

# Phagosome–Lysosome Fusion Is a Calcium-independent Event in Macrophages

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**Abstract.** Phagosome–lysosome membrane fusion is a highly regulated event that is essential for intracellular killing of microorganisms. Functionally, it represents a form of polarized regulated secretion, which is classically dependent on increases in intracellular ionized calcium ( $[Ca^{2+}]_i$ ). Indeed, increases in  $[Ca^{2+}]_i$  are essential for phagosome–granule (lysosome) fusion in neutrophils and for lysosomal fusion events that mediate host cell invasion by *Trypanosoma cruzi* trypomastigotes. Since several intracellular pathogens survive in macrophage phagosomes that do not fuse with lysosomes, we examined the regulation of phagosome–lysosome fusion in macrophages. Macrophages (MØ) were treated with 12.5  $\mu$ M bis-(2-amino-*S*-methylphenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester (MAPT/AM), a cell-permeant calcium chelator which reduced resting cytoplasmic  $[Ca^{2+}]_i$  from 80 nM to  $\leq 20$  nM and completely blocked increases in  $[Ca^{2+}]_i$  in response to multiple stimuli, even in the presence of extracellular calcium. Subsequently, MØ phagocytosed serum-opsonized zymosan, staphylococci, or *Mycobac-*

*terium bovis*. Microbes were enumerated by 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining, and phagosome–lysosome fusion was scored using both lysosome-associated membrane protein (LAMP-1) as a membrane marker and rhodamine dextran as a content marker for lysosomes. Confirmation of phagosome–lysosome fusion by electron microscopy validated the fluorescence microscopy findings. We found that phagosome–lysosome fusion in MØ occurs normally at very low  $[Ca^{2+}]_i$  ( $\leq 20$  nM). Kinetic analysis showed that in MØ none of the steps leading from particle binding to eventual phagosome–lysosome fusion are regulated by  $[Ca^{2+}]_i$  in a rate-limiting way. Furthermore, confocal microscopy revealed no difference in the intensity of LAMP-1 immunofluorescence in phagolysosome membranes in calcium-buffered vs. control macrophages. We conclude that neither membrane recognition nor fusion events in the phagosomal pathway in macrophages are dependent on or regulated by calcium.

**P**HAGOCYTOSIS is a tightly regulated process leading to the internalization of particulate ligands such as bacteria and fungi. Its initiation depends on the engagement of specific plasma membrane receptors expressed on macrophages and neutrophils. In a manner analogous to that of hormones and growth factors, binding of particulate ligands to phagocytic receptors triggers intracellular signals. One common intracellular signaling event that accompanies phagocytosis is an increase in in-

tracellular ionized (i.e., cytoplasmic-free) calcium ( $[Ca^{2+}]_i$ )<sup>1</sup> (7, 12, 19, 25). An increase in  $[Ca^{2+}]_i$  is not required for phagocytosis per se, therefore  $Ca^{2+}$  may regulate one or more events subsequent to phagocytosis (7, 12, 16).

In one type of phagocytic cell, the neutrophil, transient increases in  $[Ca^{2+}]_i$  are essential for phagosome–granule (lysosome) fusion (16). It is this fusion event that allows lysosome contents access to the phagosome, which contributes to the killing and digestion of phagocytosed microorganisms. The importance of  $[Ca^{2+}]_i$  transients for ly-

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1. *Abbreviations used in this paper:* BCG, *M. bovis* BCG;  $[Ca^{2+}]_i$ , intracellular ionized calcium; CMFHHBSS, HBSS containing 10 mM HEPES, pH

7.4, and lacking calcium and magnesium; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; em, emission; ex, excitation; HHBSS, HBSS containing  $Ca^{2+}$ ,  $Mg^{2+}$ , and 10 mM HEPES, pH 7.4; LAMP, lysosome-associated membrane protein; MAPT/AM, bis-(2-amino-*S*-methylphenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetraacetoxymethyl ester; MØ, macrophages; MDMØ, monocyte-derived MØ; RD, rhodamine dextran.

sosomal fusion events is further illustrated by the observation that host cell invasion by *Trypanosoma cruzi* trypomastigotes, which is mediated by lysosomal fusion, depends on parasite-induced transient increases in  $[Ca^{2+}]_i$  (28). Transient increases in cytoplasmic  $[Ca^{2+}]_i$  are also required for membrane fusion in regulated secretory events such as neurotransmitter release (5), insulin (9), and pancreatic enzyme (29) secretion. Therefore  $Ca^{2+}$  is a crucial mediator of membrane fusion in numerous and diverse regulated systems.

Certain intracellular pathogens, e.g., *Mycobacterium tuberculosis* (1), *Toxoplasma gondii* (17, 18), and *Legionella pneumophila* (15), persist in macrophages (MØ) within phagosomes that fail to fuse with lysosomes. Therefore, the elucidation of the mechanisms and regulation of phagosome-lysosome fusion in MØ is important for our understanding of the survival strategies of intracellular pathogens. To determine whether the transient increases in  $[Ca^{2+}]_i$  that accompany phagocytosis are essential for phagosome-lysosome fusion in MØ, we examined fusion of phagosomes with lysosomes in MØ whose  $[Ca^{2+}]_i$  was profoundly reduced by chelation. We found phagosome-lysosome fusion in human monocyte-derived macrophages (MDMØ) and murine J774 macrophages to be independent of increases in  $[Ca^{2+}]_i$  and thus regulated in a fundamentally different way from that in neutrophils.

## Materials and Methods

### Isolation and Culture of Macrophages

Peripheral blood mononuclear cells were isolated from buffy coats received together with the corresponding plasma from the Irving Memorial Blood Bank (San Francisco, CA). The method of Böyum (3) was used to separate mononuclear cells from granulocytes and erythrocytes. In a subsequent step, monocytes were separated from lymphocytes on Percoll gradients (Pharmacia Biotech, Inc., Piscataway, NJ) as described by Gmelig-Meyling (10). This procedure yielded a mean of  $1.3 \pm 0.8 \times 10^8$  mononuclear cells per buffy coat ( $n = 90$ ). 96% of the cells were viable by trypan blue exclusion, and 86% were monocytes by neutral red uptake. MDMØ used for immunofluorescence were cultured for 6–8 d on 12-mm round cover glasses (Fisher Scientific Co., Pittsburgh, PA) in flat-bottom 24-well plates ( $1 \times 10^6$  cells/well; Corning Glass Works, Corning, NY) in RPMI 1640 supplemented with 2 mM L-glutamine and 10% autologous serum (culture medium) at 37°C in 95% air–5%  $CO_2$ . Autologous serum was recovered by clotting plasma after adding  $CaCl_2$  (10 mM final). Culture medium was exchanged after 5 d of culture. MDMØ were enumerated by counting nuclei in a hemacytometer after lysis with 2% (wt/vol) cetyltrimethylammonium bromide (United States Biochemical Corp., Cleveland, OH) in 0.5 M citric acid, pH 3.8. After 6–8 d of culture, representative wells contained  $3.1 \pm 1.3 \times 10^5$  MDMØ.

The murine macrophage cell line, J774A.1 (TIB 67; American Type Culture Collection, Rockville, MD), was grown in DME supplemented with 10% FBS and 2 mM L-glutamine. Cells were used for experiments 1 d after seeding  $3 \times 10^5$  cells on 12-mm cover glasses in flat-bottom 24-well plates.

### Preparation of Staphylococci and Zymosan

A laboratory strain of coagulase-negative staphylococcus which lacks protein A was grown in tryptic soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) overnight at 37°C with constant shaking. After vortexing with glass beads to disrupt clumps, bacteria were washed once by centrifugation and opsonized in 50% fresh human serum in HBSS containing 10 mM Hepes (HHBSS) for 30 min at 37°C. The bacteria were washed three times in HBSS lacking calcium and magnesium and containing 10 mM Hepes (CMFHBBSS), counted in a Petroff-Hausser chamber, and resuspended in CMFHBBSS at  $5 \times 10^8$ /ml.

Zymosan particles (Sigma Chemical Co., St. Louis, MO) were op-

sonized for 30 min at 37°C in 50% autologous serum in HHBSS, washed, counted, and resuspended in CMFHBBSS at  $5 \times 10^8$ /ml.

### Fura-2 and bis-(2-amino-S-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (MAPT/AM) Loading of Macrophages

For assays of intracellular calcium in cell populations, MDMØ cultured on 9 mm  $\times$  22 mm rectangular glass coverslips were loaded by incubation with Fura-2/AM (2.5  $\mu$ M) (Molecular Probes, Eugene, OR) in CMFHBBSS at room temperature. After 1 h, dye-containing buffer was replaced by fresh CMFHBBSS, and the cells were held at room temperature until assays were performed. To load cells with the nonfluorescent intracellular chelator, MAPT, MAPT/AM (12.5  $\mu$ M; Calbiochem-Novabiochem Corp., La Jolla, CA) was added to the incubation medium simultaneously with Fura-2/AM. In some studies, Fura-2/AM was added to the incubation medium containing MAPT/AM during the final 30 min of incubation. Loading MØ with MAPT/AM did not decrease loading of cells with Fura-2, as indicated by the identical fluorescence emission intensity (at 510 nm) of cells loaded with Fura-2 with and without MAPT when excited at 360 nm (where fluorescence is independent of  $[Ca^{2+}]_i$ ). To monitor  $[Ca^{2+}]_i$  in adherent MDMØ, coverslips were held vertically in cuvettes using Teflon coverslip holders constructed as described (7). Fluorescence emission at 510 nm was monitored at ambient temperature in a spectrofluorimeter (800C; SLM-AMINCO, Urbana, IL) equipped with a motorized excitation monochromator alternating between excitation wavelengths of 340 and 380 nm. Ratio data were obtained directly with the software supplied by SLM-AMINCO, and free calcium concentrations were calculated using Microsoft Excel. Calibration of intracellular calcium concentrations in MDMØ was performed in three ways. In adherent MDMØ,  $R_{min}$  and  $R_{max}$  were obtained by treating cells with ionomycin (5.0  $\mu$ M) in buffer containing 4 mM EGTA/30 mM Tris base or containing 10 mM  $CaCl_2$ , respectively. The same method was also applied to MDMØ ( $4.75 \times 10^5$ /ml) put in suspension by scraping after loading with Fura-2/AM. In addition, Fura-2-loaded MDMØ in suspension were calibrated by lysis with 0.5% Triton X-100 in buffer containing EGTA/Tris or  $CaCl_2$ . All three methods gave comparable values for  $R_{min}$  and  $R_{max}$ . For studies of the effects of MAPT/AM in single MØ during phagocytosis,  $[Ca^{2+}]_i$  was measured as described previously (13, 14, 21).

