Inhibition of Ca\(^{2+}\) Signaling by Mycobacterium tuberculosis Is Associated with Reduced Phagosome-Lysosome Fusion and Increased Survival within Human Macrophages

By Zulfikar A. Malik,*‡ Gerene M. Denning,*§ and David J. Kusner*†§

Abstract

Complement receptor (CR)-mediated phagocytosis of Mycobacterium tuberculosis by macrophages results in intracellular survival, suggesting that M. tuberculosis interferes with macrophage microbicidal mechanisms. As increases in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) promote phagocyte antimicrobial responses, we hypothesized that CR phagocytosis of M. tuberculosis is accompanied by altered Ca\(^{2+}\) signaling. Whereas the control complement (C)-opsonized particle zymosan (COZ) induced a 4.6-fold increase in [Ca\(^{2+}\)]\(_c\) in human macrophages, no change in [Ca\(^{2+}\)]\(_c\) occurred upon addition of live, C-opsonized virulent M. tuberculosis. Viability of M. tuberculosis and ingestion via CRs was required for infection of macrophages in the absence of increased [Ca\(^{2+}\)]\(_c\), as killed M. tuberculosis or antibody (Ab)-opsonized, live M. tuberculosis induced elevations in [Ca\(^{2+}\)]\(_c\), similar to COZ. Increased [Ca\(^{2+}\)]\(_c\) induced by Ab-opsonized bacilli was associated with a 76% reduction in intracellular survival, compared with C-opsonized M. tuberculosis. Similarly, reversible elevation of macrophage [Ca\(^{2+}\)]\(_c\) with the ionophore A23187 reduced intracellular viability by 50%. Ionophore-mediated elevation of [Ca\(^{2+}\)]\(_c\) promoted the maturation of phagosomes containing live C-opsonized bacilli, as evidenced by acidification and accumulation of lysosomal protein markers. These data demonstrate that M. tuberculosis inhibits CR-mediated Ca\(^{2+}\) signaling and indicate that this alteration of macrophage activation contributes to inhibition of phagosome-lysosome fusion and promotion of intracellular mycobacterial survival.

Key words: calcium • macrophages • tuberculosis • bacterial pathogenesis • immunology

Introduction

Tuberculosis is a global health problem with enormous impact on human morbidity and mortality (1). Approximately one-third of the world’s population is infected with Mycobacterium tuberculosis, and three million people die of active disease each year. An essential virulence characteristic of M. tuberculosis is its ability to successfully parasitize monocytes and macrophages, despite the presence of multiple microbicidal mechanisms within these cells (2). The molecular mechanisms responsible for the intracellular survival of M. tuberculosis are unknown.

Multiple host-pathogen interactions may impact the fate of M. tuberculosis within human monocytes and macrophages and, consequently, the presence or absence of disease in infected individuals. The earliest interaction between M. tuberculosis and mononuclear phagocytes is the binding and uptake of the bacilli by plasma membrane phagocytic receptors (3). Phagocytosis of M. tuberculosis, in either the presence or absence of serum, is predominantly mediated by the complement receptor (CR)1, CR3, and CR4 (4–6). In human monocytes and monocyte-derived macrophages (MDMs), the \(\beta_2\)-integrin CR3 is the major phagocytic receptor for M. tuberculosis, and anti-CR3 Abs inhibit ingestion of tubercle bacilli by \(\sim\)80% (5). In serum-free conditions, the macrophage mannose receptor also mediates mycobacterial phagocytosis, although its contribution to ingestion of M. tuberculosis is much less in the presence of complement proteins (7).

The ability of M. tuberculosis to enter macrophages via the CR-mediated phagocytic pathway may contribute to its intracellular survival, as, in many cases, CR ligation does not trigger phagocyte microbicidal responses (8, 9). Studies with murine macrophages demonstrate that the class of phago-
cytic receptor that mediates ingestion of \( M. \) \( tuberculosis \) has a strong influence on the extent of phagosomal maturation. \( CR \) -mediated phagocytosis of \( M. \) \( tuberculosis \) results in a phagosome that is unable to fuse with lysosomes (10). Conversely, if the bacillus is opsonized with \( M. \) \( tuberculosis \)-specific \( Ab \), its ingestion is mediated by macrophage \( Fc \) \( R \) \( s \), and the mycobacterial phagosome undergoes full maturation to a phagolysosome (11). These results suggest that \( Fc \) \( R \) -mediated ingestion of \( M. \) \( tuberculosis \) must mobilize signaling pathways that are distinct from those that are activated by \( CR \) \( s \), which are responsible for the difference in phagosome maturation. The relevance of these observations to human disease has been questioned, because the antimycobacterial activity of murine macrophages is much more easily demonstrated in vitro than that of human macrophages. Although multiple investigators have demonstrated that \( CR \) -dependent ingestion of \( M. \) \( tuberculosis \) by human macrophages is also followed by defective phagosomal maturation (12), to our knowledge, no data is available on the effects of \( Ab \) opsonization on survival of \( M. \) \( tuberculosis \) within human macrophages. Furthermore, the biochemical mechanisms responsible for incomplete maturation of \( M. \) \( tuberculosis \)-containing phagosomes are unknown.

Many distinct signal transduction pathways contribute to the activation of phagocyte antimicrobial defenses, but their integrative function and relative priority in the killing of specific pathogens is unknown. Stimulation-induced increases in cytosolic \( Ca^{2+} \) concentration \( ([Ca^{2+}]_c) \) are essential for activation of the phagocyte respiratory burst, production of nitric oxide, secretion of microbialicid granule constituents, and synthesis of proinflammatory mediators, including \( TNF-\alpha \) (13-17). Based on these considerations, three questions of specific relevance to the pathogenesis of \( tuberculosis \) were investigated in this study: (a) Does virulent \( M. \) \( tuberculosis \) alter \( Ca^{2+} \)-mediated signal transduction in human macrophages? If so, (b) Do these alterations in macrophage \( Ca^{2+} \) signaling contribute to incomplete phagosomal maturation and intracellular survival of \( M. \) \( tuberculosis \), and (c) Does the route of entry into human macrophages, i.e., via \( CR \) -versus \( Fc \) \( R \)-mediated phagocytosis, affect the intracellular viability of \( M. \) \( tuberculosis \)?

**Materials and Methods**

Chemicals. \( Hepes \), zymosan, and collagen were obtained from Sigma Chemical Co. RPMI 1640 medium with \( I \)-glutamine and PBS was purchased from Gibco BRL. Middlebrook 7H9 broth was obtained from BBL Microbiology Systems, and 7H11 agar, oleic acid-albumin-dextrose-catalase enrichment medium, and auramine-rhodamine stain were from Difco Labs, Inc. Teflon wells were obtained from Savillex Corp. Tissue culture plates were purchased from Linbro Flow Labs, and Fura2 and A23187 were from Molecular Probes, Inc. Bis (2-amino-5-methylphenox) ethane-N,N,N,N' ,N'-tetracetic acid tetracetoxyethyl ester (\( MAPTAM \)) was obtained from Calbiochem Corp., and dipalmitolphyadicholine (DPDC) was from Avanti, Inc.

\( Ab \)s. Polyclonal (A-188) and monoclonal \( Ab \)s (CS-40, CS-35) to lipoarabinomannan (LAM \( ) \) from \( M. \) \( tuberculosis \) were provided by Drs. Patrick Brennan and John Belisle (Colorado State University, Fort Collins, CO; National Institutes of Health grant AI-75320). A-188 and CS40 are specific to LAM from the virulent Erdman strain of \( M. \) \( tuberculosis \), whereas CS35 recognizes an epitope common to LAM \( s \) from several strains of \( M. \) \( tuberculosis \). m\( Ab \)s to CD18 (H52) and lysosome-associated membrane protein \( (LAMP) \) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). F(\( ab \)\(^\prime \)) fragments of CD18 were prepared by digestion with pepsin as previously described (20) and partially purified by protein \( G \) -Sepharose chromatography. Goat anti-human C3 IgG was obtained from Atlantic Antibodies, Inc.

Preparation of M. acrophage M onlays. PBM Cs were isolated from healthy, purified protein derivative (PPD)-negative, adult volunteers and cultured in Teflon wells for 5 d in RPMI 1640 with 20% fresh autologous serum as previously described (21). M acrophages were purified by adherence to chromic acid-cleaned, collagen-coated glass coverslips for 2 h at \( 37^\circ \)C in 5% \( CO_2 \). M onlays were washed repeatedly and incubated in RPMI 20 mM \( Hepes \) (R H), pH 7.4, 2.5% \( \% \) serum for use in experiments. Effects of experimental manipulations on macrophage viability were assessed by exclusion of trypan blue, and monolayer density was determined by nuclei counting with naphthol blue-black stain (22).

