is indicated in the figure.

(18). Phagocytosis was carried out under two conditions: (*a*) phagocytosis of yeast particles in suspension (under identical condition of the electron microscopy experiments) at a ratio of one particle to one neutrophil; surface phagocytosis of yeast particles adherent to culture petri dishes (under identical condition of immunofluorescence experiments) (16, 18).

Statistical Analysis

Data were analyzed with one of the following tests: *t* test, Mann-Whitney-U test, or one-way analysis of variance.

Results

Ca^{2+} Activity in Single Adherent Neutrophils during Phagocytosis

To study the role of extracellular and intracellular Ca^{2+} during phagocytosis, opsonized yeast particles adherent to coverslips were incubated with neutrophils under various buffer conditions (neutrophils in the presence or absence of extracellular Ca^{2+} or Ca^{2+}-depleted neutrophils in Ca^{2+}-free medium). The $[Ca^{2+}]_i$ resting level before the ingestion of the yeast particle is significantly lower in Ca^{2+}-depleted cells: 19 $\pm$ 2 nM (n = 26) in Ca^{2+}-depleted cells versus 89 $\pm$ 6 nM (n = 16) and 84 $\pm$ 6 nM (n = 34) in nondepleted cells in the presence or absence of extracellular Ca^{2+}, respectively (mean $\pm$ SEM) ($P < 0.001$).

In the presence of 1 mM extracellular calcium, neutrophils exhibited a rapid increase in $[Ca^{2+}]_i$ upon contact with an opsonized yeast particle (Fig. 1, *Aa* and *Ab*). This $[Ca^{2+}]_i$ elevation is repeated upon contact with a second particle (Fig. 1 *Aa*). After an initial rapid rise, there is also a sustained and/or an oscillatory response (Fig. 1, *A* and *Ba*) for 1–2 min, correlating with the ingestion of the particle; thereafter the $[Ca^{2+}]_i$ returns to baseline. When 1 mM EGTA was present in the medium to reduce the extracellular calcium concentration ($[Ca^{2+}]_o$) to 10^{-9} M, phagocytosis still proceeded with a rapid but transient $[Ca^{2+}]_i$ change (Fig. 1 *Bb*). No clear rapid oscillations were observed under this condition. When the neutrophils were Ca^{2+}-depleted by preloading the cells with 25 μM MAPT/AM, the basal $[Ca^{2+}]_i$ level was drastically reduced to <20 nM and the $[Ca^{2+}]_i$ transient completely abolished during phagocytosis (Fig. 1 *Bc*).

Fig. 2 analyzes the Ca^{2+} oscillatory pattern observed in phagocytosing neutrophils in the presence or absence of extracellular Ca^{2+}. Peaks were rejected if shorter than 5 s, or of smaller amplitude than 30 nM as described previously (13). Out of 22 cells in Ca^{2+} medium, 18 had more than one Ca^{2+} peak, whereas 17 out of 28 cells had only 1 peak in Ca^{2+}-free medium (Mann-Whitney-U test, $P < 0.001$). Similarly, the median of the total Ca^{2+} activity duration was respectively, 145 s (range 90–350 s) for Ca^{2+} medium and 75 s (range 0–200 s) for Ca^{2+}-free medium ($P < 0.05$, Mann-Whitney-U test). Moreover, the maximal $[Ca^{2+}]_i$ transient level for these two conditions was also significantly different: 1,586 $\pm$ 373 nM (n = 22) for cells in Ca^{2+} medium and 386 $\pm$ 60 nM (n = 25) for cells in Ca^{2+}-free medium ($P < 0.01$).

A proportional reduction of the Ca^{2+} transient triggered during phagocytosis could be obtained by increasing the cytosolic calcium buffering capacity with increasing concentrations of the Ca^{2+}-chelator MAPT/AM in the presence of extracellular Ca^{2+}. Fig. 3 shows examples of the Ca^{2+} transient occurring in these Ca^{2+}-buffered cells (none, 10, or 25 μM MAPT/AM). The $[Ca^{2+}]_i$ baseline in Ca^{2+}-buffered cells with 10 or 25 μM MAPT/AM in Ca^{2+} medium is not significantly different from that of nonbuffered cells: 107 $\pm$ 20 nM for 10 μM and 97 $\pm$ 9 nM for 25 μM MAPT/AM (n = 15, mean $\pm$ SEM). The maximal $[Ca^{2+}]_i$ transients are significantly decreased to 274 $\pm$ 46 nM (n = 13) with 10 μM MAPT/AM compared with unbuffered cells (1,586 $\pm$ 373 nM, $P < 0.001$) and further decreased to 138 $\pm$ 8 nM (n =

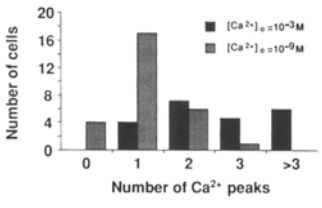

Figure 2. Number of $[Ca^{2+}]_i$ oscillations during phagocytosis of serum-opsonized yeast particles in neutrophils in the presence or absence of extracellular Ca^{2+} (Ca^{2+} or Ca^{2+}-free medium). The cells were loaded with fura2/AM as described in Materials and Methods in the presence of extracellular Ca^{2+}. Black bars represent the number of Ca^{2+} peaks occurring in single neutrophils during phagocytosis in Ca^{2+}-medium; shaded bars represent the Ca^{2+} peaks occurring in cells phagocytosing in Ca^{2+}-free medium. Ca^{2+} peaks were defined as previously described in reference 13.

