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Localization of intracellular Ca^{2+} stores in HeLa cells during infection with *Chlamydia trachomatis*

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SUMMARY

Chlamydia trachomatis **elementary bodies (EBs) enter epithelial cells within membrane-bound endosomes that aggregate with each other in a calcium-regulated process, but avoid fusion with lysosomes. Annexin III but not I translocates to chlamydial aggregates and inclusions. In this study, we localize the intracellular Ca^{2+} stores during the course of infection by analyzing the distribution of three intracellular Ca^{2+} store proteins: calreticulin, type-1 inositol-1,4,5-trisphosphate receptor (IP3-R), and Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase type 2 (SERCA2) in HeLa cells infected with *C. trachomatis* serovar L2. In uninfected cells, immunofluorescence staining of the proteins showed a fine granular distributed pattern for all three proteins. After infection with *C. trachomatis*, calreticulin was found at the periphery of chlamydial aggregates and inclusions from 3 to 48 hours post-infection. In infected cells, SERCA2 was intimately associated with chlamydial inclusions after 3 and 24 hours, but not after 48 hours. Moreover, IP3-R was translocated to and colocalized with EB aggregates and chlamydial inclusions and had a distribution very similar to that of SERCA 2.**

After 24 hours incubation with chlamydiae, there was a local accumulation of $[Ca^{2+}]_i$ (105±17 nM) in the proximity of chlamydial inclusions, compared to 50±13 nM in other parts of the cell cytoplasm. In the absence of extracellular Ca^{2+}, this local accumulation of Ca^{2+} increased to 295±50 nM after adding 50 μM ATP, and to a similar extent after adding 100 nM thapsigargin (Tg). These data indicate that during infection of HeLa cells with chlamydiae, intracellular Ca^{2+} stores are redistributed, causing local accumulation of Ca^{2+} in the vicinity of chlamydial inclusions. These changes may trigger the association of certain proteins such as annexins with chlamydia-containing vesicles, and thereby regulation of membrane-membrane interaction during endosome aggregation and inclusion formation.

Key words: Intracellular Ca^{2+} store protein, Chlamydia, Thapsigargin, Elementary body, HeLa cell

INTRODUCTION

Chlamydiae are a group of obligate intracellular gram-negative bacteria with a unique biphasic life cycle (Schachter, 1988). The genus *Chlamydia* comprises four species: *C. trachomatis*, *C. psittaci*, *C. pneumoniae* (TWAR), and a recently recognized species which infects animals, *C. pecorum* (Fukushi and Hirai, 1992). The importance of *C. trachomatis* as a human pathogen is well documented. It causes a variety of diseases in both women, such as cervicitis, endometritis and salpingitis, and men, such as urethritis and epididymitis. These diseases are usually successfully treated and cured. However, reinfection or reactivation of a persistent infection may provoke severe and sustained local inflammatory responses that can lead to blindness or infertility.

Infection of target cells is initiated by a nonmetabolic life form, the elementary body (EB), while intracellular multiplication proceeds by the reticulate body (RB), a metabolically active, noninfectious form. The life cycle of chlamydiae starts when EBs attach to the host cell surface. This binding is mediated by both specific (Joseph and Bose, 1991) and non-specific interactions (Su and Caldwell, 1990). Once internalized, EBs remain separated from the cytoplasm in a membrane-bound vesicle where they reorganize into RBs. Fusion of the vesicle with host cell lysosomes is avoided during infection with chlamydiae (Eissenberg and Wyrick, 1981; Eissenberg et al., 1983). Viable chlamydiae are not required to avoid this fusion; indeed, EB envelopes can avoid phagolysosome formation (Eissenberg et al., 1983). In contrast, heat-treated or antibody-coated chlamydiae fuse with lysosomes in macrophages (Wyrick and Brownridge, 1978). After multiple divisions, RBs transform back to new EBs, which are released from the host cell to initiate a new infection.