Bacteria. The Erdman, H37R v, and H37R at a strain of \( M. \) \( tuberculosis \) were obtained from the American Tissue Type Culture Collection and were cultured and prepared for use in experiments as noted previously (5, 7, 21). In brief, aliquots of frozen \( M. \) \( tuberculosis \) stocks in 7H9 broth were thawed, cultured for 9 d on 7H11 agar at \( 37^\circ \)C in 5% \( CO_2 \)/95% air, scraped from agar plates, and suspended in R H by vortexing briefly in an Eppendorf tube containing two glass beads. After settling, the supernatant was transferred to a new tube and allowed to settle once again. Heat killing was accomplished by incubating this final suspension at \( 100^\circ \)C for 10 min and confirmed by absence of CFUs (23, 24). Gamma-irradiated (killed) \( M. \) \( tuberculosis \) was provided by Drs. Patrick Brennan and John Belisle (Colorado State University). For experiments requiring complement-opsonized (C-op) bacilli, aliquots of \( M. \) \( tuberculosis \) (live, heat-killed, or gamma-irradiated) were preopsonized in 50% human serum for 30 min at \( 37^\circ \)C and then washed three times in PBS. Ab opsonization of \( M. \) \( tuberculosis \) was achieved by incubating the bacilli with 10 \( \mu \)g/ml CS-40 or CS-35 or 10 \( \mu \)l of A-188 for 30 min, followed by washing in PBS. After opsonization, \( M. \) \( tuberculosis \) preparations were resuspended in H BSS using glass beads, and clumped organisms were allowed to settle, as described above. \( M. \) \( tuberculosis \) suspensions were counted in a Petroff-Hauser chamber, and the concentration of bacteria was adjusted for use in experiments. Final \( M. \) \( tuberculosis \) preparations contained \( \geq \)95% single bacteria, with \( \geq \)75% viability by determination of CFUs (5, 21). The effects of various experimental manipulations on the viability of \( M. \) \( tuberculosis \) were also determined by analysis of CFUs.

Analysis of Phagocytosis. Phagocytosis of \( M. \) \( tuberculosis \) was determined as previously described (5, 21). In brief, macrophage monolayers adherent to glass coverslip \( s \) (~2 \( \times \) 10\(^5\) \( \mu \)M \( d \)\( m \)s per coverslip) in 24-well tissue culture plates were incubated with \( M. \) \( tuberculosis \) (multiplicity of infection \( [M:O] \) of 10:1 in \( R \) \( H \), 2.5% autologous nonimmune serum. After incubation for various intervals, monolayers were washed repeatedly to remove nonadherent bacteria, fixed in 10% formalin, and stained with auramine-rhodamine for 20 min (5, 21). Coverslip \( s \) were washed with distilled water and incubated with acid alcohol for 3 min, washed, and incubated in KMnO\(_4\) for 2 min. Aherent bacteria were quantitated by fluorescence microscopy of triplate cover-
slips for each experimental condition (50–200 MDMs per coverslip), and results of a set of experiments were expressed as the mean (± SEM) number of adherent M. tuberculosis per 100 macrophages (phagocytic index). Previous electron microscopic studies of this assay have indicated that all adherent mycobacteria are phagocytosed, both under control conditions and in experiments in which phagocytosis is inhibited or augmented (5, 21).

Western Blot to Detect C3 Fixation to M. tuberculosis. Heat-killed or live M. tuberculosis was incubated in 50% human serum for 30 min at 37°C. The bacteria were recovered by centrifugation at 12,000 g for 10 min, washed twice, and solubilized in SDS sample buffer (62.5 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 75 mM β-ME, 0.0025% bromphenol blue). After SDS-PAGE on 10% gels, proteins were transferred to polyvinylidene difluoride membranes. Western blotted with goat anti-human C3 IgG, and detected by enhanced chemiluminescence as described (5, 21).

Determination of Intracellular C alan. Calcium measurements were performed at the Cell Fluorescence Core Facility (Veterans Affairs Medical Center, Iowa City, IA). MDMs were adhered to collagen-coated glass coverslips and incubated with 10 μM Fura2-AM in HBSS for 30 min at 37°C. [Ca²⁺]i, in single MDMs or the mean [Ca²⁺]i of groups of 10–20 cells, was determined using a Photon scan II spectrofluorometer (Photon Technology Intl.) with a Nikon monolayers were washed and repleted with 20 μl equivalent volume of ethanol solvent (0.1%). After 20 min, the ratios of the corrected fluorescence intensities (F/F₀) at each excitation wavelength were subtracted from each data point. The ratios of the corrected fluorescence intensities (R) were then converted to the actual calcium concentration using the formula, [Ca²⁺]i = Kd × (R – R_mic)/(R_max – R) (reference 25), where the maximum and minimum ratios, as well as the dissociation constant, were empirically derived from [Ca²⁺]i curves generated with the instrument. In certain experiments, the effects of the absence of extracellular Ca²⁺ were determined by incubation of MDMs in Ca²⁺-free HBSS with 3 mM EGTA. To chelate cytosolic Ca²⁺, MDMs were preincubated with MAPTAM (15–25 μM) for 30 min at 37°C (26, 27).

A analysis of CFUs. MDMs adherent to collagen-coated glass coverslips were infected at an M OI of 1:1 with Erdman M. tuberculosis (precomplemented with complement or anti-LAM Abs) in R H, 2.5% heat-inactivated autologous serum. After 1 h, the monolayers were washed and repleted with buffer containing 1% heat-inactivated serum. 24, 48, and 96 h after infection, supernatants were transferred to sterile microfuge tubes, monolayers were lysed with ice cold sterile water, and SDS was added to a final concentration of 0.25%. Lysates were combined with their corresponding supernatants and resuspended in TH9, and serial dilutions were plated in duplicate on 7H11 agar. Colonies were counted 2 wk after plating. To determine the effect of elevation of MDM intracellular [Ca²⁺]i on mycobacterial survival, monolayers were infected at a 1:1 ratio with C-op M. tuberculosis in HBSS containing the Ca²⁺ ionophore A23187 (1 μM) or an equivalent volume of ethanol solvent (0.1%). After 20 min, monolayers were washed and repleted with 20 μg/ml phosphatidylocholine vesicles, 1% autologous serum in R H, to reverse the A23187-mediated influx of extracellular Ca²⁺ (28). DPPC vesicles were prepared by evaporation of a chloroform/methanol (2:1) solution under N₂ and resuspension in HBSS by sonication for 10 min at 25°C (21). CFUs were counted as described above.

Confocal microscopy. The acidophilic dye LysoTracker Red (Molecular Probes, Inc.) was incubated at a 1:10,000 dilution with MDM monolayers in R H, 2.5% autologous serum, for 2 h at 37°C. Unincorporated dye was removed by washing, followed by infection with M. tuberculosis for 1 h. After removal of nonadherent bacilli, LysoTracker Red was added to each well at the same concentration used for initial labeling. 24 h after infection, MDMs were fixed in 3.75% paraformaldehyde for 15 min and permeabilized with ice cold methanol/acetic (1:1). The localization of M. tuberculosis was ascertained by incubating monolayers with auramine for 20 min at 25°C, followed by a 3-min incubation in acid alcohol. After thorough washing, monolayers were blocked with a PBS, 5% BSA, 10% goat serum for 1 h. In parallel experiments using Abs to the lysosomal protein markers LAM P-1, cathepsin D, and CD63, coverslips were incubated with the appropriate primary Abs (diluted in blocking solution) for 1 h at 25°C, washed, and then incubated with the corresponding fluorophore-conjugated secondary anti-IgG Ab for 1 h. After repeated washings, coverslips were mounted with buffered glycerol solution and sealed with nail polish. Confocal microscopy was performed on a Zeiss Laser Scan Inverted 510 microscope (Carl Zeiss, Inc.). An argon/krypton laser (excitation, 488 nm; emission, 505–530 nm) was used for detection of auramine fluorescence, and a helium/neon laser (excitation, 543 nm; emission, >585 nm) for detection of Texas Red and LysoTracker Red. The percentage of M. tuberculosis phagosomes colocalizing with the marker of interest was determined by counting >25 phagosomes from at least 10 different fields per condition.

Analysis of data. Data from each experimental group were subjected to an analysis of normality and variance. Differences between experimental groups composed of normally distributed data were analyzed for statistical significance using Student’s t-test. Nonparametric evaluation of other data sets was performed with the Wilcoxon Rank Sum test (29).

Results

C-op zymosan Induces an Increase in Cytosolic Ca²⁺ in Human Macrophages. As binding and phagocytosis of M. tuberculosis by macrophages in the presence or absence of serum is primarily mediated by CR s (4, 5, 30), we first characterized macrophage Ca²⁺ signaling induced by the model particulate CR-ligand, C-op zymosan (COZ) (31, 32). Previous studies in neutrophils have demonstrated that COZ induces a significant increase in [Ca²⁺]i, due to stimulation of CR3 and, to a lesser extent, CR1 (33–37). In addition to CR3 and CR1, macrophages, unlike neutrophils, also express high levels of CR4 (38). Therefore, it was necessary to characterize in detail the effects of COZ on [Ca²⁺]i, in human macrophages to serve as a control for subsequent experiments with M. tuberculosis.