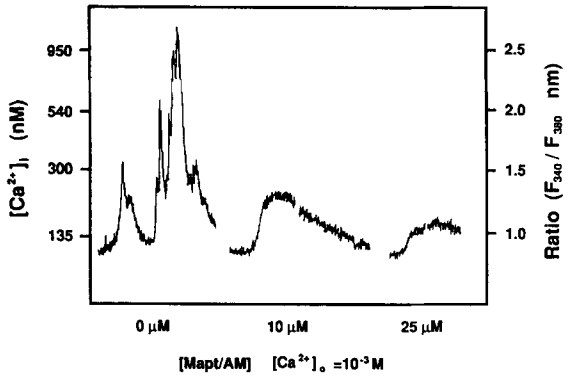

Figure 3. $[Ca^{2+}]_i$ transients occurring in normal cells or in Ca^{2+}-buffered cells with 10 or 25 μM of MAPT/AM in Ca^{2+} medium during phagocytosis ($P < 0.001$ when comparing a series of experiments under these different conditions, see Results).

10) with 25 μM MAPT/AM ($P < 0.05$ compared with 10 μM MAPT/AM, Kruskal-Wallis test).

Surface Phagocytosis in the Presence and Absence of $[Ca^{2+}]_i$ Changes

The same number of neutrophils were able to phagocytose adherent yeast particles irrespective of intracellular or extracellular Ca^{2+} concentrations. In the presence of extracellular Ca^{2+}, 100% of the neutrophils phagocytosed yeast particles after 10 min compared with 97 ± 2% and of 95 ± 3% in the absence of extracellular Ca^{2+} and in Ca^{2+}-depleted cells, respectively. For these two last conditions, phagocytosis reached 100% after 20 min. When the kinetics of early phagocytic uptake was assayed, a slight reduction was observed in the ingestion rate both in cells without extracellular Ca^{2+}, and in Ca^{2+}-depleted cells. Assessment of extracellular enzyme release triggered by phagocytosis showed that both in suspension at a ratio of one yeast particle to one neutrophil or in the adherent state release of secondary granule content was minimal (<6% in three experiments for both conditions), whereas release of primary granules content was undetectable when neutrophils during phagocytosis were compared with control neutrophils.

Localization of Lactoferrin during Phagocytosis

Lactoferrin is selectively localized in secondary granules in human neutrophils. Most quantitation of lactoferrin localization during phagocytosis was performed by indirect immunofluorescence with anti-human lactoferrin antibody using standard epifluorescence. Quantification by confocal and electron microscopy confirmed the results assessed by standard epifluorescence (see below).

Fig. 4 shows a typical staining pattern for lactoferrin after phagocytosis in the presence (Fig. 4, *A–C*) and absence (Fig. 4, *D–F*) of a $[Ca^{2+}]_i$ rise. Apart from the granular staining of the cytoplasm, a distinct continuous fluorescent ring is observed close to the phagocytosed yeast particle in a normal cell (Fig. 4, *A–C*). In the MAPT/AM-loaded neutrophils and Ca^{2+}-depleted neutrophils (Fig. 4, *D–F*) no such fluorescence in apposition to the phagocytosed yeast was observed, only the granular fluorescence of the cytoplasm. To show the striking difference in these two conditions more convincingly, a three-dimensional computer representation of the lactoferrin fluorescence is shown in Fig. 5. *A* and *B* also show fluorescent cells at one confocal level and the line *a* to *b*, across the cell and through the phagosome along which the relative fluorescent intensity is measured. A clear increase in relative fluorescence is observed as two peaks at the periphery of the particle in control cells (normal cells in Ca^{2+} medium) (Fig. 5 *C*). In contrast, in Ca^{2+}-depleted cells (Fig. 5 *D*) there is no increase in fluorescence close to the phagocytosed particle; only varying granular fluorescence is observed. To visualize the fluorescence intensity of a confocal picture, a three-dimensional plot is shown in Fig. 5, *E* and *F*. In contrast to Ca^{2+}-depleted cells, a localized increase in fluorescence is observed around the phagocytosed particle in control cells. Control experiments with preimmune rabbit IgG showed very weak fluorescence in both cell preparations. These results suggest the absence of translocation of lactoferrin from the secondary granules into the phagosomes during the ingestion process in Ca^{2+}-depleted cells. The P–L fusion was routinely assayed after 10 min, since no significant enhancement in fusion was observed after more prolonged incubation. A very rapid degranulation during phagocytosis has previously been reported (23).