For routine studies of phagosome-lysosome fusion in calcium-chelated cells, MDMØ were loaded by incubation with MAPT/AM exactly as described above except for the omission of Fura-2/AM from the incubation medium. Experiments in which cells loaded with both MAPT/AM and Fura-2/AM were compared with cells loaded with MAPT/AM alone before phagocytosis revealed no difference in the rate of phagocytosis or phagosome-lysosome fusion. This indicates that the addition of Fura-2 to MAPT did not contribute to calcium buffering or other activity that measurably influenced phagocytosis or phagosome-lysosome fusion.

Adherent murine J774 cells were loaded with Fura-2/AM and MAPT/AM using a procedure identical to that needed for human MDMØ, with the exception of 200  $\mu$ g/ml gemfibrozil included in all buffers to prevent sequestration or efflux of Fura-2 or MAPT from the cytoplasm (26).

### Labeling Macrophages with Rhodamine Dextran

To visualize the lysosomal contents of MDMØ, the cells were labeled by incubation with 0.5 mg/ml lysine-fixable rhodamine-isothiocyanate-conjugated dextran (mol wt 70,000; Molecular Probes) in culture medium for 24 h. Two washes with warm PBS to remove the noninternalized rhodamine dextran (RD) were followed by a 2-h incubation in RD-free culture medium to ensure transfer of the marker to a late endosomal-lysosomal compartment (8).

### Phagocytosis Assay with Staphylococci and Zymosan

RD-prelabeled or -unlabeled MDMØ were washed twice with CMFHBBSS (MAPT/AM-treated cells) or HHBSS (MØ with normal  $[Ca^{2+}]_i$ ). 0.3 ml of CMFHBBSS or HHBSS containing serum-opsonized coagulase-negative staphylococci (hereafter termed staphylococci) at a ratio of 10 bacteria to 1 MDMØ or zymosan particles at a ratio of 5:1 were added to each well and incubated for 60 min in 95% air–5%  $CO_2$  at 37°C. The ratios were chosen to result in ~70% of the cells having associated bacteria or particles. After two washes with CMFHBBSS (MAPT/AM-treated MØ), or HHBSS (MØ with normal  $[Ca^{2+}]_i$ ) to remove nonadherent bacteria or

particles, the cells were fixed in 4% paraformaldehyde, pH 7.3, for 15 min at room temperature.

For studies of the kinetics of particle binding, phagocytosis, and phagosome-lysosome fusion, MDMØ were incubated with staphylococci exactly as described above except that the particle/cell ratio was reduced to 5:1 and the cells were incubated for 15, 30, 45, 60, 90, and 120 min.

### Immunofluorescence

Paraformaldehyde was washed off in three washes with PBS. To localize lysosome-associated membrane protein (LAMP)-1, 0.4 ml H4A3 hybridoma supernatant (Developmental Studies Hybridoma Bank, Iowa City, IA) diluted 1:10 in RPMI 1640 with 0.1% saponin was added to each well. After 15 min, unbound primary antibody was removed in three washes with PBS followed by incubation with either 0.4 ml FITC-conjugated affinity-purified goat anti-mouse IgG (37.5 µg/ml final; Zymed Laboratories, Inc., South San Francisco, CA) or Cy3-conjugated affinity-purified goat anti-mouse IgG (2.9 µg/ml final; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS containing 5% goat serum, 0.1% Triton X-100, 0.02% SDS, and 0.5 µg/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) for 15 min. The cover glasses were then washed three times with PBS and two times with dH<sub>2</sub>O, air dried, and mounted on glass slides with a drop of SlowFade (Molecular Probes).

When J774 macrophages were used, the same protocol was followed, except that LAMP was detected using a rat anti-mouse LAMP-1 mAb (1D4B, hybridoma supernatant; Developmental Studies Hybridoma Bank) followed by Cy3-conjugated affinity-purified goat anti-rat IgG (2.9 µg/ml final) (Jackson ImmunoResearch Laboratories, Inc.).

When the kinetics of ligand binding, phagocytosis, and phagosome-lysosome fusion were studied, MDMØ were stained exactly as described above except that after fixation the nonpermeabilized cells were incubated for 15 min with 0.4 ml FITC-conjugated affinity-purified goat anti-human IgG (14 µg/ml final) (Jackson ImmunoResearch Laboratories, Inc.) to label extracellular opsonized staphylococci. After three washes with PBS to remove unbound antibody, the cells were fixed again in 4% paraformaldehyde for 15 min before adding anti-LAMP-1 and secondary antibodies as detailed above.

MDMØ were evaluated using a microscope (DMRB; E. Leitz, Inc., Rockleigh, NJ) equipped with a Wild photo system and fluorescence filters from Chroma Corp. (Branford, VT). For optimum selectivity between probe signals when a specimen was stained with multiple dyes, all filter cubes were equipped with band pass excitation (ex) and emission (em) filters with the following characteristics: DAPI: ex 360 ± 50 nm, em 460 ± 50 nm; fluorescein: ex 480 ± 40 nm, em 535 ± 50 nm; rhodamine/Cy3: ex 540 ± 25 nm, em 630 ± 60 nm. Photographs were taken on Fujichrome 400D Professional film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Cells with greater than or equal to one bacterium or particle projected over their body were counted as cells with bacteria or particles. Bacteria or particles in projection over a cell were considered to be in phagolysosomes when they were surrounded by a thin, smooth, and brightly fluorescent ring in cells stained with anti-LAMP antibodies. The same criteria were used when lysosomes were labeled with RD. In the experiments studying the kinetics of ligand binding, phagocytosis, and phagosome-lysosome fusion, staphylococci in projection over a cell were considered to be adherent to the outside of the plasma membrane when surrounded by a thin, green, fluorescent ring (fluorescein), to reside in a phagolysosome when enveloped in a red fluorescent ring (Cy3), and to reside in a non-fused phagosome when not surrounded by a ring. In each experiment, ≥100 consecutive cells on at least two coverslips were evaluated for each time point and condition.

For analysis of the fluorescence intensity of LAMP-1 in phagolysosome membranes, cells were examined by fluorescence microscopy using a laser scanning confocal imaging system (MRC-600; Bio-Rad Laboratories, Hercules, CA) equipped with an argon laser and high sensitivity blue excitation filter set (excitor 488 DF 10; dichroic reflector 510 LP; emission filter, 515 LP). The images were scanned with a PlanApo 63×, NA 1.4 objective (Nikon, Inc., Garden City, NJ) mounted on a Nikon Optiphot microscope. Phagolysosome profiles, selected at random in cells that had phagocytosed bacteria, were identified by their characteristic morphology: an empty circular space (the unstained bacterium) of diam ~1 µm surrounded by a ring of LAMP-1-specific fluorescence. Optical sections were made by scanning the equatorial plane of phagolysosome profiles under standardized instrument settings, i.e., laser neutral density filter (No. 2), confocal aperture (smallest), black level and gain positions locked, slow scan rate,

zoom at ×3, and Kalman filtering with four scans. These settings were not changed during the entire data collection process, thus ensuring that reliable relative intensity measurements could be made. Digital images were collected sequentially and stored on an optomagnetic disk. To perform image analysis, each phagolysosome profile was enclosed within a frame of 150 × 100 pixels and zoomed to fill the full screen. Using the image analysis capability built into CoMOS software (Version 6.05.4; Bio-Rad Microsciences, Cambridge, MA), the fluorescent ring surrounding the phagolysosome was outlined by tracing with the cursor. The area, average pixel intensity value (0–254), number of pixels sampled, and the sum of all pixel values sampled were automatically determined using the Histogram function of CoMOS. The average fluorescence intensity of the entire profile was indexed by the average pixel value measured. By subtraction of the total pixel value of the unstained phagolysosome luminal space (containing phagocytosed bacteria) from that of the entire phagolysosome profile, the total pixel values relating to the LAMP-1 fluorescence ring surrounding the bacterial dark space were calculated. By dividing this by the number of pixels in the ring region, the average pixel value and thus the relative fluorescence intensity of the ring could be determined. Care was taken to ensure that standard instrument conditions resulted in unsaturated images, i.e., maximum pixel values never exceeded 254. Thus, relative fluorescence intensities could be correlated with average pixel values for the rings measured.