As obligate intracellular parasites, chlamydiae have successfully adapted to their intracellular environment, probably by utilizing and modifying the host cell regulating machinery for their own benefits. However, the exact mechanisms by which chlamydiae subvert host cell functions are still not well understood.

We have previously studied the early events following chlamydial infection of eucaryotic cells. Upon entry, EBs become enclosed in membrane-bound endosomes and aggregate intracellularly; filamentous actin (F-actin) and clathrin are involved in this process (Majeed and Kihlström, 1991). A physiological concentration of intracellular ionized calcium ($[Ca^{2+}]_i$) is required for the intracellular formation of chlamydial aggregates and inclusions (Majeed et al., 1993), and certain Ca^{2+}-binding proteins, annexins, are selectively translocated to the proximity of intracellular chlamydiae (Majeed et al., 1994).

As intracellular compartments, Ca^{2+} stores are characterized by their high intraluminal Ca^{2+} content and their involvement in the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through rapid Ca^{2+} release and accumulation (Pozzan et al., 1994). While Ca^{2+} release from these stores is mediated by two families of Ca^{2+} release channels: ryanodine receptors and inositol-1,4,5-trisphosphate receptors (IP3-R) (Berridge, 1993), the Ca^{2+} accumulation into stores is controlled by a family of proteins known as Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPases (SERCAs), with different isoforms (Burk et al., 1989; Bobe et al., 1991; Wuytack et al., 1994). All the SERCA isoforms share the property of being selectively inhibited by thapsigargin (Tg), a tumor-promoting sesquiterpene lactone (Thapstrup et al., 1990; Lytton et al., 1991). Calreticulin, a protein that binds Ca^{2+} with high capacity and low affinity, is a multifunctional endoplasmic reticulum (ER) protein that has been shown to be involved in cellular Ca^{2+} storage (Mery et al., 1996; Krause and Michalak, 1997).

In this study we have evaluated whether the Ca^{2+} stores reorganize during endocytosis of chlamydiae, as has been shown during phagocytosis in human neutrophils (Stendahl et al., 1994). Upon infection, calreticulin was redistributed and concentrated around the chlamydial aggregates and inclusions, whereas SERCA2 and IP3-R became intimately associated with chlamydial aggregates and inclusions 24 hours post-infection. At the same time there was a local accumulation of Ca^{2+} to chlamydial inclusions. These results indicate that mobilization of intracellular Ca^{2+} stores may have an important role in both regulating local Ca^{2+} gradients and subsequent binding of certain fusogenic cofactors such as annexins.

MATERIALS AND METHODS

Polyclonal antibodies against calreticulin, IP3-R and SERCA2

Antibodies were raised against recombinant human calreticulin, and against synthetic peptides corresponding to the 20 N-terminal amino acids of the human non-muscle Ca^{2+}-ATPase (SERCA2) and the 15 C-terminal amino acids of the type-1 Ins(1,4,5)P3 receptor (IP3-R), as described previously (Van Delden et al., 1992; Denning et al., 1997). When IP3-R was evaluated, anti-IP3-R antibody was absorbed with purified chlamydiae for 2 hours at 4°C. The suspension was spun down at 14,000 ***g*** at 4°C for 15 minutes using Eppendorf centrifuge 5415C (Hamburg, Germany). The preparation was subjected to immunofluorescence staining and western blot analysis and showed no binding to chlamydiae.

Microorganisms

Chlamydia trachomatis serovar L2 was used. Stock organisms were propagated in HeLa cells, in 75 cm^2 plastic flasks, essentially as described previously (Söderlund and Kihlström, 1982; Majeed and Kihlström, 1991).

Preparation of monolayers

HeLa cells were grown and maintained in minimal essential medium (MEM) from Flow Laboratories (Irvine, Scotland), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1% non-essential amino acids and 10 mg/l of gentamicin. Fresh monolayers were grown to subconfluency on glass coverslips before infection with *C. trachomatis* (Majeed et al., 1993). Cells were tested for mycoplasma contamination periodically by staining with 4-6-diamine-2-phenyl-indole dihydrochloride (Boehringer Mannheim, Germany).