MDMs were purified from PBMCs of healthy, PPD-negative adult donors after 5-d culture in RPMI, 20% autologous serum, by adherence to collagen-coated glass coverslips (21). After loading of MDMs with the Ca²⁺-sensitive dye Fura2 (10 μM), monolayers were washed and placed in Ca²⁺-, Mg²⁺-containing HBSS (CHBSS). Levels of [Ca²⁺]i in single MDMs were determined by fluorescence ratio imaging of Fura2 (25). The basal level of [Ca²⁺]i in resting MDMs ranged from ~50 to 150 nM (Fig. 1 A). Incubation with COZ at a particle/cell ratio of 10:1 resulted in a rapid increase in macrophage [Ca²⁺]i, which peaked in the 300–800 nM range and gradually returned to basal levels over the next 8–10 min (Fig. 1 A; fold-increase...
in $[\text{Ca}^{2+}]_c = 4.6; \text{range, 2.4–6.5-fold; } n = 20$). Subsequent addition of thapsigargin (1 $\mu$M), which inhibits the $\text{Ca}^{2+}$/ATPase responsible for reaccumulation of $[\text{Ca}^{2+}]_c$, into endoplasmic reticulum stores (39), resulted in a further increase in macrophage $[\text{Ca}^{2+}]_c$. This thapsigargin-induced increase in $[\text{Ca}^{2+}]_c$, provided verification of the intact capacity of the intracellular $\text{Ca}^{2+}$ storage pool and the functional integrity of the capacitative $\text{Ca}^{2+}$ entry mechanism (40, 41).

The COZ-induced increase in macrophage $[\text{Ca}^{2+}]_c$, was due to both release of $\text{Ca}^{2+}$ from intracellular stores and influx of extracellular $\text{Ca}^{2+}$, as the average magnitude and duration of the elevated $[\text{Ca}^{2+}]_c$ was significantly attenuated, but not abolished, by incubation of MDMs in $\text{Ca}^{2+}$-free HBSS containing 3 mM EGTA (data not shown). Under these conditions, the residual elevation in $[\text{Ca}^{2+}]_c$ is due to release from intracellular $\text{Ca}^{2+}$ stores in the endoplasmic reticulum, as evidenced by the increase in Fura2 fluorescence upon addition of thapsigargin. Preincubation of MDMs with the intracellular $\text{Ca}^{2+}$ chelator MAPTAM (12.5 $\mu$M), followed by placement in $\text{Ca}^{2+}$-free HBSS, 3 mM EGTA (EH BSS), completely inhibited the increase in $[\text{Ca}^{2+}]_c$, due to COZ (data not shown). To test the hypothesis that COZ-induced $[\text{Ca}^{2+}]_c$, elevations were dependent on stimulation of the $\beta_2$-integrins C3 (CD11b/CD18) and CR 4 (CD11c/CD18), MDMs were preincubated with F(ab')2, fragments of $\alpha$-CD18 mAb (H52). Subsequent addition of COZ did not cause a significant change in $[\text{Ca}^{2+}]_c$, (Fig. 1 B). Inhibition of the increase in $[\text{Ca}^{2+}]_c$ by $\alpha$-CD18 F(ab')2 fragments was specific for CR-dependent stimuli, as there was no effect on the $[\text{Ca}^{2+}]_c$ elevation stimulated by platelet activating factor (PAF, Fig. 1 B). These experiments demonstrate that, similar to neutrophils (35, 36, 42), CR-dependent stimulation of human macrophages with COZ results in a marked increase in $[\text{Ca}^{2+}]_c$, which is derived from both intracellular and extracellular $\text{Ca}^{2+}$ pools. Furthermore, the $\beta_2$-integrins, C3 and CR 4, are responsible for the majority of macrophage CR-stimulated $\text{Ca}^{2+}$ signaling.

Phagocytosis of M. tuberculosis does not cause a significant change in macrophage cytosolic calcium. In the presence of serum, M. tuberculosis is opsonized with C3bi and, to a lesser extent, C3b via the alternative pathway of complement (5, 43, and data not shown). The effect of binding and phagocytosis of the virulent Erdman strain of M. tuberculosis on MDM $[\text{Ca}^{2+}]_c$, levels was determined exactly as noted above for COZ. Incubation of MDM with live, C-op M. tuberculosis at an MOI of 10:1 did not result in any significant change in macrophage $[\text{Ca}^{2+}]_c$ (Fig. 2 A; fold-increase in $[\text{Ca}^{2+}]_c = 1.01; \text{range, 1.0–1.25-fold; } n = 18$). To determine whether the failure of M. tuberculosis to elicited an increase in MDM $[\text{Ca}^{2+}]_c$ was due to a defect in either intracellular $\text{Ca}^{2+}$ stores or capacitative $\text{Ca}^{2+}$ entry via macrophage plasma membrane $\text{Ca}^{2+}$ channels, we analyzed the effect of thapsigargin on M. tuberculosis-infected MDMs. Addition of 1 $\mu$M thapsigargin resulted in a prompt rise in MDM $[\text{Ca}^{2+}]_c$, (Fig. 2 A), the magnitude and duration of which were comparable to that of uninfected MDMs. This response to thapsigargin confirmed the adequacy of both intracellular $\text{Ca}^{2+}$ stores and the capacitative coupling of store depletion to the influx of extracellular $\text{Ca}^{2+}$ in M. tuberculosis-infected MDMs.

The lack of an increase in macrophage $[\text{Ca}^{2+}]_c$, was not due to a failure to bind or ingest M. tuberculosis. At an MOI of 10:1, the mean (± SEM) number of ingested bacilli per macrophage was 5.36 ± 0.41, and 73 ± 4% of MDMs ingested at least one tubercle bacillus (21). To ensure that each MDM phagocytosed at least one tubercle bacillus, select single-cell $[\text{Ca}^{2+}]_c$, determinations were conducted with increased MOIs of 30:1 and 100:1. At these higher levels of infection, each MDM phagocytosed at least one bacillus, as determined by subsequent staining of cell monolayers with auramine-rhodamine (data not shown). However, even at MOIs of 30:1 (data not shown) and 100:1 (Fig. 2 B), M. tuberculosis did not result in any change in macrophage $[\text{Ca}^{2+}]_c$. To complement the single-cell determinations of $[\text{Ca}^{2+}]_c$, the aperture of the spectrofluorometer was adjusted to encompass a population of 15–20 MDMs per sample to determine the average $[\text{Ca}^{2+}]_c$ in resting and M. tuberculosis-infected macrophages. Similar to the results of the sin-
gle-cell analysis, the mean $[\text{Ca}^{2+}]_c$ of a population of macrophages did not exhibit a significant change in $[\text{Ca}^{2+}]_c$ upon incubation with M. tuberculosis (Fig. 2 C).

The defect in macrophage $\text{Ca}^{2+}$ signaling was not restricted to the Erdman strain of M. tuberculosis. Infection of MDMs with a second, well characterized virulent strain, H37Rv M. tuberculosis, also occurred without a significant alteration of $[\text{Ca}^{2+}]_c$ (Fig. 2 D; fold-increase in $[\text{Ca}^{2+}]_c$ = 1.0; n = 4). These results suggest that the lack of initiation of $\text{Ca}^{2+}$ signaling may be a general property of pathogenic M. tuberculosis. The ability to test this hypothesis is limited somewhat by the lack of an avirulent strain of M. tuberculosis (Fig. 2 C).

Figure 2. Macrophage phagocytosis of live M. tuberculosis does not induce a significant rise in cytosolic $\text{Ca}^{2+}$. (A) Virulent M. tuberculosis Erdman strain (MTb) was opsonized in 50% autologous nonimmune serum for 30 min at 37°C, washed three times, and resuspended in CHBSS. More than 95% of each M. tuberculosis preparation consisted of single bacilli. MTb were added to Fura2-loaded MDMs at an MOI of 10:1, and $[\text{Ca}^{2+}]_c$ of single MDMs was determined as noted in the legend to Fig. 1. At the indicated time, 1 μM thapsigargin (Tg) was added to the monolayer to evaluate intracellular $\text{Ca}^{2+}$ stores. (B) The effect of Erdman M. tuberculosis on macrophage $[\text{Ca}^{2+}]_c$ was determined at an MOI of 100:1. (C) The average basal level of $[\text{Ca}^{2+}]_c$ in a group of 20 macrophages was determined by expanding the aperture of the spectrofluorometer. Erdman M. tuberculosis was added at an MOI of 10:1 at the indicated time. (D) MDMs were infected with the virulent H37Rv strain of M. tuberculosis, and macrophage $[\text{Ca}^{2+}]_c$ was determined as above. Figures are representative of 18 identical experiments for A and at least 4 experiments for B–D.

Ca$^{2+}$-mediated signal transduction is characterized by a complex series of positive and negative regulatory circuits, as well as distinct temporal and spatial determinants of signal propagation (49–52). To determine whether the defect in $\text{Ca}^{2+}$ signaling accompanying infection with M. tuberculosis resulted in a global depression of macrophage $\text{Ca}^{2+}$-dependent signal transduction, we tested the response of infected MDMs to PAF, a potent $\text{Ca}^{2+}$-mobilizing ligand that binds to a G protein–coupled receptor (53). 10 min after infection with Erdman M. tuberculosis, macrophages were incubated with 100 nM PAF, and levels of $[\text{Ca}^{2+}]_c$ were determined via fluorescence of Fura2. As demonstrated in Fig. 3 A, PAF induced a rapid and significant rise in $[\text{Ca}^{2+}]_c$, in infected macrophages. H37Rv, and H37Ra, were similar in terms of their lack of effect on basal levels of $[\text{Ca}^{2+}]_c$ in human macrophages.
effect of M. tuberculosis that could be specific to CR-induced elevations in [Ca\(^{2+}\)]\(_i\), we determined whether infected macrophages exhibited an altered [Ca\(^{2+}\)]\(_i\) response to COZ. Similar to the PAF-stimulated MDMs noted above, COZ-induced elevations in macrophage [Ca\(^{2+}\)]\(_i\) occurred normally in the presence of prior (Fig. 3C) or concurrent (data not shown) infection with M. tuberculosis. Therefore, the lack of increase in [Ca\(^{2+}\)]\(_i\) levels during M. tuberculosis infection is not accompanied by alterations in [Ca\(^{2+}\)]\(_i\) signaling by either particulate or soluble stimuli, which utilize the same or different classes of macrophage cell-surface receptors for their initiation. Our results do not exclude the possibility that M. tuberculosis may alter more distal aspects of Ca\(^{2+}\)-mediated signal transduction by these or other stimuli.