These observations were verified at the electron microscopic level localizing lactoferrin by immunocytochemical methods. Fig. 6 shows segments of yeast particles phagocytosed by neutrophils in Ca^{2+} medium and Ca^{2+}-depleted neutrophils in Ca^{2+}-free medium. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde, embedded in Lowicryl K4M, sectioned and then incubated with anti-lactoferrin and an anti-IgG-gold complex. The labeling of secondary granules is evident and in Ca^{2+} medium (Fig. 6 *b*) the peripheral rim of the phagosomal space (see above) is intensively labeled. In contrast, in Ca^{2+}-depleted cells, this labeling is drastically reduced (Fig. 6 *a*). These observations were confirmed by a quantitative analysis, showing that the density of gold particles localizing lactoferrin is reduced to much lower levels than control values in MAPT-AM-loaded neutrophils (Fig. 7). In Ca^{2+}-free medium the density of gold particles is intermediate between those measured in Ca^{2+} medium and cells loaded in Ca^{2+}-free medium supplemented with MAPT/AM (data not shown).

Modulation of Phagosome–Lysosome Fusion by Changing the Extracellular and Cytosolic Free Calcium Concentration

The $[Ca^{2+}]_i$ in the neutrophils was manipulated during surface phagocytosis by loading the cells with different concentrations of MAPT/AM in Ca^{2+}- or Ca^{2+}-free medium, and subsequently performing the phagocytosis experiments in either Ca^{2+} or Ca^{2+}-free buffer.

P–L fusion was quantified by standard epifluorescence under these different $[Ca^{2+}]_i$ conditions. The number of cells showing P–L fusion during phagocytosis under control conditions (presence of Ca^{2+}, no MAPT/AM) was 80 ± 4% (Fig. 8 *a*). Increasing the cytosolic Ca^{2+} buffering capacity with 10 and 25 μM MAPT/AM in the presence of extracellular Ca^{2+} (16) reduced the percentage of P–L fusion to 49 ± 7 and 30 ± 9%, respectively (Fig. 8 *a*). When phagocytosis was performed in Ca^{2+}-free medium, thus decreasing the

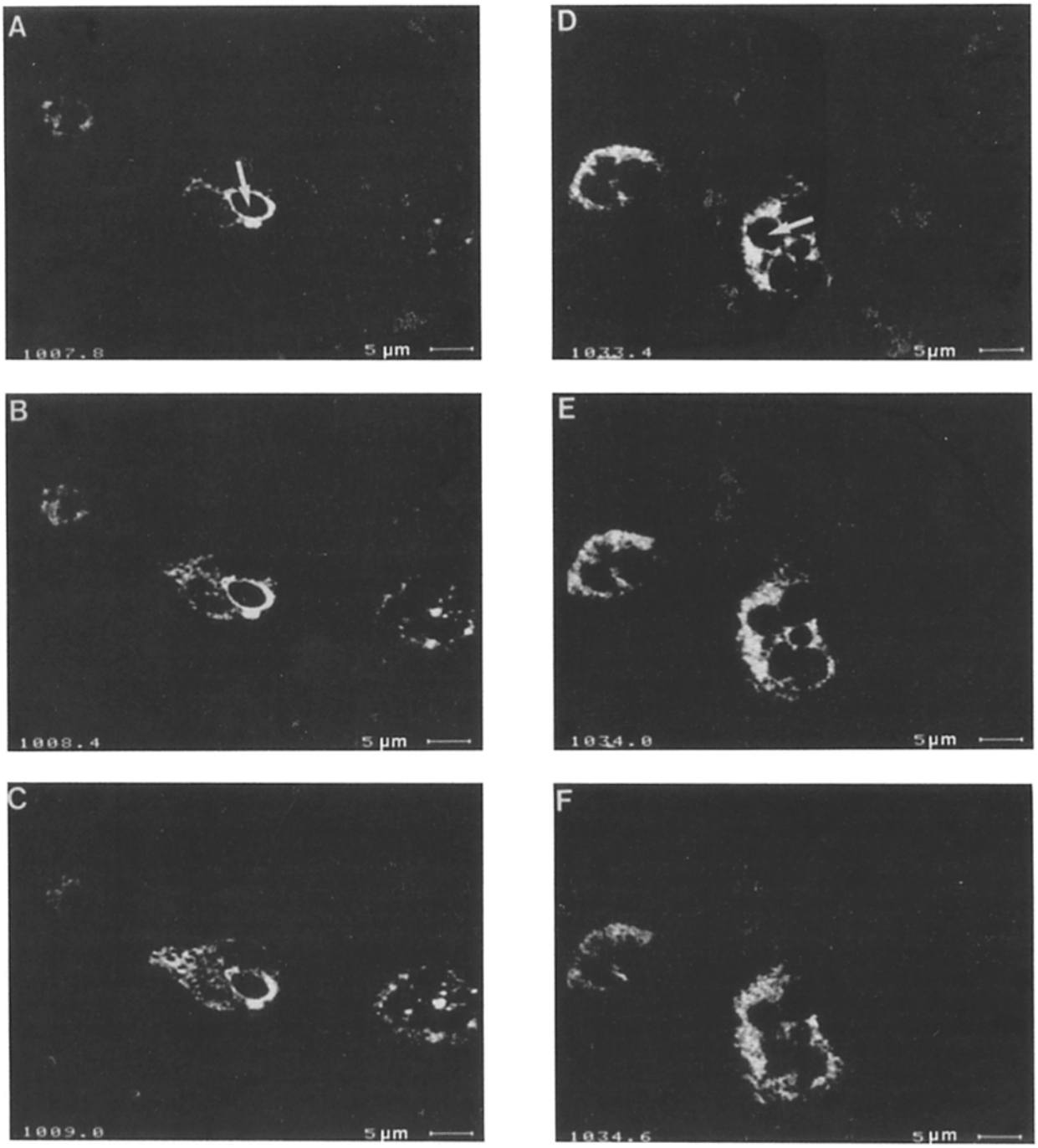

Figure 4. Localization of lactoferrin immunofluorescence in phagocytosing neutrophils by laser scanning confocal microscopy. Three thin sections (0.6 μm) of fluorescence distribution in a neutrophil phagocytosing one yeast particle (indicated by the arrow) in Ca^{2+} medium (*A–C*), and in a Ca^{2+}-depleted cell in Ca^{2+}-free medium (*D–F*).