Inoculation with *C. trachomatis*

After washing the monolayers twice in Ca^{2+}-free Krebs-Ringer phosphate buffer containing 120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.7 mM KH_2PO_4, 8.3 mM $Na_2HPO_4.2H_2O$, 10 mM Hepes (Flow) and 10 mM glucose, pH 7.4 (Ca^{2+}-free KRG), HeLa cells were infected with 70 µl of a multiplicity of infection (MOI) of about 300 EBs of *C. trachomatis* serovar L2 per cell in Ca^{2+}-free KRG. This inoculum made the local aggregates of chlamydiae easy to detect and did not cause host cell cytotoxicity, as determined by exclusion of Trypan Blue, as previously described (Majeed and Kihlström, 1991). During the subsequent 2-hour adsorption period at 4°C, the culture tubes were gently shaken 15 times per minute on a rocking device (Rockomat; Tecnomara AG, Zürich, Switzerland) to ensure even dispersion of EBs in the inoculum. After decanting the inoculum, infected cells were washed twice at 4°C with Ca^{2+}-free KRG to remove nonadherent chlamydiae. Then fresh MEM supplemented with 10 mM Hepes (Flow), warmed to 37°C was added. The infected cells were incubated at 37°C in a waterbath for 3 hours. Infected cells were fixed in methanol for 10 minutes and stained for 30 minutes at 37°C with a fluorescein isothiocyanate (FITC)-labeled anti-*C. trachomatis* monoclonal antibody reacting with the major outer membrane protein (MOMP) (Syva Microtrak, San Jose, CA). Coverslips were then washed in phosphate-buffered saline, pH 7.3 (PBS). 100 cells per coverslip were examined for randomly distributed or aggregated *C. trachomatis* organisms at ×1000 magnification in a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Randomly distributed and aggregated chlamydiae were defined as described previously (Majeed and Kihlström, 1991). Uninfected cells were treated and stained identically and showed neither randomly distributed nor aggregated fluorescence. The viability of infected cells was evaluted by exclusion of 0.2% Trypan Blue (Sigma Chem. Co., St Louis, MO, USA) for 5 minutes.

Loading the cells with MAPT/AM and thapsigargin (Tg)

After the 2-hour adsorption period at 4°C, infected HeLa cells were washed twice in Ca^{2+}-free KRG, and then incubated either with Ca^{2+}-containing medium (Gibco, Paisley, Scotland; Cat. No. 041-01380) containing 1 mM $CaCl_2$, 2 mM glutamine, 10 mg/l of gentamicin and 1% non-essential amino acids (Ca^{2+}-containing MEM), or with Ca^{2+}-free MEM containing 12.5 µM MAPT/AM(1,2-bis-5-methyl-amino-phenoxylethane-N,N,n′-tetra-acetoxymethylacetate) (Calbiochem-Behring Corp. AG, La Jolla, CA, USA) for 3 hours at 37°C. In Tg-treated cell preparations, the cells were pretreated with the drug (100 nM) for 30 minutes at 37°C. Then the cells were washed twice in Ca^{2+}-free KRG, infected and treated as in MAPT/AM-treated cells, with Tg present during the 3-hour incubation time at 37°C. In some experiments, infected cells were treated with Tg for 24 hours post-infection.