Inhibition of Macrophage Ca\(^{2+}\) Signaling Is Dependent on the Viability of M. tuberculosis. The specific virulence determinants that enable M. tuberculosis to survive within the phagosomes of human macrophages are unknown. In addition, there are no avirulent strains of M. tuberculosis that may be used to define the molecular mechanisms that regulate essential pathogenic interactions between tubercle bacilli and mononuclear phagocytes. Despite these limitations, considerable evidence indicates that the failure of M. tuberculosis-containing phagosomes to mature into acidic microbicidal phagolysosomes is an important component of tuberculous pathogenesis (24, 54, 55). Clemens and Horwitz have demonstrated that this inhibition of phagosomal maturation is dependent on the viability of M. tuberculosis, as phagosomes containing heat-killed M. tuberculosis develop into mature phagolysosomes (23, 24). We tested the hypothesis that the M. tuberculosis-induced inhibition of macrophage Ca\(^{2+}\) signaling would demonstrate a similar requirement for bacterial viability. Erdman M. tuberculosis was killed by heating to 100°C for 10 min, followed by opsonization in autologous, nonimmune serum as described above for live bacilli (23, 24). Particular care was taken to ensure that the preparation of heat-killed M. tuberculosis consisted of >95% single bacilli, as noted in M. aterials and Methods. The loss of viability of heat-killed M. tuberculosis was verified by absence of growth on 7H11 agar. Heat-killed Erdman M. tuberculosis induced a rapid and significant rise in macrophage [Ca\(^{2+}\)]\(_i\). (Fig. 4A; fold-increase in [Ca\(^{2+}\)]\(_i\) = 3.8; range, 2.1–6.5-fold; n = 16), which closely resembled that induced by COZ. Utilization of an alternate protocol for heat killing (80°C, 60 min; reference 5) resulted in similar stimulation of increased [Ca\(^{2+}\)]\(_i\), by dead, C-op M. tuberculosis (data not shown). The increase in levels of macrophage [Ca\(^{2+}\)]\(_i\) induced by heat-killed M. tuberculosis was completely inhibited by preincubation of these cells with F(ab\(^\prime\))\(_2\), fragments of α-C D 18 mAb (Fig. 4B), indicating a major role for CR 3 and/or CR 4 in the initiation of this response. Studies with Ca\(^{2+}\)-free media (Fig. 4C) and intracellular Ca\(^{2+}\) buffering (Fig. 4D) indicated that the increase in [Ca\(^{2+}\)]\(_i\), stimulated by heat-killed M. tuberculosis resulted from both release of Ca\(^{2+}\) from intracellular stores as well as influx of extracellular Ca\(^{2+}\).

As heat killing of M. tuberculosis may induce changes in mycobacterial surface structures that could alter MDM Ca\(^{2+}\) signaling by mechanisms other than the loss of bacterial viability, similar studies were conducted with M. tuberculosis that had been killed by gamma irradiation. Incubation of MDMs with gamma-irradiated M. tuberculosis resulted
Figure 4. Viability of \( M. \) tuberculosis is required for inhibition of macrophage \( \text{Ca}^{2+} \) signaling. (A) An aliquot of Erdman \( M. \) tuberculosis was heated to 100°C for 10 min, and efficiency of killing was demonstrated by absence of growth on 7H11 agar. After opsonization in 50% autologous serum, heat-killed bacilli (Hk-MTB) were incubated with Fura2-loaded macrophages at a bacteria/MDM ratio of 10:1. Levels of macrophage \( [\text{Ca}^{2+}]_c \) were determined from the ratio of fluorescence of Fura2, as noted in Materials and Methods. (B) MDMs were incubated with \( \text{F(ab')}_2 \) fragments of \( \alpha-\text{CD18} \) mAb for 30 min at 15°C, followed by repeated washing to remove unbound Ab. MDMs were warmed to 37°C and incubated with heat-killed \( M. \) tuberculosis. At the indicated time, 100 nM PAF was added to evaluate CD18-independent \( \text{Ca}^{2+} \) signaling. (C) After loading with Fura2-AM, macrophages were incubated in EBSS for 5 min before addition of Hk-MTB and determination of \( [\text{Ca}^{2+}]_c \). Thapsigargin (1 \( \mu \)M) was added at the indicated time. (D) MDMs were incubated in EBSS with 25 \( \mu \)M MAPTAM for 30 min at 37°C, followed by addition of Hk-MTB (arrow). (E) Gamma-irradiated H37Rv, preopsonized in 50% autologous serum, was incubated with MDMs in CHBSS, and \( [\text{Ca}^{2+}]_c \) levels were determined via fluorescence of Fura2. Results in A are representative of data from 16 identical experiments; at least 4 separate experiments were conducted for each of the other conditions.

In a prompt increase in \( [\text{Ca}^{2+}]_c \) (Fig. 4E; fold-increase in \( [\text{Ca}^{2+}]_c \) = 2.9; range, 2.6-3.4; \( n = 6 \) that was indistinguishable from that induced by heat-killed tubercle bacilli. These studies support the hypothesis that mycobacteria-induced inhibition of macrophage \( \text{Ca}^{2+} \) signaling requires viability of \( M. \) tuberculosis.

Inhibition of Macrophage \( \text{Ca}^{2+} \) Signaling by \( M. \) tuberculosis Is Dependent on the Class of Receptor That Mediates Mycobacterial Phagocytosis. To test the hypothesis that the receptors that mediate phagocytosis of \( M. \) tuberculosis are determinants of macrophage \( \text{Ca}^{2+} \) signaling, Erdman \( M. \) tuberculosis was incubated with polyclonal rabbit Ab to its cell wall glycolipid, LAM, to confer phagocytosis by macrophage FcyRs. After opsonization with anti-LAM Ab, mycobacteria were washed in PBS, resuspended in CHBSS, and counted to ensure that single bacilli comprised at least 95% of the preparation. Addition of Ab-op \( M. \) tuberculosis induced a prompt and significant increase in macrophage \( [\text{Ca}^{2+}]_c \) (Fig. 5A, fold-increase in \( [\text{Ca}^{2+}]_c \) = 3.3; range, 2.1-5.0-fold; \( n = 9 \)). In control experiments conducted with MDMs from the same donors, \( M. \) tuberculosis incubated with preimmune serum or irrelevant rabbit polyclonal Ab (anti-myeloperoxidase Ab) did not induce a change in macrophage \( [\text{Ca}^{2+}]_c \) (data not shown). Opsonization of \( M. \) tuberculosis with either of two mAbs to LAM (CS-35 or CS-40) resulted in similar elevations in macrophage \( [\text{Ca}^{2+}]_c \) when added to Fura2-loaded macrophages (Fig. 5B and data not shown). Parallel control experiments conducted with \( M. \) tuberculosis that had been incubated with isotype-matched irrelevant mAb (mouse myeloma IgG1) did not result in stimulation of MDM \( \text{Ca}^{2+} \)
Elevation of Macrophage Cytosolic Ca\textsuperscript{2+} Signaling in Tuberculosis

Defective Macrophage Ca\textsuperscript{2+} Signaling contributes to the intracellular survival of \textit{M. tuberculosis}, we compared the viability of C- and Ab-op \textit{M. tuberculosis} at 37°C for 1 h. The level of phagocytosis did not differ between these two groups (data not shown). 1 h after infection, monolayers were washed and replenished with fresh medium. Detergent lysates were prepared from infected MDMs (monolayers plus supernatants) 1, 48, and 96 h after infection, and viability of intracellular \textit{M. tuberculosis} was determined by analysis of growth on 7H11 plates, as described in Materials and Methods. For each time point, CFUs derived from monolayers infected with Ab-op \textit{M. tuberculosis} were shown as a percentage of the growth observed in the same donors' MDMs infected with C-op \textit{M. tuberculosis}. Results are mean (± range) from six experiments, and cells from each donor are represented by a different symbol. The difference in viability between Ab- and C-op \textit{M. tuberculosis} was statistically significant at all time points tested: P < 0.01 at 1 h and P < 0.001 at 48 and 96 h.