### Immunoelectron Microscopy

MDMØ and staphylococci were prepared as described except that monocytes were seeded on 10-cm petri dishes and, after 7 d, incubated with an excess of 20:1 staphylococci for 60 min. Cells were fixed in 0.05% glutaraldehyde and 2% paraformaldehyde in buffer containing 100 mM NaCl, 0.5 mM CaCl<sub>2</sub>, and 30 mM Hepes, pH 7.25, scraped off with a rubber policeman, washed, resuspended in the same fixative for 60 min, and rinsed in PBS three times for 5 min each. MDMØ were dehydrated by progressive lowering of temperature in ethanol according to the instructions in the Lowicryl HM20 kit (Electron Microscopy Sciences, Fort Washington, PA) and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences) at –35°C overnight. The resin was polymerized by indirect irradiation with 360-nm UV light for 24 h at –35°C, followed by 2 d of direct UV irradiation at room temperature. Sections (70 nm thick) were cut on an Ultracut E microtome (Reichert Scientific Instruments, Buffalo, NY) and picked up either on 200-mesh nickel grids covered with carbon-stabilized Formvar film (E. F. Fullam, Inc., Latham, NY), or on bare 400-mesh nickel grids. For immunolabeling, grids/sections were soaked for 30 min at room temperature in blocking buffer consisting of 0.1% teleostean gelatin (Sigma Chemical Co.), 0.8% BSA (Sigma Chemical Co.), and 0.02% Tween-20 (Sigma Chemical Co.) in PBS. They were incubated in undiluted H4B4 hybridoma supernatant (mAb against LAMP-2) overnight at 4°C, rinsed in PBS with 0.02% Tween-20 at room temperature for 5 min and three changes of PBS over 15 min, then incubated for 1–2 h in goat anti-mouse IgG conjugated to 10 nm gold (Ted Pella, Inc., Redding, CA), diluted 1:20 in blocking buffer, rinsed as above with PBS-Tween and PBS, fixed for 5 min with 0.5% glutaraldehyde in PBS, rinsed in PBS and dH<sub>2</sub>O for 5 min, and air dried. An mAb against LAMP-2 instead of LAMP-1 was used because the latter failed to recognize its target after glutaraldehyde fixation. Both mAbs reveal the same intracellular compartment. Grids were counterstained for 4 min in 1% uranyl acetate in 70% methanol and for 2 min in lead citrate before viewing in an electron microscope operating at 80 kV (Jeol USA, Peabody, MA). Control sections stained with secondary antibody alone showed no gold labeling.

### Statistical Analysis

Statistical analyses were performed using InStat (GraphPad Software, Inc., San Diego, CA) on a Macintosh computer.

## Results

### Assay of Intracellular Calcium in Human Macrophages

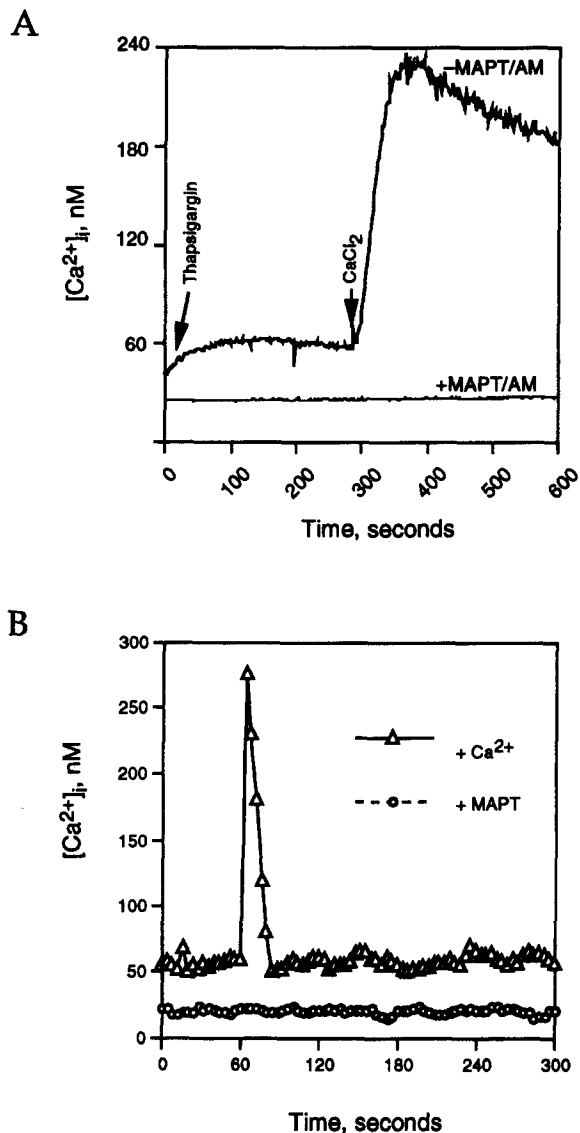
Human MDMØ were readily loaded with Fura-2. No compartmentalization of the dye was evident under the loading conditions used, as indicated by fluorescence microscopy as well as by finding that Fura-2 was fully released by

treatment of MDMØ with digitonin (50 µg/ml). Murine macrophages possess an efflux pump that extrudes Fura-2 and other organic anions from the cytosol (26, 27); however, we found no evidence of such activity in human MDMØ: the intracellular content of Fura-2 was constant in loaded cells  $\geq 30$  min, as reflected by a stable fluorescence emission intensity (at 510 nm) upon excitation at 360 nm. Furthermore, neither probenecid nor gemfibrozil increased the intracellular concentration of Fura-2 achieved by the routine loading procedure.

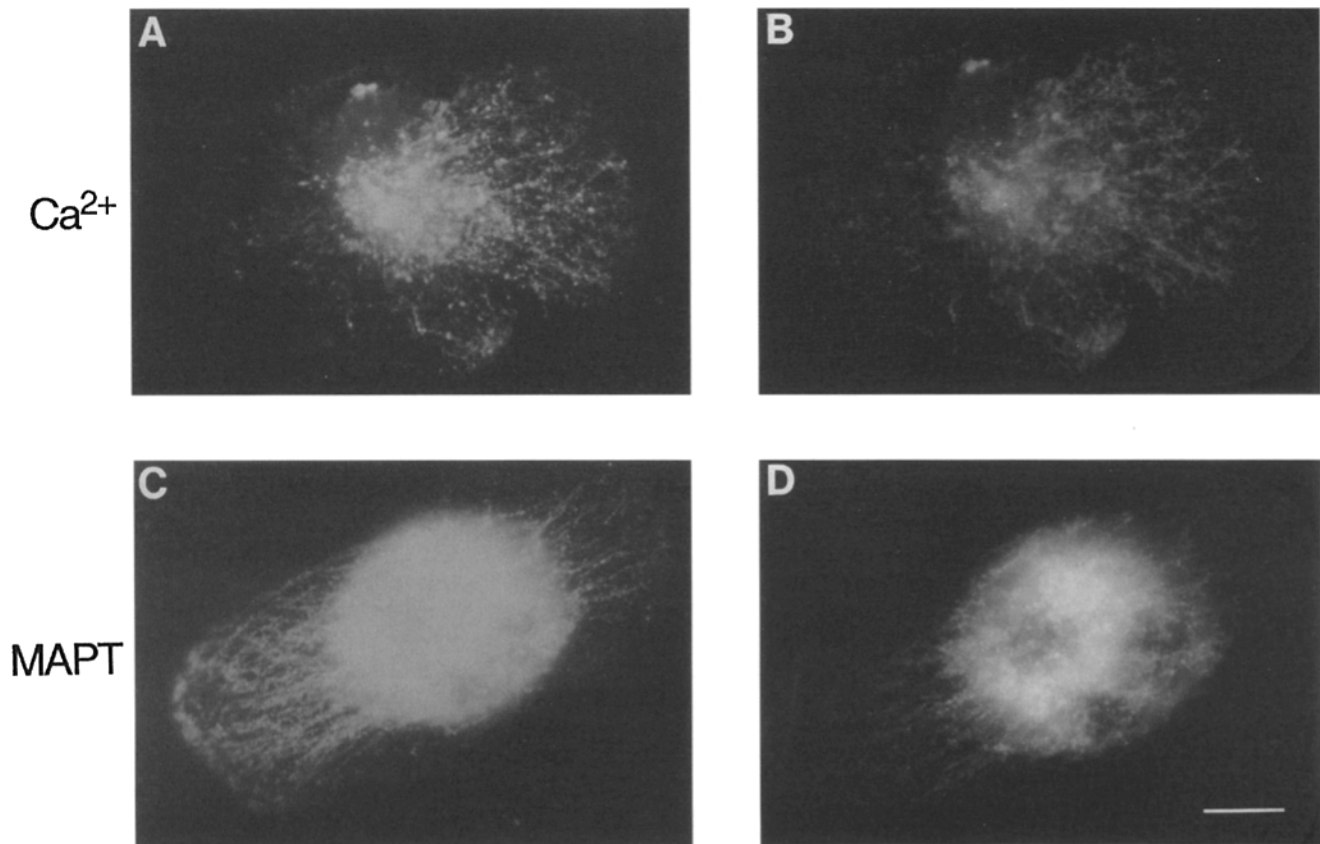
To study phagosome-lysosome fusion in the presence of extremely low  $[Ca^{2+}]_i$ , MDMØ were treated with the acetoxymethyl ester of the nonfluorescent calcium chelator, MAPT. Incubation of MDMØ with MAPT/AM reduced baseline  $[Ca^{2+}]_i$  to  $\leq 20$  nM and completely blocked the increase in  $[Ca^{2+}]_i$  that occurred in cell populations in response to thapsigargin, even in the presence of extracellular calcium (Fig. 1 A). In addition, this concentration of MAPT/AM was sufficient to completely block increases in  $[Ca^{2+}]_i$  in response to f-Met-Leu-Phe and to monocyte/macrophage chemotactic protein-1. MAPT/AM loading also inhibited increases in  $[Ca^{2+}]_i$  in response to ionomycin (5 µM) in the presence of 2 mM extracellular  $Ca^{2+}$  by  $\sim 85\%$ . Finally, MAPT/AM completely blocked increases in periphagosomal  $[Ca^{2+}]_i$  during phagocytosis of IgG-opsonized yeast as revealed by calcium imaging (Fig. 1 B). Therefore, incubation of MDMØ with 12.5 µM MAPT/AM reduces resting  $[Ca^{2+}]_i$ , and provides considerable cytoplasmic calcium buffering capacity. Simultaneous loading with MAPT/AM did not reduce the efficiency of loading or hydrolysis of Fura-2/AM as indicated by equivalent fluorescence emission at 510 nm when excited at 360 nm ( $Ca^{2+}$ -independent wavelength) and by identical excitation spectra for Fura-2 after cell lysis.