Immunofluorescence staining of calreticulin, SERCA2, IP3-receptor and chlamydiae

C. trachomatis L2-infected or uninfected HeLa cells were fixed in methanol at −20°C for 30 minutes, washed three times in PBS containing 1% BSA, and incubated with normal swine serum (NSS) (Dakopatts, Copenhagen, Denmark) diluted 1:5 in PBS, pH 7.3, for 30 minutes at room temperature in a moist chamber. Excess NSS was removed and rabbit polyclonal antibody, raised against calreticulin protein and diluted 1:200 in PBS, plus 1% BSA was added to the cells and incubated for 30 minutes at 37°C. The monolayers were rinsed three times in PBS plus 1% BSA for 3 minutes each. Cells were overlaid with

rhodamine-conjugated swine anti-rabbit IgG (Dakopatts) diluted 1:150. After incubation for 30 minutes at 37°C and rinsing, monolayers were overlaid with mouse IgG3 monoclonal antibody to chlamydial MOMP (Washington Research Foundation, Seattle, WA) diluted 1:2,048 and incubated as above. The cells were then washed and FITC-conjugated donkey anti-mouse IgG (heavy plus light chains) (Jackson Immunoresearch, West Grove, PA) diluted 1:60 was added and the cells were incubated as above. Cells were washed three times in PBS plus 1% BSA, once in PBS and once in absolute ethanol for 5 minutes before observation in the fluorescence microscope, while alternating between FITC- and rhodamine-isothiocyanate (RITC) filter combinations with excitation at 450-490 nm and 546 nm, and emission at 520 nm and 590 nm, respectively. For SERCA2 or IP3-R staining, the cells were handled as above, except that rabbit polyclonal antibodies, raised against SERCA2 protein or type 1 IP3-R, diluted 1:100 in PBS plus 1% BSA was added to the cells. Some cell monolayers were also stained separately for calreticulin, IP3-R or SERCA2. Control experiments revealed that anti-MOMP antibody did not react with calreticulin, IP3-R or SERCA2 and nor did anti-calreticulin or anti-SERCA2 antibodies with chlamydiae. Furthermore, conjugated secondary antibodies did not unspecifically react with host cells or chlamydiae. However, the anti-IP3-R antibody reacted with purified chlamydiae. This antibody was therefore absorbed with purified chlamydiae and then used to detect cellular IP3-R. This absorbed preparation did not react with isolated chlamydiae, as revealed by western blot analysis (Fig. 2).

Chlamydial inclusions

Confluent monolayers of HeLa cells were washed twice in PBS, pH 7.3. The cells were infected with 70 µl of a multiplicity of infection (MOI) of about 2 EBs of *C. trachomatis* serovar L2 per cell in Ca^{2+}-free KRG. An inoculum with more than 12 EBs/cell caused host cell cytotoxicity after 12 hours incubation, as determined microscopically. Infected cells were incubated for 1 hour at 4°C on the rocking device. After washing twice in Ca^{2+}-free KRG to remove nonadherent chlamydiae, infected cells were incubated for 24 hours or 48 hours in MEM at 37°C in an atmosphere of 5% CO_2 to permit the development of chlamydial inclusions. Infected cells were fixed and stained for chlamydiae, calreticulin, SERCA2 or IP3-R as described above. Chloramphenicol-treated cells were handled the same way, but the drug (100 mg/l) was added 2-12 hours after infection at 2 hour intervals. Some cell preparations were treated with emetine (0.5 mg/l) added 2 hours after infection.

Confocal microscopy

Fluorescent specimens were observed with a confocal fluorescence imaging system, the Sarastro 2000 microscope (Molecular Dynamics, Sunnyvale, CA) with a ×100 (numerical aperture = 1.4) objective as described previously (Majeed et al., 1994). This technique allows observation of cell structures within a narrow section (about 0.6 µm thick). It allows several colocalization measurements of adjacent structures within a 0.2 µm focal range (Carlsson and Åslund, 1987).

Heat-inactivation of chlamydiae

C. trachomatis serovar L2 was heat-inactivated for 3 minutes at 56°C. This treatment effectively destroys the chamydial infectivity, as determined by inclusion formation.