Figure 5. Ab-op, live \textit{M. tuberculosis} stimulates an increase in macrophage cytosolic Ca\textsuperscript{2+} and exhibits decreased survival compared with C-op bacilli. (A) Erdman \textit{M. tuberculosis} was opsonized with 5 μg/ml of rabbit polyclonal Ab to LAM for 30 min at 37°C (Ab-MTB). After extensive washing of the bacilli to remove unbound Ab, Fura2-loaded MDMs were infected at an MOI of 10:1. Single-cell determinations of macrophage [Ca\textsuperscript{2+}]\textsubscript{i} levels were calculated from the fluorescence of Fura2. In parallel experiments with MDMs from the same donors, opsonization of live \textit{M. tuberculosis} with either autologous serum or control, irrelevant Ab (rabbit polyclonal Ab to myeloperoxidase) did not result in any change in macrophage [Ca\textsuperscript{2+}]\textsubscript{i} (data not shown). (B) Similar analyses of macrophage [Ca\textsuperscript{2+}] showed that differentiation of CR-dependent Ca\textsuperscript{2+} signal transduction contributes to the intracellular survival of \textit{M. tuberculosis}, we compared the viability of C- and Ab-op \textit{M. tuberculosis} at 37°C for 1 h. The level of phagocytosis did not differ between these two groups (data not shown). 1 h after infection, monolayers were washed and replenished with fresh medium. Detergent lysates were prepared from infected MDMs (monolayers plus supernatants) 1, 48, and 96 h after infection, and viability of intracellular \textit{M. tuberculosis} was determined by analysis of growth on 7H11 plates, as described in Materials and Methods. For each time point, CFUs derived from monolayers infected with Ab-op \textit{M. tuberculosis} were shown as a percentage of the growth observed in the same donors' MDMs infected with C-op \textit{M. tuberculosis}. Results are mean (± range) from six experiments, and cells from each donor are represented by a different symbol. The difference in viability between Ab- and C-op \textit{M. tuberculosis} was statistically significant at all time points tested: P < 0.01 at 1 h and P < 0.001 at 48 and 96 h.

Figure 5. Ab-op, live \textit{M. tuberculosis} stimulates an increase in macrophage cytosolic Ca\textsuperscript{2+} and exhibits decreased survival compared with C-op bacilli. (A) Erdman \textit{M. tuberculosis} was opsonized with 5 μg/ml of rabbit polyclonal Ab to LAM for 30 min at 37°C (Ab-MTB). After extensive washing of the bacilli to remove unbound Ab, Fura2-loaded MDMs were infected at an MOI of 10:1. Single-cell determinations of macrophage [Ca\textsuperscript{2+}]\textsubscript{i} levels were calculated from the fluorescence of Fura2. In parallel experiments with MDMs from the same donors, opsonization of live \textit{M. tuberculosis} with either autologous serum or control, irrelevant Ab (rabbit polyclonal Ab to myeloperoxidase) did not result in any change in macrophage [Ca\textsuperscript{2+}]\textsubscript{i} (data not shown). (B) Similar analyses of macrophage [Ca\textsuperscript{2+}] showed that differentiation of CR-dependent Ca\textsuperscript{2+} signal transduction contributes to the intracellular survival of \textit{M. tuberculosis}, we compared the viability of C- and Ab-op \textit{M. tuberculosis} at 37°C for 1 h. The level of phagocytosis did not differ between these two groups (data not shown). 1 h after infection, monolayers were washed and replenished with fresh medium. Detergent lysates were prepared from infected MDMs (monolayers plus supernatants) 1, 48, and 96 h after infection, and viability of intracellular \textit{M. tuberculosis} was determined by analysis of growth on 7H11 plates, as described in Materials and Methods. For each time point, CFUs derived from monolayers infected with Ab-op \textit{M. tuberculosis} were shown as a percentage of the growth observed in the same donors' MDMs infected with C-op \textit{M. tuberculosis}. Results are mean (± range) from six experiments, and cells from each donor are represented by a different symbol. The difference in viability between Ab- and C-op \textit{M. tuberculosis} was statistically significant at all time points tested: P < 0.01 at 1 h and P < 0.001 at 48 and 96 h.

Takeda and coworkers, these results demonstrated that both the viability of \textit{M. tuberculosis} and the receptors that mediate its phagocytic signaling were significant determinants of macrophage Ca\textsuperscript{2+}-mediated signal transduction. Ingestion of live \textit{M. tuberculosis} via CRs was not accompanied by detectable changes in levels of macrophage [Ca\textsuperscript{2+}]\textsubscript{i} when phagocytosis of either dead bacilli via CRs or live, Ab-op \textit{M. tuberculosis} by FcγR was associated with significant and prolonged increases in [Ca\textsuperscript{2+}]\textsubscript{i}.

Elevation of Macrophage Cytosolic Ca\textsuperscript{2+} by Ab-op \textit{M. tuberculosis} is associated with decreased intracellular survival. To evaluate the hypothesis that mycobacteria-induced inhibition of CR-dependent Ca\textsuperscript{2+} signal transduction contributes to the intracellular survival of \textit{M. tuberculosis}, we compared the viability of C- and Ab-op \textit{M. tuberculosis} at 37°C for 1 h. The level of phagocytosis did not differ between these two groups (data not shown). 1 h after infection, monolayers were washed and replenished with fresh medium. Detergent lysates were prepared from infected MDMs (monolayers plus supernatants) 1, 48, and 96 h after infection, and viability of intracellular \textit{M. tuberculosis} was determined by analysis of growth on 7H11 plates, as described in Materials and Methods. For each time point, CFUs derived from monolayers infected with Ab-op \textit{M. tuberculosis} were shown as a percentage of the growth observed in the same donors' MDMs infected with C-op \textit{M. tuberculosis}. Results are mean (± range) from six experiments, and cells from each donor are represented by a different symbol. The difference in viability between Ab- and C-op \textit{M. tuberculosis} was statistically significant at all time points tested: P < 0.01 at 1 h and P < 0.001 at 48 and 96 h.
Erdman M. tuberculosis was opsonized with autologous human serum (C-op) or 5 μg/ml of rabbit polyclonal Ab to LAM (Ab-op). After extensive washing of the bacilli, MDMs were infected at an MOI of 1:1. 1 h after infection, monolayers were washed and repleted with fresh media. Detergent lysates were prepared from infected MDMs (monolayers plus supernatants) 1, 48, and 96 h after infection, and viability of intracellular M. tuberculosis was analyzed by determination of CFUs on 7H11 plates as described in Materials and Methods.

Donor | CFUs 1 h | CFUs 48 h | CFUs 96 h
--- | --- | --- | ---
1 | 36,000 | 19,500 | 406,500 | 73,500 | 531,750 | 192,750
2 | 4,125 | 2,250 | 19,500 | 9,375 | 203,625 | 33,750
3 | 13,688 | 8,250 | 403,875 | 68,625 | ND | ND
4 | 10,125 | 6,938 | 123,000 | 45,375 | 840,375 | 181,500
5 | 7,500 | 4,313 | 187,125 | 25,500 | 841,125 | 182,625
6 | 43,688 | 18,188 | 383,250 | 80,625 | 784,875 | 110,625

Table I. Opsonization of M. tuberculosis with Polyclonal Abs to LAM Results in Decreased Survival within Human M. tuberculosis.

Donor | CFUs 1 h | CFUs 48 h | CFUs 96 h
--- | --- | --- | ---
1 | 36,000 | 19,500 | 406,500 | 73,500 | 531,750 | 192,750
2 | 4,125 | 2,250 | 19,500 | 9,375 | 203,625 | 33,750
3 | 13,688 | 8,250 | 403,875 | 68,625 | ND | ND
4 | 10,125 | 6,938 | 123,000 | 45,375 | 840,375 | 181,500
5 | 7,500 | 4,313 | 187,125 | 25,500 | 841,125 | 182,625
6 | 43,688 | 18,188 | 383,250 | 80,625 | 784,875 | 110,625

Table II. Survival of C-op M. tuberculosis in Macrophages Following Incubation with A23187.

| Donor | CFUs 1 h | CFUs 48 h | CFUs 96 h |
--- | --- | --- | ---
1 | 36,000 | 19,500 | 406,500 | 73,500 | 531,750 | 192,750
2 | 4,125 | 2,250 | 19,500 | 9,375 | 203,625 | 33,750
3 | 13,688 | 8,250 | 403,875 | 68,625 | ND | ND
4 | 10,125 | 6,938 | 123,000 | 45,375 | 840,375 | 181,500
5 | 7,500 | 4,313 | 187,125 | 25,500 | 841,125 | 182,625
6 | 43,688 | 18,188 | 383,250 | 80,625 | 784,875 | 110,625

Elevation of M macrophage [Ca2+]c correlates with A23187-induced elevation of macrophage [Ca2+]c, which results in rapid equilibration of the intracellular and extracellular Ca2+ concentrations (Fig. 6 A). To mimic the temporally restricted elevation of [Ca2+]c, initiated by the particular stimuli noted above, the effects of A23187 were reversed after 20 min by addition of phosphatidylcholine vesicles (20 μg/ml) (28), which resulted in a rapid return of [Ca2+]c to a level approximating that of resting macrophages (Fig. 6 A). To examine the effects of cytosolic Ca2+ levels on survival of intracellular M. tuberculosis, parallel sets of infected MDM monolayers were lysed and viable mycobacteria quantitated 24 and 48 h after infection by analysis of CFU s C-op and Ab-op respectively (Fig. 6 B and Table II). These results were not due to a direct bactericidal effect of A23187, as incubation of M. tuberculosis suspensions in the calcium ionophore, followed by addition of phosphatidylcholine vesicles under the exact conditions applied to infected macrophages did not result in alteration of mycobacterial viability (data not shown). These results indicated that ionophore-induced elevation of macrophage [Ca2+]c, during phagocytosis of C-op M. tuberculosis was associated with decreased intracellular survival of the bacilli.