amplitude and duration of the Ca^{2+} transient in a more pronounced manner (18), P-L fusion in nonloaded cells was reduced to 52 ± 14%, and further down to 29 ± 4 and 19 ± 6% in neutrophils previously loaded with 10 or 25 μM MAPT/AM, respectively (Fig. 8 *b*). To reduce the basal level of $[Ca^{2+}]_i$ to <20 nM, cells were loaded with MAPT/AM in Ca^{2+}-free medium and allowed to phagocytose in Ca^{2+}-free medium. Under these conditions, P-L fusion was drastically reduced; only 10 ± 2 and 6 ± 3% of the cells showed significant translocation of lactoferrin staining (Fig. 8 *c*). As mentioned above quantification by confocal microscopy confirmed the results assessed by standard epifluorescence and electron microscopy, i.e., positive cells for P-L fusion counted by confocal microscopy were 75 ± 5% in presence of Ca^{2+} and 18 ± 6% in Ca^{2+}-depleted cells in the absence of extracellular Ca^{2+} (n = 25, number cells studied, three slides per cell).

Depletion of the Ca^{2+} stores by the ionophore ionomycin

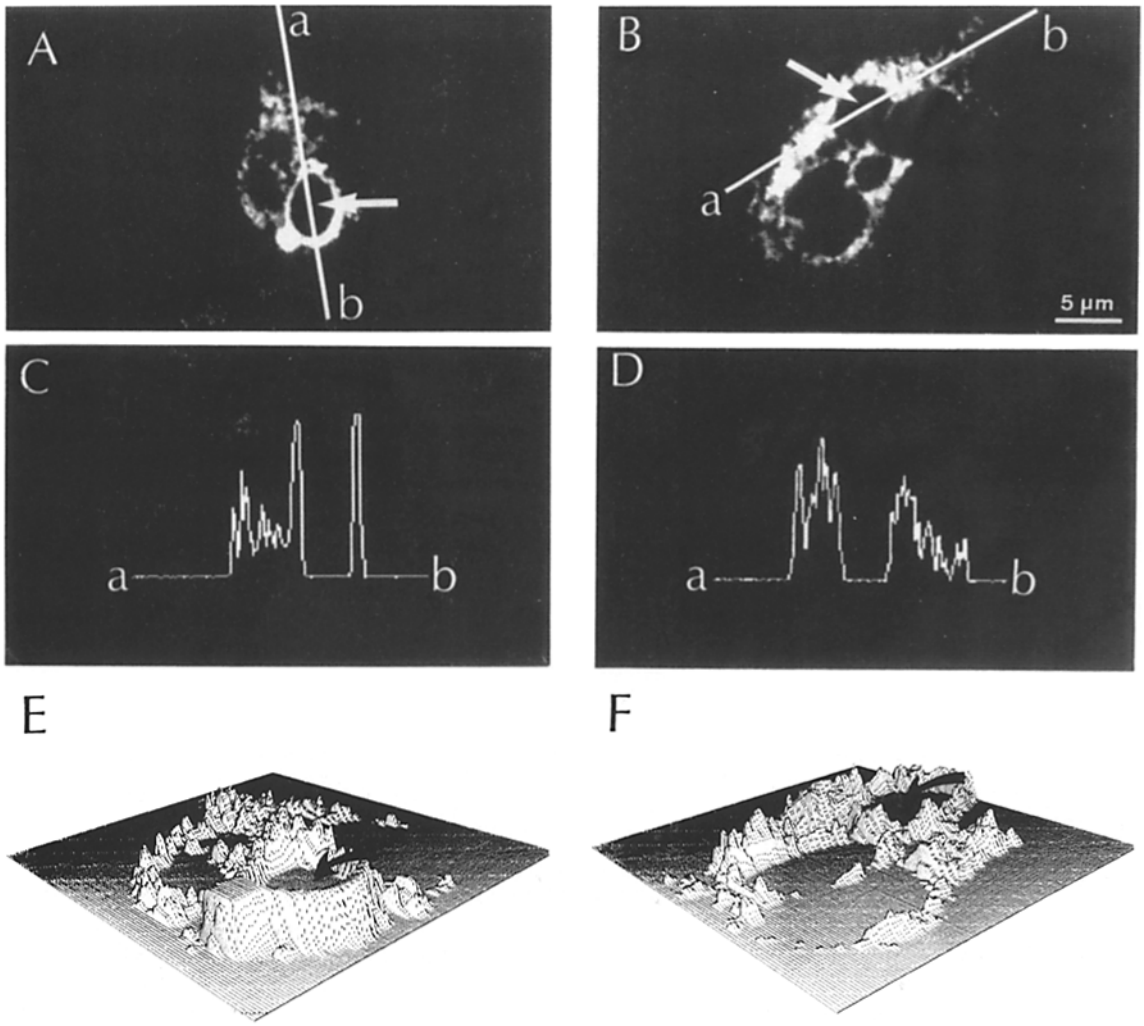

Figure 5. Relative quantitation of lactoferrin immunofluorescence in phagocytosing neutrophils using confocal laser scanning microscopy. (*A*, *C*, and *E*) Control cells in Ca^{2+} medium; (*B*, *D*, and *F*) Ca^{2+}-depleted cells. (*A* and *B*) Fluorescent cell and the line (*a* to *b*) along which the fluorescence intensity was measured. (*C* and *D*) Relative fluorescence intensity along the line *a* to *b*. (*E* and *F*) Three-dimensional plot of the fluorescence intensity over the whole cell, as shown in *A* and *B*. The arrows indicate the position of the ingested yeast particles. The other nonfluorescent parts of the cells correspond to the nuclei.