#### Anti-LAMP-1 and Internalized Rhodamine Dextran Stain the Same Compartment of Human Macrophages

We used an antibody against LAMP-1 to label lysosomal membranes and rhodamine dextran as a lysosomal content marker. The latter was internalized via fluid phase endocytosis during a loading period of 24 h. Subsequent incubation in RD-free culture media for 2 h allowed passage of the dye through the endocytic pathway to a late endosomal-lysosomal compartment (8). By phase-contrast microscopy, the RD labeling procedure did not alter the MDMØ monolayer or the appearance of individual cells. The rhodamine/Cy3 filter set revealed a punctate red fluorescence that appeared most concentrated in the perinuclear area of the macrophages. Double fluorescence labeling of RD-loaded MDMØ with an anti-LAMP-1 antibody resulted in a pattern that very closely resembles the lysosomal compartment defined by rhodamine dextran (Fig. 2). At high magnification it could occasionally be seen that a rhodamine-labeled lysosome was surrounded by a ring indicative of LAMP staining. Although MAPT-treated MDMØ tend to contract and round up without detaching from the surface, the close association of lysosomal content markers and membrane markers remained unchanged in calcium-depleted cells. In addition, MDMØ loaded with RD and stained with an anti- $\beta$ -tubulin antibody revealed that the alignment of lysosomes on microtubules was not disrupted by chelation of intracellular  $Ca^{2+}$  (not shown).



**Figure 1.** MAPT blocks increases in  $[Ca^{2+}]_i$  in MDMØ. Adherent MDMØ were loaded with Fura-2/AM in the presence (+MAPT/AM) or absence of MAPT/AM (–MAPT/AM) (12.5 µM).  $[Ca^{2+}]_i$  was assayed as described in the text. (A) Studies of cell populations. For these experiments, coverslips containing cells were held in cuvettes containing 2.0 ml CMFHHBSS. Thapsigargin (20 µl of 20 µg/ml, final concentration, 100 ng/ml) was injected at the time indicated by the first arrow, and  $CaCl_2$  (2.0 µl of a 1.0 M stock solution) was added at the time indicated by the second arrow. Loading of MDMØ with MAPT reduced baseline  $[Ca^{2+}]_i$  and completely blocked the increase in  $[Ca^{2+}]_i$  in response to thapsigargin, even in the presence of 2.0 mM extracellular  $Ca^{2+}$ . In addition, MAPT/AM treatment completely blocked increases in  $[Ca^{2+}]_i$  that occurred in response to f-Met-Leu-Phe and to monocyte/macrophage chemotactic protein-1 (MCP-1). (B) Calcium imaging studies of single cells during phagocytosis. Macrophages loaded with Fura-2 with (+MAPT) or without (+ $Ca^{2+}$ ) MAPT/AM were monitored by phase-contrast (to detect phagocytosis) and fluorescence microscopy (to quantitate  $[Ca^{2+}]_i$  as described in the text). When macrophages that were not loaded with MAPT/AM phagocytosed IgG-opsonized yeast,  $[Ca^{2+}]_i$  in the periphagosomal region increased from  $59 \pm 5$  nM (mean  $\pm$  SD,  $n = 5$ ) to  $75 \pm 25$  nM. In contrast, MAPT/AM loading reduced resting  $[Ca^{2+}]_i$  to  $17 \pm 6$  nM and completely blocked increases in periphagosomal  $[Ca^{2+}]_i$  ( $20 \pm 4$  nM). Results shown are for representative experiments with and without MAPT/AM.



**Figure 2.** Anti-LAMP-1 and internalized RD stain the same compartment of human MDMØ. Cells were incubated with RD for 24 h, washed, and kept in RD-free media for 2 h to allow passage of the marker to a lysosomal compartment. After fixation cells were labeled with an mAb against LAMP-1 followed by FITC-conjugated anti-mouse IgG. MDMØ were kept in HHBSS ( $Ca^{2+}$ ), or, to chelate intracellular calcium, loaded with MAPT/AM for 60 min and kept in CMFHHBSS for an additional 60 min before fixation (*MAPT*). For each condition, fluorescein (*A* and *C*) and rhodamine (*B* and *D*) fluorescence images of the same field are shown. Rhodamine dextran labeling generally resulted in less intense fluorescence than immunostaining LAMP-1; nevertheless, in all cells examined the distribution of the two labels was indistinguishable. The close similarity of the fluorescence patterns is not due to crossover fluorescence since cells labeled with either RD or anti-LAMP antibody alone did not emit a detectable fluorescence signal when observed through the narrow bandpass filter set appropriate for the other fluorochrome. Bar, 20  $\mu$ m.

### ***Phagosomes Containing Zymosan Fuse with Lysosomes in the Presence of Intracellular Ionized Calcium Concentrations $\leq 20$ nM***

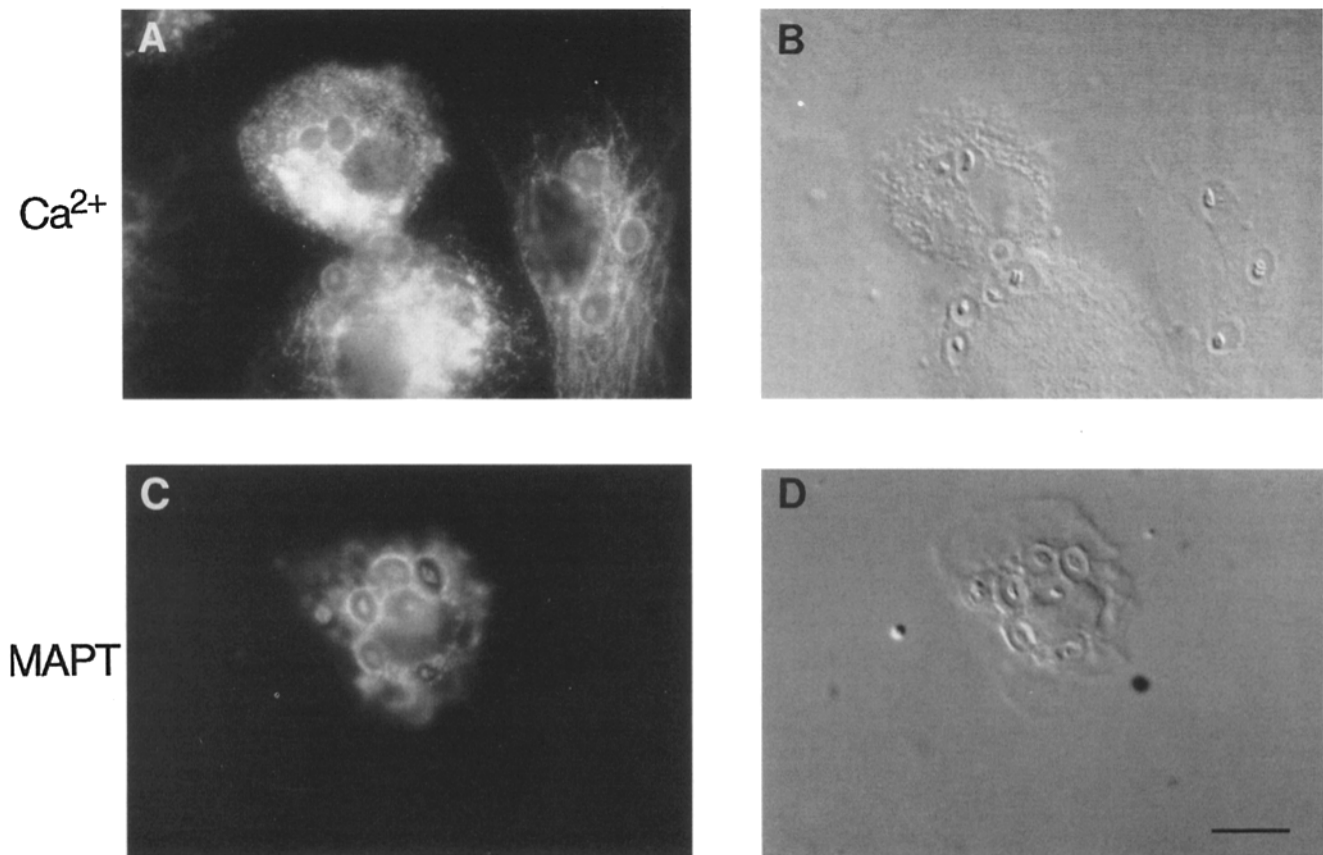
To study phagosome-lysosome fusion, we incubated MDMØ with zymosan, serum-opsonized *Saccharomyces cerevisiae* yeast cell wall particles. Adding a zymosan suspension at a ratio of five particles to one MDMØ resulted in  $\sim 70\%$  cells associated with particles after an incubation time of 60 min. Approximately 95% of the particles were surrounded by a thin, smooth, continuous and intensely fluorescent ring after staining with an anti-LAMP-1 antibody (Fig. 3 and Table I). We interpreted the smooth appearance of the ring and its tight apposition to the zymosan particles as evidence that this colocalization of LAMP-1 and particle reflects phagosome-lysosome fusion, a finding that was confirmed by electron microscopy (see below). Additional validation of this assay of phagosome-lysosome fusion was provided by the observation that treatment of cells with ammonium chloride before and during phagocytosis exerted a concentration-dependent inhibition of ring formation (phagosome-lysosome fusion). The presence of periphagosomal LAMP-1 rings was

inhibited by 63% and 87% by 5 and 10 mM  $NH_4Cl$ , respectively. When  $[Ca^{2+}]_i$  was reduced to  $\leq 20$  nM, MDMØ assumed a rounder shape and ingested fewer opsonized zymosan particles. Nevertheless, profound reduction of  $[Ca^{2+}]_i$  did not significantly affect the number of cells associated with particles, nor did intracellular calcium chelation significantly reduce the proportion of cell-associated zymosan that was contained in phagolysosomes.

The same results was obtained when the lysosomal content marker RD was used instead of the LAMP-1 antibody (data not shown).