Measurement of association and proteinase K-resistant fraction of ^{14}C-labelled *C. trachomatis*

Monolayers of HeLa cells in 50 mm diameter plastic culture Petri dishes (Nunc, Roskilde Denmark), were infected essentially as described previously (Söderlund and Kihlström, 1983a). Briefly, cells were washed twice in Ca^{2+}-free KRG and inoculated with 0.6 ml of heat-inactivated ^{14}C-labelled *C. trachomatis* serovar L2 suspended in Ca^{2+}-free KRG. The dishes were incubated for 2 hours at 4°C with gentle rocking. Then the inoculum was decanted, the monolayer washed three times with ice-cold Ca^{2+}-free KRG and incubated with 1% sodium dodecyl sulfate. The lysate was transferred to scintillation vials and the Petri dishes were washed three times in Ca^{2+}-free KRG. The samples were counted in a beta scintillation counter (Söderlund and Kihlström, 1983b).

To determine the extent of proteinase K-resistant (= intracellular chlamydiae) and -sensitive (= extracellular adherent chlamydiae) radioactivity, cells were infected and incubated for 2 hours at 4°C, the inoculum decanted and the monolayer rinsed as above. The cells were then incubated for 3 hours at 37°C in fresh MEM, and then at 4°C with 1 mg/ml proteinase K (Boehringer Mannheim, Germany) for 45 minutes. Then the cells were lysed and the radioactivities counted as above.

Measurements of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ were done essentially as described previously (Majeed et al., 1993). Briefly, an area of infected cells was selected in the micropscope and the mean $[Ca^{2+}]_i$ of that area was measured and calculated. Subconfluent monolayers of HeLa cells (2×10^5 cells/ml), were grown on glass coverslips (42 mm in diameter) (Bachofer Laboratory Equipment, Reutling, Germany) placed in a coverslip holder (Bachofer Laboratory Equipment). The cells were infected as described for chlamydial inclusions. After a 24-hour incubation time at 37°C, the infected cells were washed twice in Ca^{2+}-free KRG and loaded with 2 µM of fura-2/AM (Molecular Probes, Eugene, OR) in Ca^{2+}-containing KRG and incubated for 30 minutes at 37°C. Loaded cells were washed twice in Ca^{2+}-free KRG and 1 ml of fresh Ca^{2+}-free KRG containing 2 mM EGTA and 10 µM Lathanum nitrate hexahydrate ($La(NO_3)_3.6H_2O$ (Sigma) was added. After 20 initial $[Ca^{2+}]_i$ measurements at 2-second intervals, 50 µM ATP (sodium salt) (Sigma) or Tg (100 nM) (Sigma) was added and the measurements were continued for 2 minutes. When heat-inactivated EBs were used, cells were infected and loaded with fura-2/AM during the last 30 minutes of the 1-hour adsorption period at 4°C. Infected cells were washed twice in Ca^{2+}-containing KRG and measurements of $[Ca^{2+}]_i$ in the whole cells were taken during 2 hours with 30-second intervals. To rule out the possibility of fura-2 sequestration, identical measurements were done using 30 µM digitonin (Sigma) and 1% w/v Triton X-100 (Kao et al., 1989).

Protein determination

All protein concentrations were determined by the method of Bradford (1976) using a commericial kit provided by Bio-Rad Labs (Richmond, CA). Standard curves were obtained with a stock solution (1.5 mg/ml) of BSA.

SDS-PAGE and western blotting

Samples of infected or uninfected HeLa cells, or isolated chlamydiae, were subjected to 4-12% SDS gradient polyacrylamide gel electrophoresis (SDS-4-12%PAGE) (Novex, San Diego, CA) under reducing conditions essentially as described by Laemmli (1970). Proteins on SDS-polyacrylamide were electrophoretically transferred to Immobilon P membranes (Millipore) using a Tris-glycine buffer system for 3.5 hours in a transfer apparatus (Pharmacia, LKB, Bromma, Sweden) at a constant current of 200 mA. The membranes were blocked overnight at 4°C in a solution of PBS with 5% BSA and PBS with 5% BSA plus 1% fat-free dried milk for 45 minutes at 37°C. Polyclonal antibody to calreticulin, IP3-R or SERCA2 was added and the membranes were incubated for 2 hours at room temperature. The blots were washed five times in PBS with 1% BSA, 0.05% Tween 20 and 1% fat-free dried milk. The secondary antibody (horseradish peroxidase-goat anti-rabbit immunoglobulin G; Dakopatts), diluted 1:6,000 in PBS with 1% BSA, 0.05% Tween 20 and 1% fat-free dried milk, was added, and the membranes were incubated for 1 hour and washed as described above. Before developing, the membranes were treated with 5% H_2O_2 for 15 minutes at room temperature, a treatment that removes almost all nonspecific binding, as recommended by the manufacturer (Amersham, Cardiff, UK). The membrane were developed essentially as described previously (Majeed et al., 1994).