Elevations of M macrophage Ca2+ occur with maturation of phagosomes to acidic pH conditions. A key aspect of tuberculous pathogenesis is the ability of M. tuberculosis to limit the maturation of its phagosomelysosome complex, thereby preventing the development of microbicidal phagosomelysosomes (10–12, 23, 24, 54, 55). We tested the hypothesis that mycobacterial inhibition of macrophage Ca2+ signaling contributes to retardation of phagosomal maturation (inhibition of phagosome-lysosome [P-L] fusion) by (a) characterizing the degree of maturation of phagosomes containing either live or killed C-op M. tuberculosis and (b) determining the effects of modulation of [Ca2+]c on P-L fusion. The extent of maturation of M. tuberculosis-containing phagosomes 24 h after infection was characterized by confocal microscopy, using three lysosomal protein markers (cathepsin D, LAMP-1, CD63), combined with the determination of phagosomal pH with the acidophilic fluorophore, LysoTracker Red. The three protein markers were used in combination, because use of a single marker can provide ambiguous results. For example, LAMP-1 localizes to both late endosomes and lysosomes (24). LysoTracker Red was employed for assessment of phagosomal acidification, as this fluorophore is stable to fixation, ensuring that biosafety conditions are maintained during confocal microscopy.

24 h after infection of human MDMs with C-op M. tuberculosis, phagosomes were observed in mature and lysosomes that exhibited low amounts of the lysosomal protein markers. The percentage of phagosomes positive for cathepsin D, LAMP-1, and CD63 were 32, 37, and 25%, respectively (Fig. 7). Additionally, only 41% of phagosomes containing live M. tuberculosis colocalized with LysoTracker Red. These results are indicative of the extent of phagosomal maturation.
in agreement with previous characterizations of the maturational state of M. tuberculosis-containing phagosomes in macrophages, as determined by epifluorescence, confocal immunofluorescence, and cryoimmunoelectron microscopy (10–12, 23, 24, 54, 55). To further evaluate the potential causal role of macrophage cytosolic Ca\(^{2+}\) in P–L fusion, the maturation of phagosomes containing live, C-op M. tuberculosis was determined after transient elevation of [Ca\(^{2+}\)], with A23187, followed by quenching with phosphatidylcholine vesicles. Ionophore-induced elevation of [Ca\(^{2+}\)] to ~500 nM for 20 min during phagocytosis of live, C-op M. tuberculosis resulted in a striking reversal of the block in phagosomal maturation. The percentage of phagosomes positive for cathepsin D increased from 32 (control) to 92%, LAMP-1 positivity increased from 37 to 82%, and CD63 positivity increased from 25 to 83% (Fig. 7). Elevation of [Ca\(^{2+}\)], also promoted increased phagosomal localization of LysoTracker Red, from a control value of 41 to 89% in MDMs treated with A23187. Elevation of [Ca\(^{2+}\)] was required for the A23187-induced increase in P–L fusion, as incubation of macrophages in EHBSS during ionophore treatment resulted in a profile of phagosomal staining for the lysosomal protein markers and LysoTracker Red that was indistinguishable from values for control, untreated MDMs (Fig. 7).

In marked contrast to the intracellular compartmentation of live tubercle bacilli, phagosomes containing dead (gamma-irradiated) M. tuberculosis progressed to fully mature phagolysosomes, as determined by high levels of all three lysosomal protein markers (Fig. 8). 88% of phagosomes containing killed M. tuberculosis were positive for cathepsin D, whereas the corresponding values for LAMP-1 and CD63 were 77 and 76%, respectively. 88% of these phagosomes accumulated LysoTracker Red, consistent with their acidification. Incubation of macrophages in EHBSS or chelation of cytosolic Ca\(^{2+}\) with MAPTAM resulted in failure of phagosomes containing dead M. tuberculosis to accumulate lysosomal protein markers (Fig. 8). Compared with the percentage of phagosomes positive for cathepsin D, LAMP-1, and CD63 in Ca\(^{2+}\)-containing media noted above, removal of extracellular Ca\(^{2+}\) resulted in significantly less colocalization with all three lysosomal protein markers 66, 30, and 47%, respectively. Chelation of intracellular Ca\(^{2+}\) with 12.5 μM MAPTAM resulted in even more pronounced reductions in phagosomal accumulation of lysosomal markers cathepsin D, 37%; LAMP-1, 24%; and...
CD63, 38%. As MAPTAM produces more significant reductions in basal and stimulated \([\text{Ca}^{2+}]_c\) compared with EGTA (Fig. 4), these results are fully consistent with the hypothesis that \([\text{Ca}^{2+}]_c\) regulates the maturation of phagosomes containing dead \(M.\) tuberculosis. Interestingly, MAPTAM but not EGTA produced significant decreases in accumulation of LysoTracker Red: untreated control, 88%; MAPTAM, 49%; and EGTA, 86% (Fig. 8). As removal of extracellular \(\text{Ca}^{2+}\) reduces but does not eliminate the increase in \([\text{Ca}^{2+}]_c\) induced by killed \(M.\) tuberculosis, these results are consistent with the hypothesis that a lesser increase in \([\text{Ca}^{2+}]_c\) is required for phagosomal acidification than for accumulation of lysosomal protein markers (especially LAMP-1 and CD63).

In summary, the results of characterization of phagosome maturation via confocal microscopy strongly support the hypothesis that levels of cytosolic \(\text{Ca}^{2+}\) regulate P–L fusion in \(M.\) tuberculosis-infected human macrophages. In all cases, elevation of macrophage \([\text{Ca}^{2+}]_c\) correlated with maturation of \(M.\) tuberculosis-containing phagosomes to phagolysosomes, and lack of elevation of \([\text{Ca}^{2+}]_c\) correlated with incomplete phagosomal maturation. Furthermore, ionophore-induced increases in \([\text{Ca}^{2+}]_c\) and the accompanying maturation of phagosomes containing live C-op \(M.\) tuberculosis correlated with decreased survival of mycobacteria within human macrophages.

**Discussion**

Macrophages possess multiple microbicidal mechanisms to eliminate phagocyted microorganisms and, consequently, represent a strategic target for inactivation by potential pathogens (56). The molecular mechanisms that allow \(M.\) tuberculosis to successfully survive and replicate within mononuclear phagocytes are unknown. Our overall hypothesis is that \(\text{Ca}^{2+}\)-dependent signaling mechanisms are potential targets for inhibition of macrophage activation by \(M.\) tuberculosis, as \([\text{Ca}^{2+}]_c\) is a critical regulator of several antimicrobial responses, including generation of reactive oxygen and nitrogen intermediates, secretion of microbicidal proteins and peptides, and synthesis of antimycobacterial cytokines, such as TNF-\(\alpha\) (13, 14, 57).
This study demonstrates that multiple strains of pathogenic M. tuberculosis inhibit Ca$^{2+}$-mediated signal transduction during infection of human macrophages. Inhibition of macrophage Ca$^{2+}$ signaling is tightly coupled to the failure of mycobacterial phagosomes to mature into acidic, microbicidal phagolysosomes and to successful intracellular survival of M. tuberculosis. Two determinants of mycobacteria-induced inhibition of macrophage Ca$^{2+}$ signaling have been defined. First, the bacilli must be viable, as killing of M. tuberculosis by heat or gamma irradiation reverses the inhibition of Ca$^{2+}$-mediated signal transduction. Although the basis of this requirement is unknown, the dependence on mycobacterial viability has previously been demonstrated for the inhibition of P–L fusion in M. tuberculosis-infected human macrophages (12, 23, 24). Second, infection of macrophages in the absence of increased [Ca$^{2+}$]c is specific for phagocytosis. Redirecting the phagocytosis of M. tuberculosis to FcγRs via opsonization with specific polyclonal or monoclonal Abs, reverses mycobacteria-induced impairment of macrophage Ca$^{2+}$ signaling, and, more importantly, reduces the intracellular survival of M. tuberculosis within human MDMs. The reduction in the intracellular survival of Ab-op bacilli was not due to a difference in phagocytosis, as both the phagocytic index (the number of bacilli ingested per macrophage) and the percentage of MDMs that phagocytosed at least one bacillus did not differ between the two groups.