### ***Phagosomes Containing Live Staphylococci or Mycobacteria Fuse with Lysosomes in the Presence of Intracellular Ionized Calcium $\leq 20$ nM***

(a) **Labeling Lysosomes with LAMP Antibodies.** Staphylococci were used to examine whether the results obtained with zymosan particles also applied when MDMØ phagocytosed live organisms. Incubation of adherent human MDMØ with an excess of 10:1 staphylococci for 60 min resulted in  $\sim 75\%$  cells associated with bacteria of which  $\sim 70\%$  were surrounded by the lysosomal membrane marker



**Figure 3.** Phagosomes containing zymosan fuse with lysosomes in the presence and absence of intracellular ionized calcium. Adherent MDMØ were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with opsonized zymosan particles at a particle/cell ratio of 5:1 for 60 min. Fixed cells were labeled with an mAb against LAMP-1 followed by FITC (A)- or Cy3 (C)-conjugated anti-mouse IgG. Fluorescein (A) or Cy3 (C) fluorescence or Nomarski micrographs (B and D) of the same cells are shown. Irrespective of the [Ca<sup>2+</sup>]<sub>i</sub>, most of the cell-associated particles are surrounded by a thin, smooth, continuous, fluorescent ring indicating phagosome-lysosome fusion. Bar, 20 µm.

(Fig. 4 and Table I). Chelation of intracellular calcium did not significantly alter the proportion of cells associated with staphylococci or the percentage of LAMP-positive bacteria, suggesting phagosome-lysosome fusion was independent of [Ca<sup>2+</sup>]<sub>i</sub>.

To further examine the calcium dependence of the fusion of lysosomes with phagosomes containing live staphylococci, phagosome-lysosome fusion (as determined by staining with anti-LAMP-1) was examined in MDMØ whose intracellular calcium stores were depleted by pretreatment of cells with thapsigargin (200 ng/ml) or iono-

mycin (5.0 µM) in buffer containing 2.5 mM EGTA for 5 min before addition of bacteria. This procedure did not affect either phagocytosis or phagosome-lysosome fusion: in two experiments in which a total of 300 phagosomes were scored for each condition, 80 ± 3.6% of phagosomes stained with anti-LAMP-1 in cells held in calcium-containing buffer, as compared to 74.3 ± 4.6% and 83.5 ± 0.1% of those in cells depleted of calcium with ionomycin or thapsigargin, respectively.

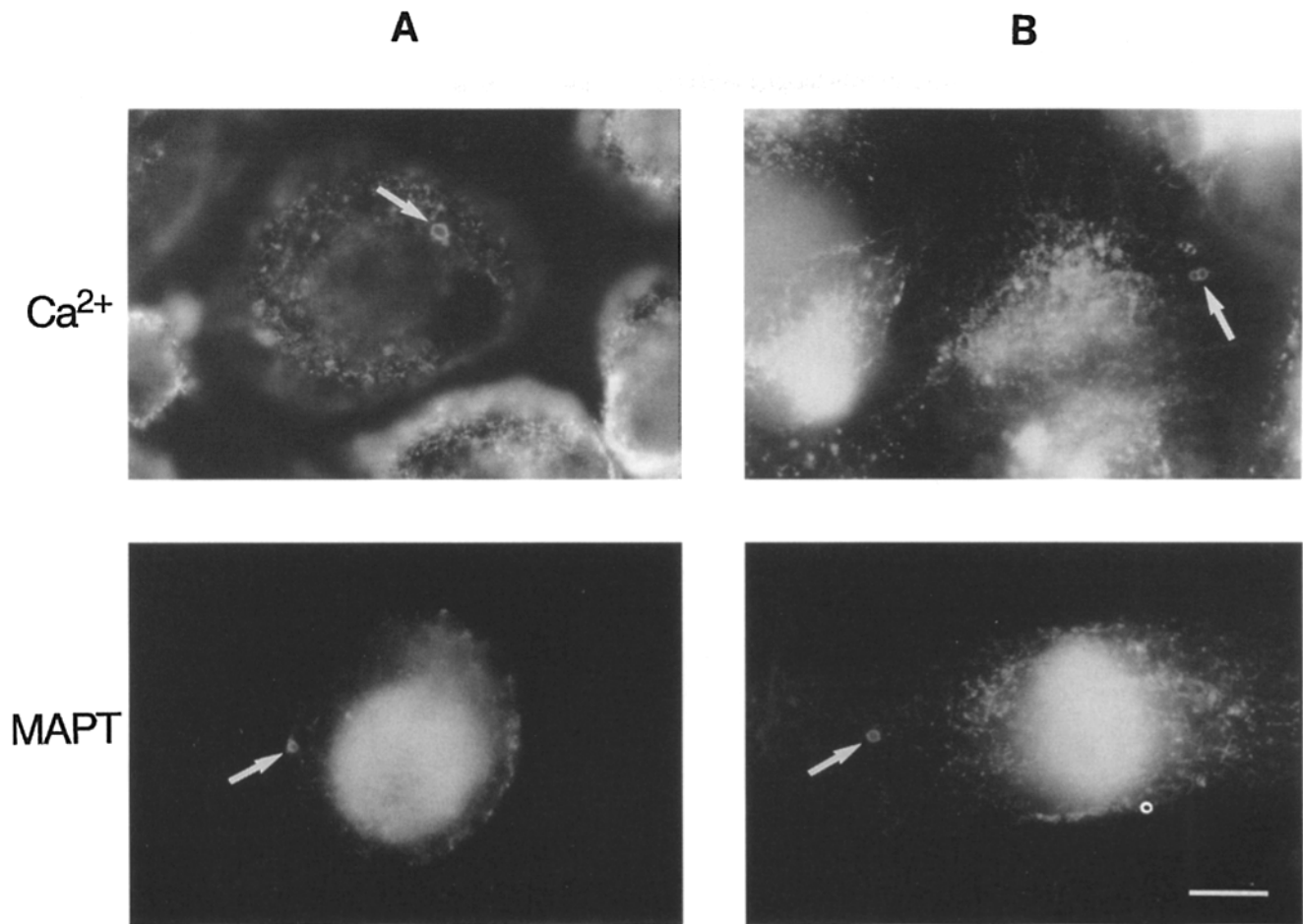
Staphylococci that are cell associated but not in phagolysosomes may be adherent to the cell surface or may be

**Table I. Phagocytosis and Phagosome-Lysosome Fusion by MDMØ with Normal and Reduced Intracellular Ionized Calcium**

MDMØ	Zymosan		Staphylococci		BCG	
	+Ca <sup>2+</sup>	+MAPT	+Ca <sup>2+</sup>	+MAPT	+Ca <sup>2+</sup>	+MAPT
Cells with bacteria /particles (percent ± SD)	68 ± 20	45 ± 18	75 ± 20	61 ± 28	67 ± 49	58 ± 40
LAMP-positive bacteria/particles (percent ± SD)	95 ± 7	73 ± 22	76 ± 21	69 ± 23	20 ± 23	19 ± 18
		NS		NS		NS
		NS		NS		NS

Adherent MDMØ were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with serum-opsonized zymosan particles (particle/cell ratio 5:1), staphylococci (10:1), or BCG (15:1) for 60 min. Fixed cells were labeled with a mAb to LAMP-1 followed by Cy3-conjugated anti-mouse IgG. DAPI was used to reveal bacterial and cellular DNA. For each condition, at least 100 consecutive cells were evaluated by fluorescence microscopy using a ×100 oil-immersion objective. Data represent the mean of at least three separate experiments. NS = *P* > 0.05 by unpaired *t* test.





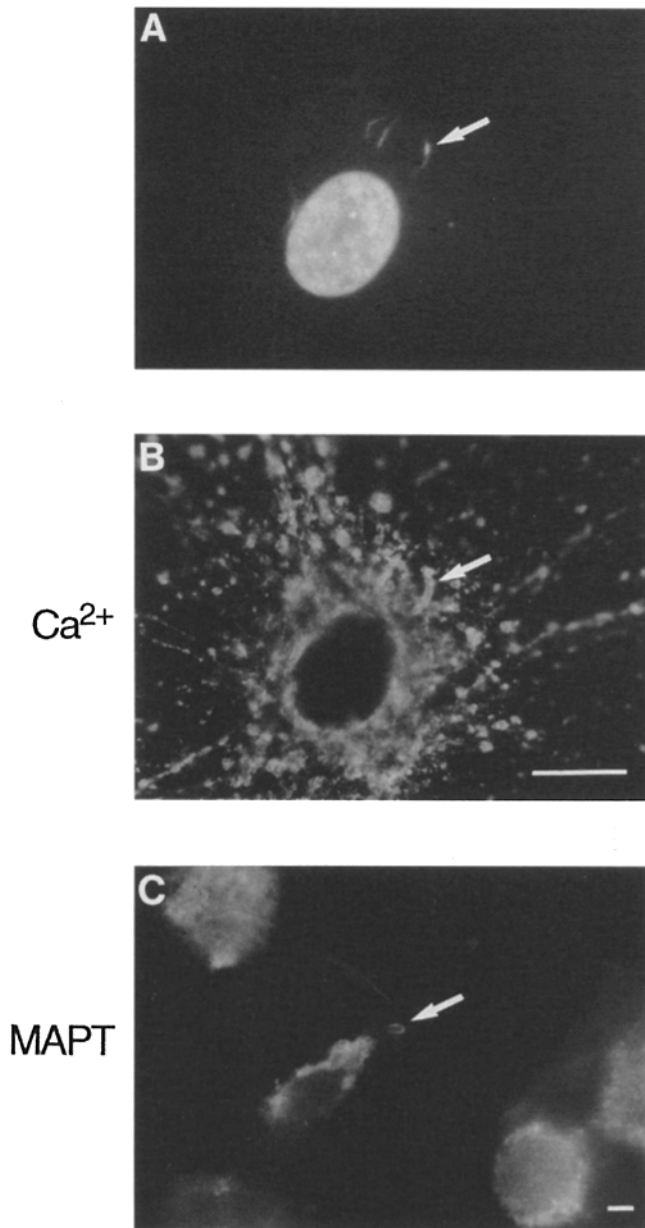
**Figure 4.** Phagosomes containing staphylococci fuse with lysosomes in the presence and absence of intracellular ionized calcium. Adherent MDM $\emptyset$  were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with opsonized staphylococci at a ratio of 10:1 for 60 min. (A) LAMP localization by immunofluorescence. Alternatively, the lysosomal compartment was visualized by incubating adherent MDM $\emptyset$  with rhodamine dextran for 24 h before further incubation in culture media (+Ca<sup>2+</sup>) or loading with MAPT/AM (+MAPT) and exposure to staphylococci as described above. (B) RD localization by fluorescence. (Arrows, phagocytosed staphylococci surrounded by fluorescent marker indicating phagosome-lysosome fusion). Bar, 20  $\mu$ m.

in phagosomes that have not fused with lysosomes. When nonadherent staphylococci were washed off after an incubation time of 60 min, and the cells incubated for an additional 30 min in culture media, 99.5% of the staphylococci were found to be LAMP positive, suggesting that eventually all bound staphylococci were internalized and trafficked to a lysosomal compartment. Most of the phagolysosomes were found in a perinuclear distribution. Nevertheless, some staphylococci located in the cell periphery were clearly LAMP positive, indicating that phagosome-lysosome fusion can occur early after phagocytosis and before internalized bacteria are transported to a perinuclear location (see below).