in Ca^{2+}-free medium provides a cell with no Ca^{2+} stores, normal basal cytosolic Ca^{2+} levels, and since there is no extracellular Ca^{2+}, $[Ca^{2+}]_i$ can not increase during phagocytosis. Neutrophils were treated with ionomycin (1 μM) in Ca^{2+}-free medium before undergoing phagocytosis: P–L fusion was reduced by such experimental procedure from 75 ± 6 to 15 ± 2% (vs. 11 ± 2% for Ca^{2+}-depleted cells with MAPT/AM), whereas the cells ingested the particle normally. These results provide clear additional evidence that a $[Ca^{2+}]_i$ elevation is necessary for fusion of the phagosomes with secondary granules and that MAPT/AM does not inhibit fusion in a manner unrelated to Ca^{2+} levels.

Reconstitution of the Impaired Degranulation

It thus appears as if the amplitude of the elevation, the number and/or the duration of the $[Ca^{2+}]_i$ transient can regulate P–L fusion in phagocytosing neutrophils. If so, any elevation of $[Ca^{2+}]_i$ in MAPT/AM-loaded cells, should reverse this inhibition, if the phagosomal membrane is still competent to fuse with the granules. Fig. 9 shows that addition of the Ca^{2+} ionophore ionomycin (1 μM after 10 min of phagocytosis) to MAPT/AM-loaded cells in the presence of extracellular Ca^{2+} enhanced significantly the number of P–L-positive cells from 21 ± 3 to 54 ± 4% ($P < 0.005$). Furthermore, if Ca^{2+}-depleted neutrophils, i.e., cells loaded with MAPT/AM in Ca^{2+}-free medium and tested in Ca^{2+}-free medium, were exposed to the phorbol ester PMA (50 nM), P–L fusion increased from 6 ± 3 to 42 ± 6% ($P < 0.001$).

Discussion

This work solves a puzzle in the areas of signaling during phagocytosis. Several reports have shown that during receptor-mediated phagocytosis both in neutrophils and macrophages (7, 15, 17, 26) the $[Ca^{2+}]_i$ elevation does not appear to be necessary for the control of the ingestion process. In this investigation, we show that the $[Ca^{2+}]_i$ elevation is, however, a necessary signal that triggers the subsequent P–L fu-