Although mechanisms other than induction of elevated [Ca$^{2+}$]c may contribute to the decreased viability of Ab-op bacilli, direct evidence for a causal role of [Ca$^{2+}$]c in regulating the survival of M. tuberculosis within human macrophages was obtained with the calcium ionophore, A23187. Thus, in this in vitro model of primary infection of human macrophages, the lack of an increase in [Ca$^{2+}$]c during infection of M. tuberculosis (H37Rv strain) for 20 min. MDMs were incubated in CHBSS (control, left column), Ca$^{2+}$-free HBSS containing 3 mM EGTA (center column), or 12.5 μM MAPTAM (right column) before infection with C-op, gamma-irradiated M. tuberculosis for 20 min. MDMs were washed and repleted with RH, 1% autologous serum and incubated at 37°C. Samples were stained with mAb to cathepsin D (top row, red) and auramine to detect M. tuberculosis (center row, green). Acquisition of both fluorescence emission maxima (bottom row) demonstrates colocalization of cathepsin D with killed M. tuberculosis in MDMs incubated in Ca$^{2+}$-containing buffer (yellow, left column), which is significantly reduced in EGTA (center column) or MAPTAM (right column). Crossover fluorescence did not contribute to the colocalization of signals, as cells labeled with either anti-cathepsin D Ab or auramine alone did not emit a detectable fluorescence signal when observed through the narrow bandpass filter appropriate for the other fluorochrome. (B) Summary of the percentage of phagosomes containing C-op, gamma-irradiated M. tuberculosis that colocalize with cathepsin D, LAMP-1, CD63, or LysoTracker Red in each of the three buffer conditions. Each value is the mean percentage (± SEM) from at least three experiments.
Immunoelectron microscopy of M. tuberculosis-containing phagosomes supports the hypothesis that the bacilli’s protected "intracellular niche" is established at a relatively early time point during infection of macrophages (24, 54). The large number of Ca\(^{2+}\)-dependent biochemical reactions and cellular functions suggests that M. tuberculosis-induced inhibition of changes in [Ca\(^{2+}\)]\(_{\text{e}}\) may compromise several components of macrophage activation and antimicrobial function.

The use of the term "inhibition" to characterize the lack of increase in [Ca\(^{2+}\)]\(_{\text{e}}\) during phagocytosis of M. tuberculosis is meant in an operational sense, as the mechanism remains unknown. Lack of initiation of a Ca\(^{2+}\) signaling pathway or its rapid termination could both yield the observed results. As CRs, especially CR 3, are the primary mediators of phagocytosis of M. tuberculosis in human MDMs (5) and because other C3b/bi-opsonized particles, including dead M. tuberculosis, stimulate a rise in [Ca\(^{2+}\)]\(_{\text{e}}\), our working model is best summarized by the question, How does live M. tuberculosis inhibit CR-mediated increases in [Ca\(^{2+}\)]\(_{\text{e}}\)? Evidence in favor of this model, particularly the comparison between live and dead C-op M. tuberculosis as a means to understand the pathogenesis of tuberculosis, include: (a) live and dead C-op M. tuberculosis are phagocytosed to the same extent by human MDMs (5), (b) the extent of phagocytosis of live and dead M. tuberculosis is inhibited to the same extent by anti-CR 3 Abs (5), (c) the level of C3 deposition does not differ between live and heat-killed bacilli (data not shown), and (d) anti-CD18 F(ab\(^{-}\))\(_{2}\) fragments eliminate the increase in [Ca\(^{2+}\)]\(_{\text{e}}\) stimulated by dead M. tuberculosis or COZ. This model encompasses the possibility that additional interactions between live M. tuberculosis and human MDMs other than ligation of mycobacterial surface-bound C3b/bi by CRs may contribute to the inhibition of Ca\(^{2+}\) signaling.

As the fluorescent detection of [Ca\(^{2+}\)]\(_{\text{e}}\) is highly sensitive, our hypothesis is that no Ca\(^{2+}\) signal is initiated during phagocytosis of live M. tuberculosis. However, the biochemical signals that normally link CRs to increases in [Ca\(^{2+}\)]\(_{\text{e}}\) are unknown, and, therefore, we cannot ascertain whether these intermediate steps are "not initiated" or "initiated but inhibited." These mechanistic uncertainties are an additional reason that we have used the more general phrase, "inhibition of Ca\(^{2+}\) signaling." However, we recognize that further definition of the mechanism(s) by which CR-induced phagocytosis of C-op M. tuberculosis occurs in the absence of a change in macrophage [Ca\(^{2+}\)]\(_{\text{e}}\), may necessitate a revision of our current model and terminology.

Comparison of our results with those recently reported by Majeed et al. (19) illustrates both the similarities and differences in the interactions of M. tuberculosis with mononuclear phagocytes versus neutrophils (PMNs). Although neutrophil ingestion of the attenuated H37Rv a stain of M. tuberculosis also occurred in the absence of a rise in [Ca\(^{2+}\)]\(_{\text{e}}\), PMNs killed 73% of phagocytosed tubercle bacilli in 2 h (19). Whether induction of a rise in Ca\(^{2+}\) via physiologic or pharmacologic intervention would augment PMN P–L fusion or bactericidal activity toward M. tuberculosis was not reported, and no virulent strains of M. tuberculosis were used (19). Furthermore, M. tuberculosis does not successfully par-
respect to the consequences for mycobacterial survival. In this study, opsonization of *M. tuberculosis* with specific monoclonal or polyclonal Abs resulted in significant decreases in intracellular viability within human macrophages. In contrast, Armstrong and Hart demonstrated that survival within murine macrophages was similar for serum- and Ab-op M. *tuberculosis* (11). As numerous investigators have documented significant differences between the tubercu-lolidal capacities of human versus murine macrophages (for review see references 3, 6, 46–48), we hypothesize that this species specificity is a major factor contributing to the contrasting effects of Ab opsonization on mycobacterial survival noted in our two studies. Zimmerli et al. recently demonstrated that Ab-mediated inhibition of individual CRs or the mannose receptor did not alter the intracellular survival of *M. tuberculosis* within human MDMs (58). This study differs from ours in two respects. First, the effect of FcγR-mediated phagocytosis on mycobacterial survival was not determined, and second, receptor-blocking re-agents were used to direct phagocytosis of M. *tuberculosis* to unblocked receptor (58). However, blocking reagents may introduce confounding effects by stimulating the receptors to which they bind, and it is often difficult to block multiple receptor classes ligated by complex particles, such as M. *tuberculosis*. In contrast, opsonization of M. *tuberculosis* with specific ligands provides a direct, physiologically rele-vant analysis of the impact of individual receptor classes on the intracellular survival of M. *tuberculosis*.

The lack of Ca²⁺ mobilization during ingestion of M. *tuberculosis* may represent an important mechanism of immune evasion that contributes to its survival within human macrophages. As the specific mechanism(s) by which human macrophages kill intracellular M. *tuberculosis* is unknown, it is difficult at present to define the means by which inhibition of Ca²⁺ signaling promotes mycobacterial survival, although inhibition of P–L fusion is likely to contribute. Despite these challenges, characterization of the molecular mechanisms responsible for M. *tuberculosis*-induced alterations in macrophage Ca²⁺ signaling and its specific contribution to intra-cellular survival will provide important insights into the pathogenesis of tuberculosis and may contribute to the development of novel therapies to treat this formidable disease.

We thank our colleagues in the Inflammation Program at the University of Iowa, particularly William M. Nauseef, Jerrold P. Weiss, and Lee Ann Allen, for their many helpful discussions and thoughtful critiques. We are especially grateful to Lee Ann Allen for her guidance with confocal microscopy. We also thank Larry S. Schlesinger and Thomas Kaufman for assistance with the determination of C3 deposition on M. *tuberculosis*, Patrick J. Brennan and John T. Belisle for their generous provision of several mycobacterial reagents, and William M. Nauseef for his kind gift of polyclonal Ab to myeloperoxidases.

Support for these studies was provided by a Veterans Affairs (VA) Merit Review Grant, VA Career Develop-ment Award, and National Institutes of Health grant (N I H) AI18571:17 to D.J. Kusner, a VA Merit Review Grant to G. Denning, and an institutional NIH National Research Service Award to the Medical Scientist Training Program at the University of Iowa to Z.A. Malik.

Submitted: 19 July 1999
Revised: 17 September 1999
Accepted: 21 September 1999

References

1. Raviglione, M.C., D.E. Snider, Jr., and A. Kochi. 1995. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA* (J. Am. M. Ed. Associ.). 273:220–226.

2. Fenton, M.J., and M.W. Vermeulen. 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. *Inf. Im mun.* 64:683–690.

3. Schlesinger, L.S. 1996. Entry of M. *yobacterium tuberculosis* into mononuclear phagocytes. *Curr. Topics Microbiol. Immunol.* 215:71–96.

4. Hirsch, C.S., J.J. Ellner, D.G. Russell, and E.A. Rich. 1994. Complement receptor-mediated uptake and tumor necrosis factor-alpha-mediated growth inhibition of M. *yobacterium tuberculosis* by human alveolar macrophages. *J. Immunol.* 152:743–753.

5. Schlesinger, L.S., C.G. Bellinger-Kawahara, N.R. Payne, and M.A. Horwitz. 1990. Phagocytosis of M. *yobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J. Im munol.* 144:2771–2780.

6. Emn, J. 1998. M. acrophage receptors for M. *yobacterium tuberculosis*. *Inf. Immun.* 66:1227–1281.

7. Schlesinger, L.S. 1993. M. acrophage phagocytosis of virulent but not attenuated strains of M. *yobacterium tuberculosis* is mediated by mannose receptors in addition to complement recep-tors. *J. Immunol.* 150:2920–2930.

8. Yamamoto, K., and R.B. Johnston, Jr. 1984. Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. *J. Exp. Med.* 159:405–416.

9. Wight, S.D., and S.C. Silverstein. 1983. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* 158:2016–2023.

10. Armstrong, J., and D. Hart. 1971. R response of cultured macrophages to M. *yobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J. Exp. Med.* 134:713–740.

11. Armstrong, J., and D. Hart. 1975. Phagosome-lysosome in-teractions in cultured macrophages infected with virulent tu-bercle bacilli. *J. Exp. Med.* 142:1–16.