(b) **Labeling Lysosomes with Rhodamine Dextran.** The lysosomal content marker rhodamine dextran was used to corroborate our findings with the membrane marker LAMP. To determine whether transfer of soluble contents of lysosomes to phagosomes also occurs in the absence of increases in [Ca<sup>2+</sup>]<sub>i</sub>, we examined the transfer of rhodamine dextran to phagosomes containing staphylococci. By labeling the lysosomal compartment of MDM $\emptyset$  with this content marker, the results were essentially the same as those

obtained with the lysosomal membrane marker LAMP (Fig. 4). At normal and at low [Ca<sup>2+</sup>]<sub>i</sub>, ingested staphylococci were surrounded by a thin rim of fluorescent dye. Double fluorescence labeling of rhodamine dextran-loaded MDM $\emptyset$  with a mAb against LAMP-1 demonstrated that many of the vesicles containing staphylococci and rhodamine dextran were confined by a membrane staining with LAMP, adding support to the impression that they were phagolysosomes.

Some strains of mycobacteria are known to survive in M $\emptyset$  in phagosomes whose interaction with intracellular organelles is distinct from that of readily digested particles. Therefore, we also examined phagosome-lysosome fusion with *Mycobacterium bovis* BCG (BCG) which can replicate in human M $\emptyset$  (Zimmerli, S., and J. D. Ernst, unpublished observations). When serum-opsonized BCG were incubated for 60 min with MDM $\emptyset$  at a ratio of 15:1, ~70% of cells were associated with mycobacteria, roughly the same proportion as observed in cells incubated with staphylococci and zymosan (Fig. 5 and Table I). At this time point, the percentage of bacteria surrounded by a clearly identifiable ring of LAMP-stained lysosomal mem-



**Figure 5.** Phagosomes containing *M. bovis* BCG fuse with lysosomes in the presence and absence of intracellular ionized calcium. Adherent MDM $\emptyset$  were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with opsonized *M. bovis* BCG at a ratio of 15:1 for 60 min. Fixed cells were labeled with a mAb against LAMP-1 followed by Cy3-conjugated anti-mouse IgG. DAPI, a fluorescent marker for DNA, was used to visualize mycobacteria and macrophage nuclei. Cy3 fluorescence (B and C) and DAPI fluorescence (A, same frame as B) micrographs are shown. Irrespective of [Ca<sup>2+</sup>]<sub>i</sub>, ~20% of cell-associated mycobacteria were surrounded by a fluorescent ring indicating phagosome-lysosome fusion. (Arrows, phagocytosed *M. bovis* BCG). Bar, 20  $\mu$ m.

brane was much lower than with zymosan or staphylococci. Nevertheless, there was no significant difference between calcium-containing and -depleted cells.

LAMP rings around all particles studied were remarkably similar: thin, smooth, continuous, and tightly apposed to the particles or bacteria. Nevertheless, it was formally

possible that the ultimate number of lysosomes that fuse with phagosomes is reduced by buffering [Ca<sup>2+</sup>]<sub>i</sub> at very low levels. If each lysosomal fusion event contributes equivalent quanta of LAMP-1 to the phagolysosome membrane, then the fluorescence intensity of LAMP-1 after immunostaining should reflect the number of lysosome-phagosome fusion events. To compare the extent of lysosome-phagosome fusion in the presence of normal and reduced [Ca<sup>2+</sup>]<sub>i</sub>, we examined the intensity of phagolysosome staining with anti-LAMP-1 by confocal microscopy and fluorescence image intensity analysis. 20 phagolysosomes (containing staphylococci) were randomly selected in each set of cells (with or without MAPT/AM), and the average fluorescence intensity (counting all pixels in the phagolysosome ring) was determined for each phagolysosome membrane. On a linear scale of fluorescence intensity in which the maximum intensity value never exceeded 254, phagolysosomes in MDM $\emptyset$  with normal [Ca<sup>2+</sup>]<sub>i</sub> exhibited 165  $\pm$  26 (mean  $\pm$  SD) fluorescence units, while phagolysosomes in MDM $\emptyset$  treated with MAPT/AM to reduce [Ca<sup>2+</sup>]<sub>i</sub> exhibited 177  $\pm$  20 fluorescence units ( $P = 0.12$  by unpaired *t* test). Therefore, the quantitative extent of LAMP-1 delivery to phagosomes is independent of [Ca<sup>2+</sup>]<sub>i</sub>. The fluorescence of rings around mycobacteria was often less intense than around staphylococci or zymosan, which may indicate that phagosomes containing mycobacteria may fuse with fewer lysosomes during the time of incubation.

#### **Phagosome-Lysosome Fusion in J774 Macrophages Is Independent of [Ca<sup>2+</sup>]<sub>i</sub>**

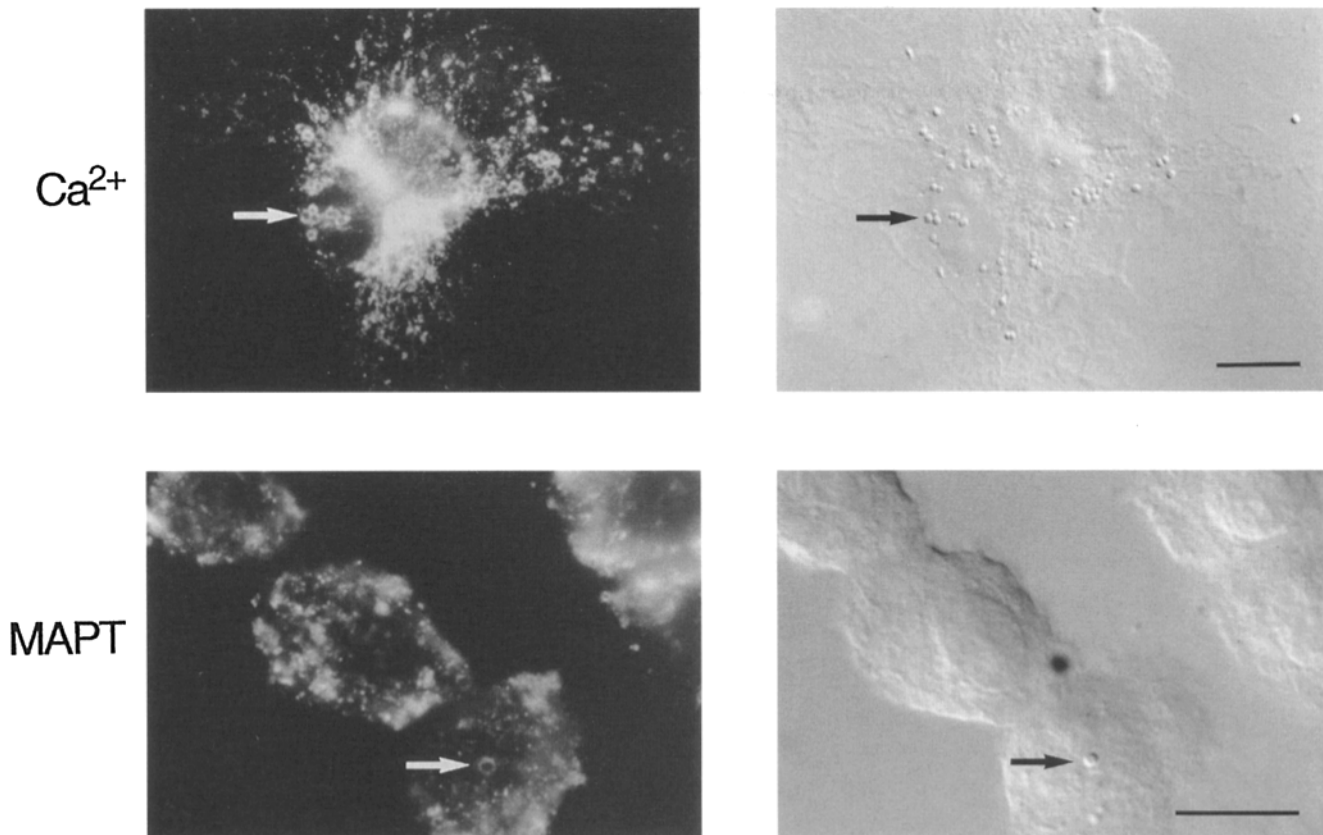
To determine whether the findings in human MDM $\emptyset$  extended to other macrophages, we repeated the experiments with the murine macrophage cell line J774. In unstimulated J774 cells, MAPT/AM effected a less profound decrease in [Ca<sup>2+</sup>]<sub>i</sub> (to ~60 nM) than in human MDM $\emptyset$ . Nevertheless, incubation of J774 cells with 12.5  $\mu$ M MAPT/AM blocked the increase in [Ca<sup>2+</sup>]<sub>i</sub> in response to thapsigargin as well as to 1.0 mM ATP<sup>4-</sup>, even in the presence of 1.0 mM extracellular calcium (data not shown).