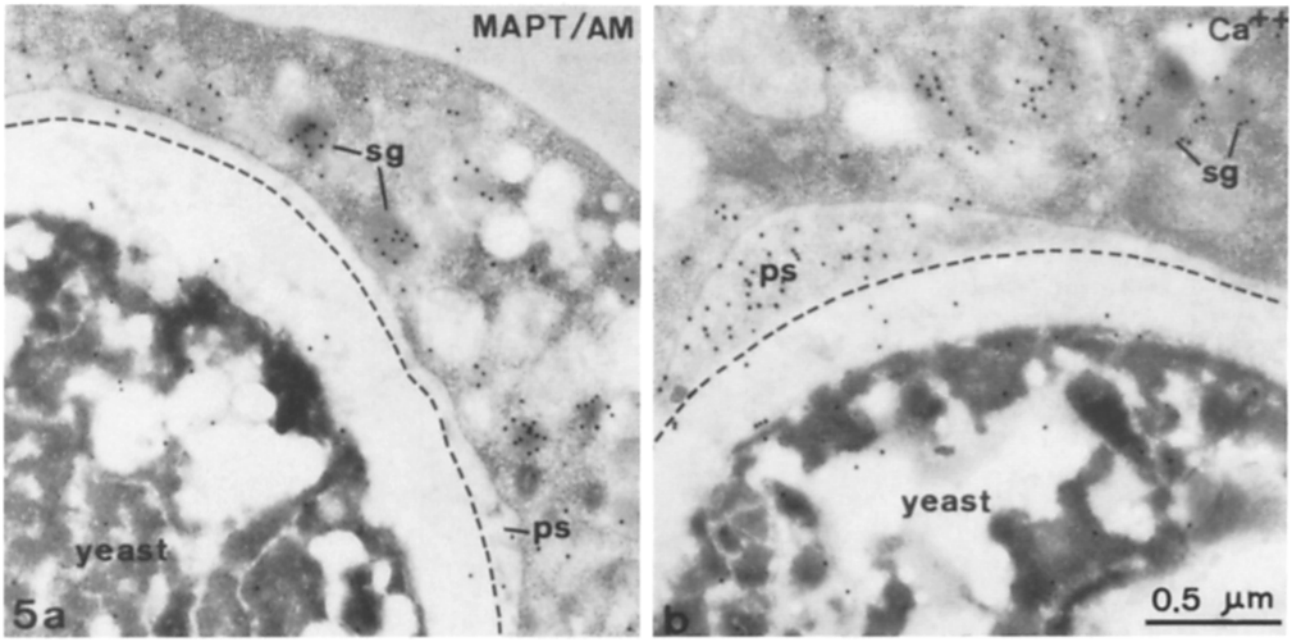

Figure 6. View of segments of yeast particles phagocytosed by human neutrophils. Following fixation in 4% paraformaldehyde and 0.1% glutaraldehyde, cells were embedded in Lowicryl K4M, thin-sectioned and the sections incubated with antilactoferrin followed by a complex of anti-IgG colloidal gold. Secondary granules (*sg*) are specifically labeled both in neutrophils incubated in Ca^{2+} medium (*b*) and in MAPT/AM loaded neutrophils incubated in Ca^{2+}-free medium (*a*). The labeling of the phagosomal space (*ps*) is more intense in *b* than in *a*.

sion event. Furthermore, the present work directly answers an additional question: are phagocytosis and P–L fusion all-or-nothing events, or can phagosome formation be dissociated from the subsequent fusion step?

Intracellular fusion events such as those involving phagosomes and granules in neutrophils are difficult to quantify under relevant experimental conditions. In cultured cells, such as macrophages and myeloid cell lines, loading with an appropriate granule indicator such as acridine orange or FITC-dextran (8, 10) offers a convenient model for studying P–L fusion. This technique is, however, less suitable for experiments in short-lived, freshly isolated human neutrophils. To study this event in individual neutrophils in parallel with $[Ca^{2+}]_i$ measurements, we have used an indirect immunostaining technique to localize lactoferrin in granules and phagocytic vacuoles. To minimize the interpretive uncertainties, we also used confocal fluorescence microscopy. With this technique it is possible to analyze the fluorescence in different planes of the cell, thereby ruling out fluorescent changes due to the form and thickness of the cell, pseudopods and phagosome. To further evaluate the fusion process, EM with immunogold technique directed against lactoferrin was also used. We were thereby able to show clear differences in the degree of P–L fusion, as a function of the nature of the $[Ca^{2+}]_i$ transient.

These results are consistent with the data indicating selective increase of $[Ca^{2+}]_i$ in the cytosol surrounding the phagocytic vesicle during ingestion (26). Our data also indicate that the Ca^{2+} spikes start during contact with the particle and initial phagosome formation.

The simple maneuver of decreasing extracellular calcium to $<10^{-9}$ M led to a significant reduction in P–L fusion. Under these conditions, only a rapid $[Ca^{2+}]_i$ transient originating from intracellular stores was detected in the majority of the phagocytosing cells. Loading the cells with increasing concentrations of MAPT/AM to increase the cytosolic Ca^{2+} buffering capacity and thereby decrease the $[Ca^{2+}]_i$ transient, led to a dose-dependent inhibition of P–L fusion even in the presence of extracellular Ca^{2+}. This buffering effect was even more pronounced in the absence of extracellular Ca^{2+} (compare Fig. 8 *b* with *a*), where Ca^{2+} influx cannot occur, and thus the Ca^{2+} transient is of shorter duration (16). When basal $[Ca^{2+}]_i$ was further reduced to very low levels and the cells were depleted from intracellular Ca^{2+} stores, i.e., under conditions where Ca^{2+}-dependent processes do not occur (6, 17), the P–L fusion was reduced to very low levels.

A further support for the role of $[Ca^{2+}]_i$ in P–L fusion is the fact that the inhibitory effect of increasing the cytosolic

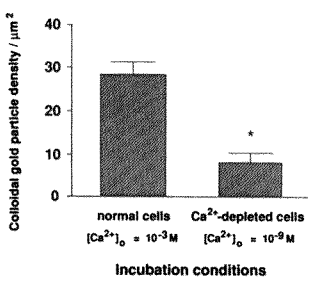

Figure 7. Density of colloidal gold particles per squared micron of phagosomal space (see Materials and Methods) in neutrophils incubated in Ca^{2+} medium or in MAPT/AM loaded neutrophils in Ca^{2+}-free medium. $n = 80$, number of pictures of different phagosomal spaces analyzed from two different experiments. * $P < 0.001$.