12. Clemens, D.L. 1996. Characterization of the M. *yobacterium tuberculosis* phagosome. *Trends Microbiol.* 4:113–118.

13. Chen, B.C., C.F. Chou, and W.W. Lin. 1998. Pyrimi-dinoceptor-mediated potentiation of inducible nitric-oxide synthase induction in J774 macrophages. *Role of intracellular calcium. J. Biol. Chem.* 273:29754–29763.

14. Watanabe, N., J. Suzuki, and Y. Kobayashi. 1996. Role of calcium in tumor necrosis factor-alphapotiation by ac-tivated macrophages. *J. Biol. Chem.* 120:1190–1195.

15. Tapper, H. 1996. The secretion of preformed granules by macrophages and neutrophils. *J. Leukoc. Biol.* 59:613–622.

16. Denlinger, L.C., P.L. Fisette, K.A. Garis, G. Kwon, A. Vazquez-Torres, A.D. Simon, B. Nguyen, R.A. Proctor, P.J. Bertics, and J.A. Corbett. 1996. R regulation of inducible nitric oxide synthase expression by macrophage purinorecep-tors and calcium. *J. Biol. Chem.* 271:337–342.

17. Kim-Park, W.K., M.A. Moore, Z.W. Hakki, and M.J. Kowalski. 1997. Activation of the neutrophil respiratory burst.
requires both intracellular and extracellular calcium. A nn. N Y A cad. Sd. 832:394–404.

18. Wilson, A., H. Lundqvist, M. Gustafsson, and O. Stendahl. 1996. Killing of phagocytosed Staphylococcus aureus by human neutrophils requires intracellular free calcium. J. Leukoc Biol. 59:902–907.

19. Majeed, M., N. Perskvist, J.D. Ernst, K. Orselius, and O. Stendahl. 1998. Roles of calcium and annexins in phagocytosis and elimination of an attenuated strain of M yobacterium tuberculosis in human neutrophils. Microbiol. Pathog. 24:309–320.

20. A hlow, E., and D. Lane. 1998. R eagents. In Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 628–631.

21. Kusner, D.J., C.F. Hall, and L.S. Schlesinger. 1996. Activation of phospholipase D is tightly coupled to the phagocytosis of M yobacterium tuberculosis or opsonized zymosan by human macrophages. J. Exp. Med. 184:585–595.

22. Nakagawara, A., and C.F. Nathan. 1983. A simple method for counting adherent cells application to cultured human monocytes, macrophages and multinucleated giant cells. J. Immunol. Methods. 56:261–268.

23. Clemens, D.L., and M.A. Horwitz. 1996. The M yobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J. Exp. Med. 184:1349–1355.

24. Clemens, D.L., and M.A. Horwitz. 1995. Characterization of the M yobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J. Exp. Med. 181:257–270.

25. Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new fluorescence indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440–3450.

26. Jaconi, M., D. Lew, J. Carpantier, K. Magnusson, M. Sjogren, M. Zimmerli, S., M. Majeed, M. Gustavsson, O. Stendahl. 1993. Redistribution of intracellular Ca2+ stores during phagocytosis in human neutrophils. Dynamics of intracellular Ca2+ stores. Blood. 85:2194–2201.

27. Helgent, C.L., M. Molony, L. Zheng, and T. Andersson. 1996. Ca2+ signaling mechanisms of the beta 2 integrin on neutrophils involvement of phospholipase gamma 2 and Ins(1,4,5)P3. Blood. 1:370:409–409.

28. Stendahl, O., K.H. Krause, J. Krischer, P. Jerstrom, J.M. Theler, R.A. Clark, J.L. Carpenter, and D.P. Lew. 1994. Redistribution of intracellular Ca2+ stores during phagocytosis in human neutrophils. Science. 265:1439–1441.

29. Fallman, M., R. Andersson, and T. Andersson. 1993. Signaling properties of CR3 and CR1 in relation to phagocytosis of complement-opsonized particles. J. Immunol. 151:330–338.

30. Arnaout, M. 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood. 75:1037–1050.

31. Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2+-ATPase. Proc. Natl. Acad. $d$. USA. 87:2466–2470.

32. Alonso, M.T., F. Mollinedo, I. Barasoin, J. Alvarez, and J. Garcia-Sancho. 1996. Transient inhibition of capacitative calcium entry in human neutrophils by a monoclonal antibody directed against a 19-kDa antigen. J. Leukoc Biol. 60:323–327.

33. Scharff, O., and B. Foder. 1996. Depletion of calcium stores by thapsigargin induces membrane depolarization by calyoxin entry in human neutrophils. Cel C 4. 20:31–41.

34. Newsholme, P.J., A.A. Adogu, M.A. Soos, and C.N. Hales. 1993. Complement-induced Ca2+ influx in cultured fibroblasts is decreased by the calcium-channel antagonist nifedipine or by some bivalent inorganic cations. Blood. 92:773–779.

35. Schlesinger, L.S., and M.A. Horwitz. 1991. Phenolic glycolipid-1 of M yobacterium leprae binds complement component C3 in serum and mediates phagocytosis by human monocytes. J. Exp. Med. 174:1031–1038.

36. Neworth, R.J., and A.A. Izzo. 1993. M ycobacterial virulence. Virulent strains of M yobacterium tuberculosis have faster in vivo doubling times and are better equipped to resist growth-inhibiting functions of macrophages in the presence and absence of specific immunity. J. Exp. Neurol. 177:1723–1733.

37. Schorey, J., M. Carroll, and E. Brown. 1997. A macrophage invasion mechanism of pathogenic mycobacteria. Science. 277:1091–1093.

38. Paul, S., P. Lauchumroonvorapong, and G. Kaplan. 1996. Comparative growth of virulent and avirulent M yobacterium tuberculosis in human macrophages in vitro. J. Infe. Dis. 174:105–112.

39. Silver, R.F., Q. Li, and J.J. Ellner. 1998. Expression of virulence of M yobacterium tuberculosis within human monocytes virulence correlates with intracellular growth and induction of tumor necrosis factor alpha but not with evasion of lymphocyte-dependent monocyte effector functions. Infect. Immun. 66:1190–1199.

40. Zhang, M., J. Gong, Y. Lin, and P.F. Barnes. 1998. Growth of virulent and avirulent M yobacterium tuberculosis strains in human macrophages. Infect. Immun. 66:794–799.

41. O'Bryn, R.C., and J.J. Ellner. 1999. Receptor-activated calcium inflow in animal cells: a variety of pathways tailored to meet different intracellular calcium signalling requirements. Biochem. J. 337:153–169.

42. Berridge, M. 1997. Elementary and global aspects of calcium signalling. J. Physiol. 499:291–306.
51. Clapham, D. 1995. Calcium signaling. Cell. 80:259–268.
52. Bootman, M., M. Berridge, and P. Lipp. 1997. Cooking with calcium: the recipes for composing global signals from elementary events. Cell. 91:367–373.
53. Hanahan, D.J. 1986. Platelet activating factor: a biologically active phosphoglyceride. Annu. Rev. Biochm. 55:483–509.
54. Sturgill-Koszycki, S., P.H. Schlesinger, P. Chakraborty, P.L. Haddix, H.L. Collins, A.K. Fok, R.D. Allen, S.L. Gluck, J. Heuser, and D.G. R ussell. 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 263:678–681.
55. Xu, S., A. Cooper, S. Sturgill-Koszycki, T. van Heyningen, D. Chatterjee, I. Orme, P. Allen, and D.G. R ussell. 1994. Intracellular trafficking in M ycobacterium tuberculosis and M ycobacterium avium-infected macrophages. J. Immunol. 153:2568–2578.
56. Reiner, N.E. 1994. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. Immunol. Today. 15:374–381.
57. Rosales, J., and J. Ernst. 1997. Calcium-dependent neutrophil secretion: characterization and regulation by annexins. J. Immunol. 159:6195–6202.
58. Zimmerli, S., S. Edwards, and J. Ernst. 1996. Selective receptor blockade during phagocytosis does not alter the survival and growth of Mycobacterium tuberculosis in human macrophages. Am. J. Respir. Cell Mol. Biol. 15:760–770.
59. Via, L.E., R.A. Fratti, M., McFalone, E. Pagan-Ramos, D. Deretic, and V. Deretic. 1998. Effect of cytokines on mycobacterial phagosome maturation. J. Cell Sci. 111:897–905.
60. May, M.E., and P.J. Spagnuolo. 1987. Evidence for activation of a respiratory burst in the interaction of human neutrophils with Mycobacterium tuberculosis. Infec. Immun. 55:2304–2307.
61. Jones, G.S., H.J. Amirault, and B.R. Andersen. 1990. Killing of Mycobacterium tuberculosis by neutrophils: a nonoxidative process. J. Infect. Dis. 162:700–704.
62. Brown, A.E., T.J. Holzer, and B.R. Andersen, 1987. Capacity of human neutrophils to kill Mycobacterium tuberculosis. J. Infect. Dis. 156:985–989.
63. Bengtsson, T., M.E. Jaconi, M. Gustafson, K.E. M agnasson, J.M. Theler, D.P. Lew, and O. Stendahl. 1993. Actin dynamics in human neutrophils during adhesion and phagocytosis is controlled by changes in intracellular free calcium. Eur. J. Cell Biol. 62:49–58.