J774 M $\emptyset$  were incubated with zymosan, staphylococci, or BCG for 60 min at 37°C to allow phagocytosis to occur. LAMP was revealed using a rat mAb (1D4B). Chelation of intracellular calcium did not significantly alter the proportion of cells associated with particles or bacteria. Moreover, the percentage of zymosan, staphylococci, or BCG found in phagolysosomes as evidenced by LAMP staining was the same in the presence or absence of Ca<sup>2+</sup> (Fig. 6 and Table II). The morphology of the LAMP rings around all studied particles was identical to that found in MDM $\emptyset$  and not influenced by [Ca<sup>2+</sup>]<sub>i</sub>. The findings in human MDM $\emptyset$  could thus be extended to a well-characterized murine macrophage cell line. Indeed, the results were remarkably similar in both cell types (Tables I and II).

#### **The Kinetics of Staphylococci Binding to MDM $\emptyset$ , Phagocytosis, and Phagosome-Lysosome Fusion Are Unaffected by Chelating Intracellular Ionized Calcium**

While the results of the above experiments indicate that Ca<sup>2+</sup> is not required for the fusion of phagosome and lyso-





**Figure 6.** In J774 macrophages, phagosomes containing staphylococci fuse with lysosomes in the presence and absence of intracellular ionized calcium. Adherent J774 cells were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with opsonized staphylococci at a ratio of 20:1 (+Ca<sup>2+</sup>) and 5:1 (+MAPT) for 60 min. Fixed cells were labeled with an mAb against LAMP-1 followed by Cy3-conjugated anti-rat IgG. Cy3 fluorescence and Nomarski micrographs of the same frame are shown for both conditions. (Arrows, phagocytosed staphylococci surrounded by fluorescent rings indicating phagosome-lysosome fusion). Bar, 20 μm.

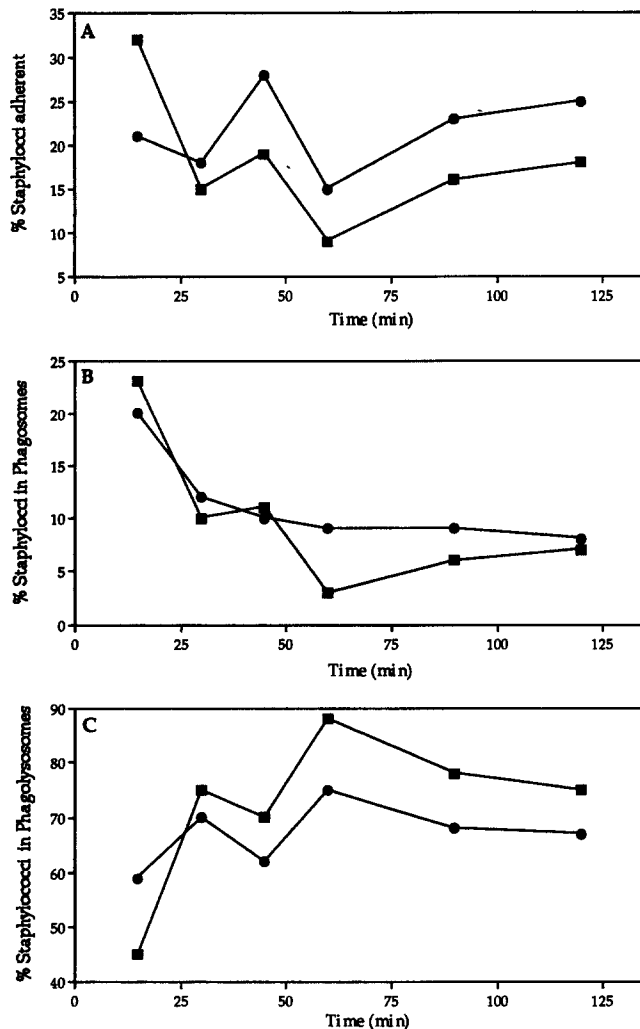
some membranes, it is possible that cytoplasmic calcium may be involved in a step preceding fusion. To test this hypothesis, we compared the rates of particle binding, phagocytosis, and phagosome-lysosome fusion over time in the presence and absence of cytoplasmic Ca<sup>2+</sup>. MDMØ loaded with MAPT/AM or kept in HHBSS were incubated with staphylococci, and the proportion of bacteria in phagosomes and phagolysosomes as well as bacteria adherent to the plasma membrane was determined at various time points from 15 to 120 min. Adherent but extracellular staphylococci were defined by a green ring (accessible to FITC-conjugated anti-human IgG), staphylococci in phagosomes by the absence of a ring (not accessible to anti-

human IgG and not surrounded by LAMP), and staphylococci in phagolysosomes by a red ring (surrounded by Cy3-labeled LAMP). Of note, in the phagocytosis assay we used, an excess of particles was present throughout the incubation. The summary of three independent experiments indicates that the proportion of adherent but extracellular staphylococci decreased up to 30 min after the start of the incubation and remained stable at ~20% of the total at later time points (Fig. 7, representative of three independent experiments). In contrast, the percentage of staphylococci found in phagolysosomes increased during the first 30 min to ~65% and remained unchanged thereafter. A slight decrease over time of the proportion of staph-

**Table II. Phagocytosis and Phagosome-Lysosome Fusion by J774 MØ with Normal or Reduced Intracellular Calcium**

J774	Zymosan		Staphylococci		BCG	
	+Ca <sup>2+</sup>	+MAPT	+Ca <sup>2+</sup>	+MAPT	+Ca <sup>2+</sup>	+MAPT
Cells with bacteria /particles (percent ± SD)	52 ± 34	45 ± 45	72 ± 17	53 ± 19	16 ± 7	14 ± 7
LAMP-positive bacteria/particles (percent ± SD)	85 ± 19	61 ± 9	79 ± 7	60 ± 10	21 ± 15	23 ± 16
		NS		NS		NS

Confluent monolayers of J774 cells were kept in HHBSS (+Ca<sup>2+</sup>) or loaded with MAPT/AM (+MAPT) before incubation with serum-opsonized zymosan (particle/cell ratio 5:1), staphylococci (10:1), or BCG (15:1) for 60 min. Fixed cells were labeled with a rat mAb (1DB4) against murine LAMP-1 followed by Cy3-conjugated anti-rat IgG. DAPI was used to reveal bacterial and cellular DNA. For each condition at least 100 consecutive cells were evaluated by fluorescence microscopy using a ×100 oil-immersion objective. Data represent the mean of at least three separate experiments. By unpaired *t* test, there were no significant differences in the proportion of bacteria/particles associated with cells and ligands enveloped by lysosomes between calcium-depleted and calcium-containing cells.



**Figure 7.** The kinetics of staphylococci binding to MDM $\phi$ , phagocytosis, and phagosome-lysosome fusion are unaffected by chelating intracellular ionized calcium. Adherent MDM $\phi$  kept in HHBSS (■) or loaded with MAPT/AM (●) were incubated with serum-opsonized staphylococci at a ratio of 5:1 for 15–120 min. Extracellular staphylococci were detected by labeling with FITC-conjugated anti-human IgG after initial fixation but without permeabilization (A). After a second fixation step, cells were permeabilized and labeled with an mAb against LAMP-1 followed by Cy3-conjugated anti-mouse IgG which allowed identification of staphylococci in phagolysosomes (C). Staphylococci in unfused phagosomes (B) were defined by the absence of a fluorescent ring. DAPI was used to reveal bacteria and cellular nuclei by staining DNA. For each condition and time point, at least 100 consecutive MDM $\phi$  were evaluated by fluorescence microscopy using a  $\times 100$  oil-immersion objective. Representative of three separate experiments shown.

staphylococci in phagosomes was noted. Particle binding, phagocytosis, and trafficking of the phagosome to a lysosomal compartment were not significantly affected by a profound reduction of  $[Ca^{2+}]_i$ .

### Colocalization of LAMP with Phagocytosed Particles Corresponds to Phagosome-Lysosome Fusion

Immunoelectron microscopy was used to determine whether the LAMP rings seen closely apposed to phagosomes by fluorescence microscopy represent phagosome-lysosome fusion or adhesion of unfused lysosomes to the phagosomal membrane. Immunogold staining showed that LAMP-2 is present in the single membrane surrounding phagocytosed staphylococci (Fig. 8). Therefore, the presence of a smooth ring of fluorescence surrounding an ingested particle genuinely reflects fusion of LAMP-containing membranes (i.e., lysosomes) with phagosome membranes. Phagosome-lysosome fusion was observed irrespective of  $[Ca^{2+}]_i$ . There was no accumulation of endosomes or lysosomes staining for LAMP-2 in the immediate vicinity of the phagosomes. The same results were obtained for zymosan and BCG (data not shown).