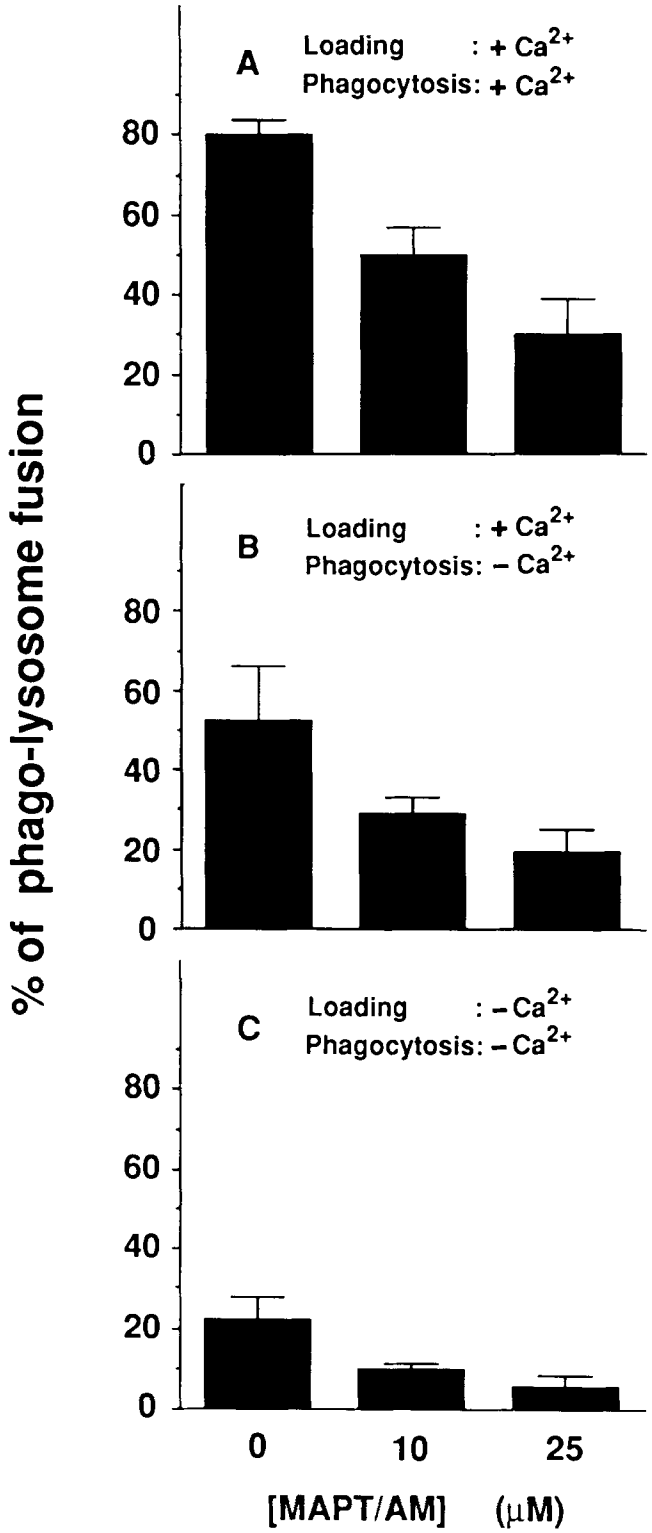

Figure 8. Quantitation of lactoferrin release into phagosomes containing yeast particles after phagocytosis at different cytosolic Ca^{2+} buffering conditions. (*A*) Ca^{2+} medium during loading with increasing concentrations of MAPT/AM and during phagocytosis. (*B*) Ca^{2+} medium during loading with increasing concentrations of MAPT/AM followed by phagocytosis in Ca^{2+}-free medium. (*C*) Ca^{2+}-free medium both during loading with increasing concentrations of MAPT/AM and during phagocytosis. The cells were loaded with MAPT/AM (10 and 25 μM) in the presence or absence of extracellular Ca^{2+} and subsequently allowed to phagocytose in the presence or absence of extracellular Ca^{2+} as described in Materials and Methods. Control cells were incubated in parallel without MAPT/AM. The Ca^{2+} conditions of the loading and the phagocytosis are indicated as $+Ca^{2+}$ or $-Ca^{2+}$ for presence or absence of extracellular Ca^{2+}, respectively. ($+Ca^{2+}$) $[Ca^{2+}]_o = 10^{-3}$ M; ($-Ca^{2+}$) $[Ca^{2+}]_o = 10^{-9}$ M. Quantitation was performed by standard epifluorescence.

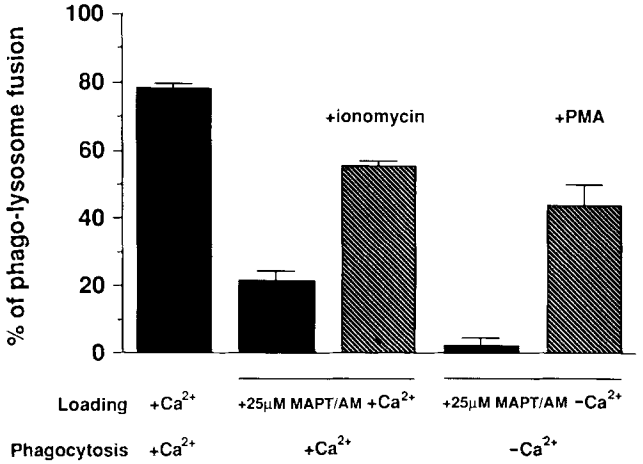

Figure 9. Effect of the Ca^{2+}-ionophore ionomycin and phorbol myristate acetate (PMA, 50 nM) on lactoferrin degranulation into the phagosome in Ca^{2+}-buffered cells. (*A*) addition of the Ca^{2+}-ionophore ionomycin (1 μM) to Ca^{2+}-buffered neutrophils (cells loaded with MAPT/AM 25 μM in Ca^{2+} medium) in Ca^{2+} medium and (*B*) addition of phorbol myristate acetate (PMA, 50 nM) to Ca^{2+}-depleted cells (cells loaded with MAPT/AM 25 μM in Ca^{2+}-free medium) in Ca^{2+}-free medium. Phagocytosis was allowed to occur under all the conditions for 10 min and the ionophore or PMA were added for another 10 min. All cells were scored for P-L fusion after 20 min. The Ca^{2+} conditions of the loading and the phagocytosis are indicated as $+Ca^{2+}$ or $-Ca^{2+}$ for presence or absence of extracellular Ca^{2+}, respectively. Quantitation was performed by standard epifluorescence.

Ca^{2+} buffering capacity can be overcome by elevating $[Ca^{2+}]_i$ with the Ca^{2+}-ionophore ionomycin in the presence of extracellular Ca^{2+}. The reversibility experiments, also induced by phorbol esters, exclude a general toxic effect induced by the experimental procedure.

These results indicate that P-L fusion is a Ca^{2+}-dependent process and very sensitive to the alterations of $[Ca^{2+}]_i$, probably within distinct regions of the cells. Further studies attempting to characterize localized variations in $[Ca^{2+}]_i$ including the amplitude, the duration and the frequency of the Ca^{2+} peaks concomitantly with P-L fusion are therefore necessary to better understand temporal relation between these two events and the regulation of P-L fusion. In addition, the results indicate that phagosome formation can not only be dissociated from lysosomal fusion but that the phagosomal membrane remains accessible to intracellular granules even if phagocytosis has been terminated.

Similarly to the present findings, it was previously shown that in human neutrophils, release of secondary (specific) granule content into the extracellular medium in response to activation of chemoattractant receptors (such as FMLP or LTB_4 [18, 19, 22]), is a very sensitive Ca^{2+}-dependent process. In fact, exocytosis can be blocked by increasing the

cytosolic free Ca^{2+} buffering capacity (16, 18). Secretion of the various granule populations in response to chemoattractants have different $[Ca^{2+}]_i$ requirements for secretion (3, 18). Furthermore, not only different types of granules, but also subpopulations of specific granules are differently regulated by Ca^{2+}. Secretion from granules containing vitamin B12-binding protein was more sensitive to elevation of $[Ca^{2+}]_i$ than those releasing lactoferrin (22). In our experimental conditions, very little degranulation appears to occur into the extracellular medium during phagocytosis as reported by others (29).

How do chemoattractant-triggered exocytosis compare to phagocytosis-induced P–L fusion? The two related processes seem to have several features in common in distinct parts of the cell: (*a*) $[Ca^{2+}]_i$ transients stimulate granular fusion both with the phagosome and the plasma membrane; (*b*) increasing the cytosolic Ca^{2+} buffering capacity diminishes this fusion, which can be restored by elevating the $[Ca^{2+}]_i$ with the Ca^{2+}-ionophore ionomycin in the presence of extracellular Ca^{2+}, and (*c*) PMA stimulates fusion at exceedingly low $[Ca^{2+}]_i$, indicating that the secretory machinery may also be triggered in a Ca^{2+}-independent manner, presumably by activation of protein kinase C. We have recently shown that DG, the endogenous activator of protein kinase C, accumulates during phagocytosis at very low $[Ca^{2+}]_i$ (9). Here we show that although this concentration of DG may function as a signal for engulfment, it does not seem to be sufficient as a fusion signal at low $[Ca^{2+}]_i$. If however, protein kinase C is maximally activated by adding exogenous PMA, no elevation of $[Ca^{2+}]_i$ is required.

Recently it was shown that GTP induces degranulation in permeabilized (2) and patched neutrophils (21) suggesting a role for a specific G-protein regulated system in the control of secretion (30). Together, the results indicate that the fusion between granules and the plasma membrane, either during exocytosis of P–L formation seems able to use a multifactorial signaling system, involving $[Ca^{2+}]_i$ increases, DG and protein kinase C activation, and probably G-regulatory proteins. This seems appropriate in the light of the different types of stimuli causing granule–membrane fusion in neutrophils.

Biochemical targets for Ca^{2+} and protein kinase C are presently unknown. The Ca^{2+}-binding synexin-like proteins may stimulate fusion, since in cell-free systems they lead to aggregation of specific granules and stimulate membrane fusion both in neutrophils and other cellular systems (1, 20). Furthermore, it is presently unclear what role the actin network plays in the intracellular fusion processes. Disrupting the actin network with cytochalasin B enhances degranulation and reduces the requirements for $[Ca^{2+}]_i$ during exocytosis (18). One mechanism for the inhibition of P–L fusion at low $[Ca^{2+}]_i$ could be sustained actin assembly close to the phagocytic vacuoles, thereby making contact between phagosome and granules less likely to occur. It is, however, at this stage not possible to explain the precise mechanism of action of $[Ca^{2+}]_i$ in the P–L fusion process.

In this report we have focussed on how the specific lactoferrin-containing granules fuse with the phagosome during ingestion of C3bi-coated yeast particles, as a model for understanding phagocytosis-induced P–L fusion. Further investigation is necessary to prove if this model is relevant for different types of granules, other phagocytic stimuli and cellular systems.

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