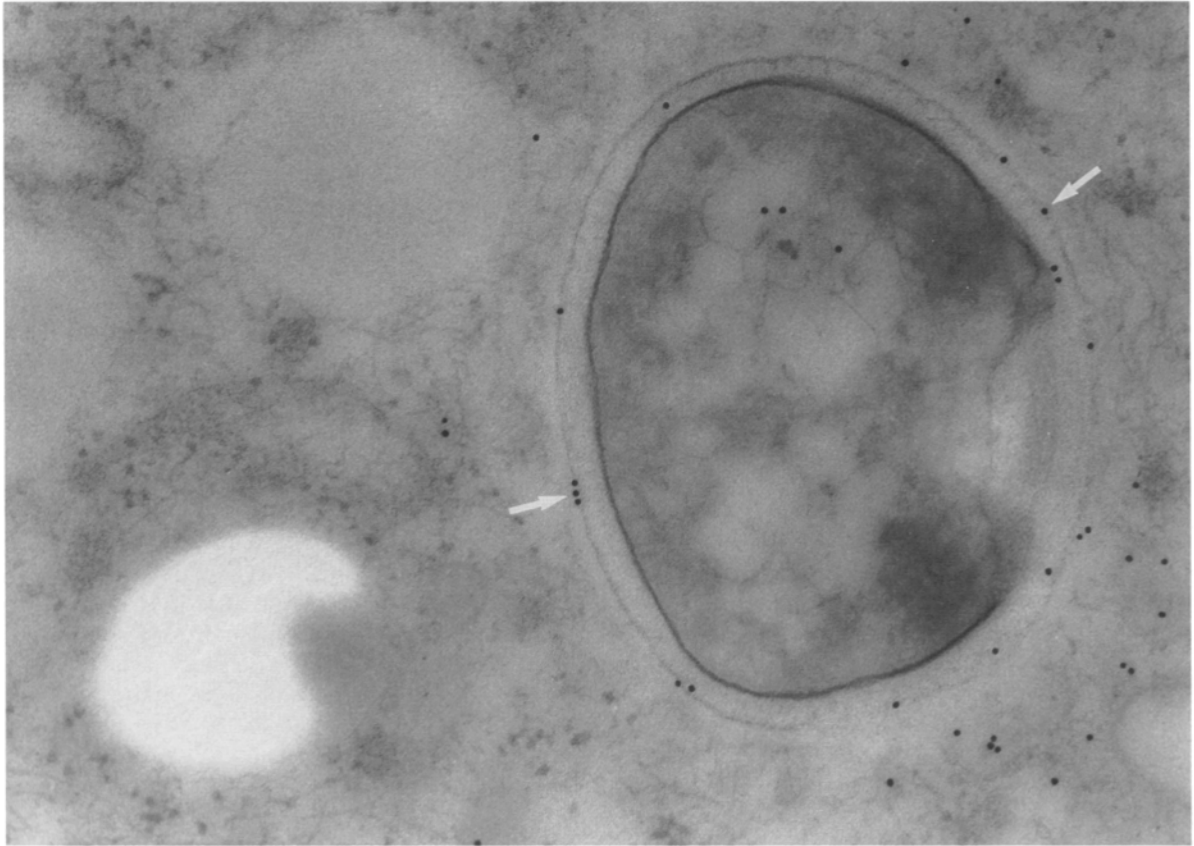
The demonstration at the electron microscopy level that LAMP rings observed in immunofluorescence represent incorporation of the lysosomal membrane marker into the phagosomal vesicle establishes that evaluating the colocalization of ingested particles and lysosomes by fluorescence microscopy is a valid measure for phagosome-lysosome fusion.

### Discussion

Both types of phagocytic cells, neutrophils and macrophages, have comparable *in vivo* functions. They also share surface receptors mediating phagocytosis, and the initial events in phagocytosis such as actin polymerization and signals generated upon receptor ligation, including increases in  $[Ca^{2+}]_i$ , appear to be similar (11). It was thus reasonable to assume that postphagocytic events were regulated in a similar manner. Jaconi et al. (16) have unambiguously established that in human neutrophils phagosome-granule (lysosome) fusion is mediated by increases in cytoplasmic free calcium. In the present work we examined whether phagosome-lysosome fusion in human M $\phi$  was also dependent on an increase in  $[Ca^{2+}]_i$ . The regulation of phagosome-lysosome fusion in M $\phi$  is of special interest since several intracellular pathogens including *M. tuberculosis* and *L. pneumophila* survive in M $\phi$  in phagosomes that do not fuse with lysosomes. It is thus far unknown at which point the microorganisms interfere with the cellular mechanisms leading to phagosome-lysosome fusion.

**Figure 8.** Colocalization of LAMP with phagocytosed bacteria and particles corresponds to phagosome-lysosome fusion. Adherent MDM $\phi$  were kept in HHBSS (+ $Ca^{2+}$ ) or loaded with MAPT/AM (+MAPT) before incubation with opsonized staphylococci at a ratio of 20:1 for 60 min and prepared for electron microscopy as described in *Materials and Methods*. Detection of LAMP-2 with the mAb H4B4 followed by an anti-mouse IgG antibody conjugated to 10-nm gold particles shows gold labeling the membranes of secondary lysosomes and phagosomal membranes surrounding staphylococci irrespective of  $[Ca^{2+}]_i$ . (Arrows, gold particles in the phagolysosomal membrane). Bar, 200 nm.

Ca<sup>2+</sup>



MAPT



By examining phagocytosis of zymosan, staphylococci, and BCG by human and murine macrophages and scoring phagosome-lysosome fusion using both a membrane and a content marker for lysosomes, we found that phagosome-lysosome fusion in macrophages occurs normally at  $[Ca^{2+}]_i \leq 20$  nM. To our knowledge this is the first example in which an important step in the processing of phagocytosed particles has proven to be fundamentally distinct in neutrophils and macrophages.

Several techniques have been used to decrease  $[Ca^{2+}]_i$  in MØ: incubation with Quin2 acetoxymethyl ester in the absence of extracellular  $Ca^{2+}$  (23), and loading EGTA into the cytoplasm via reversible permeabilization of the plasma membrane with extracellular ATP (27). We found that MAPT/AM loading in the absence of extracellular  $Ca^{2+}$  also efficiently chelates intracellular  $Ca^{2+}$  in MØ. Incubation with MAPT/AM reduces baseline  $[Ca^{2+}]_i$  of MDMØ to  $\leq 20$  nM and provides sufficient calcium-buffering capacity to inhibit increases in  $[Ca^{2+}]_i$  in response to multiple stimuli in the presence of extracellular calcium. In contrast with freshly isolated human monocytes, which require high (375  $\mu$ M) concentrations of MAPT/AM to chelate cytoplasmic calcium (20), differentiated MØ required only 12.5  $\mu$ M MAPT/AM to fully block increases in  $[Ca^{2+}]_i$ . MAPT/AM-treated MØ contract and round up without detaching from the surface. However, calcium chelation by MAPT/AM does not affect other morphologic features such as alignment of lysosomes with microtubules and colocalization of content and membrane markers of lysosomes. In addition, in MAPT-treated MØ no functional impairment was evident with respect to phagocytosis, trafficking of phagosomes to lysosomes, and phagosome-lysosome fusion. We also find no evidence, in MDMØ, for an increase in plasma membrane permeability as has been described for murine MØ with chelated cytoplasmic calcium (23). In spite of this apparent difference between MDMØ and murine MØ, the finding that phagosome-lysosome fusion is independent of  $[Ca^{2+}]_i$  in MDMØ also extends to J774 cells.

Particle binding to a receptor mediating phagocytosis leads to receptor-specific signal transduction and localized reorganization of the actin cytoskeleton, which provides the force necessary to internalize the particle (11, 12). After disassembly of the actin network surrounding it, the newly formed phagosome is rapidly transformed in a series of fusion-fission events with endosomal vesicles. In this maturation process some phagocytosed material is recycled to the cell surface, and the phagosome gradually acquires a protein composition characteristic of a phagolysosome (6, 24). Analysis of phagosome-endosome fusion in a cell-free system has revealed evidence for participation of N-ethyl-maleimide-sensitive factor as well as a role for at least two GTPases, G $\alpha$  and one or more small molecular weight GTPases (2). In addition, use of the same cell-free system has elucidated an alternative pathway of phagosome-endosome fusion that is independent of ATP, GTP, or N-ethyl-maleimide-sensitive factor but dependent on micromolar calcium. In this pathway, the calcium- and membrane-binding protein annexin II has been implicated in mediating fusion (22). While phagosome-lysosome fusion so far has not been successfully reconstituted in a cell-free system, the approach we took, chelating intracellular

calcium in intact cells, provides evidence that  $Ca^{2+}$ -regulated proteins are not essential components of the phagocytic or lysosomal membrane fusion machinery of macrophages. In addition, kinetic analysis shows that in macrophages none of the steps leading from particle binding to eventual phagosome-lysosome fusion is regulated by  $[Ca^{2+}]_i$  in a rate-limiting way.

The observations that after 60 min of incubation a much smaller proportion of BCG was found in phagolysosomes than either zymosan or staphylococci, and that prolonged incubation (3–6 d) increased the proportion of BCG in fused vesicles (data not shown), suggests that delivery of LAMP to phagosomes containing mycobacteria may be slower than to other phagosomes. This is consistent with a recent report in which quantitative analysis showed fewer LAMP-1 and -2 molecules in the membrane of vesicles containing live *M. tuberculosis* than in phagosomes containing polystyrene beads (4). Together with the finding that  $Ca^{2+}$  is not needed for phagosome-lysosome fusion in MØ, the above observations prompted us to investigate the role of  $[Ca^{2+}]_i$  in the kinetics of phagosome-lysosome fusion. Using live staphylococci, we find that a profound reduction of  $[Ca^{2+}]_i$  does not affect the rate of phagosome-lysosome fusion over time, suggesting that the reduced delivery of LAMP to phagosomes containing mycobacteria is not due to interference with intracellular calcium signaling.

Studying changes in the protein composition of latex beads containing phagosomes in J774 cells, Desjardins et al. (6) detected LAMP in the phagosomal membrane as early as 20 min after phagocytosis and showed its concentration to increase sixfold over the next 2 h. By immunofluorescence we detect LAMP-1 in the phagosomal membranes around >50% of the ingested staphylococci by 15 min and in >70% after 30 min. On the one hand, this emphasizes the sensitivity of our system to detect LAMP, and on the other hand, this shows the efficiency of MØ to traffic pathogens into a compartment designed to kill them.

Phagocytosis is generally accompanied by an increase in  $[Ca^{2+}]_i$  (7, 16). The role of this calcium transient in MØ is unknown. Our data indicate that in MØ calcium signaling is not essential for phagocytosis and phagosome-lysosome fusion, and that the rate of LAMP delivery to phagosomes is unaffected by reducing  $[Ca^{2+}]_i$  to  $\leq 20$  nM. We are currently investigating the role of calcium transients in intracellular killing mechanisms of MØ.

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