Statistics

Student's *t*-test was used.

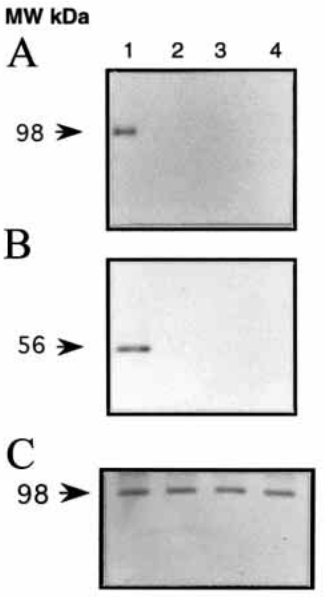

Fig. 1. Western blot analysis of calreticulin and SERCA2 from infected and uninfected HeLa cells using polyclonal antibodies that specifically recognize SERCA2 (98 kDa; A,C) and calreticulin (56 kDa; B). (A,B) Lanes 1, uninfected cells; lanes 2-4 were loaded with purified EBs (2), or purified chlamydiae harvested after 24 hours (3) or after 48 hours (4). (C) Antibody to SERCA2; uninfected cells (lane 1), infected cells incubated for 3 hours at 37°C (lane 2), infected cells incubated for 24 hours or 48 hours at 37°C (lanes 3 and 4, respectively). 5 μg of protein was loaded per well, separated by SDS-PAGE and transferred to Immobilon P membranes. After blocking, the membranes were incubated with rabbit antisera and bound antibodies were subsequently detected using ECL detection system.

RESULTS

Specificity of antibodies used to follow the distribution of three Ca^{2+} regulating proteins

To rule out the possibility that the antibodies we used against Ca^{2+}-regulating proteins also recognized antigens in *C. trachomatis*, SDS-PAGE and western blot analysis were applied. The results showed that calreticulin and SERCA2 were recognized by their respective antibodies and that no reactions were seen with purified chlamydial particles (Fig. 1A,B). Moreover, the total cellular level of SERCA2 was stable in infected cells (Fig. 1C). However, anti-IP3-R reacted with isolated chlamydiae. This antibody preparation was therefore absorbed with purified chlamydiae. After adsorption, the supernatant was tested for its ability to recognize IP3-R in HeLa cells or isolated EBs both by immunofluorescence staining and western blot analysis. The result showed that, after adsorption with chlamydiae, the anti-IP3-R preparation only recognized cellular IP3-R, but not purified chlamydiae (Fig. 2).

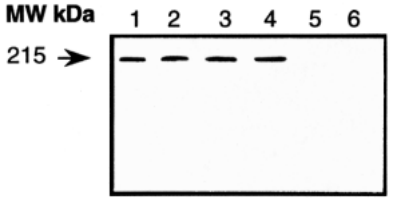

Fig. 2. Western blot analysis of IP3-R from infected and uninfected HeLa cells using antibody absorbed with chlamydiae that specifically recognizes IP3-R (215 kDa). Uninfected cells incubated for 3 hours at 37°C (lane 1). Cells infected with chlamydiae for 3 hours at 37°C (lane 2), or for 24 hours and 48 hours (lanes 3 and 4, respectively). Purified chlamydiae harvested after 24 hours or 48 hours (lanes 5 and 6, respectively).

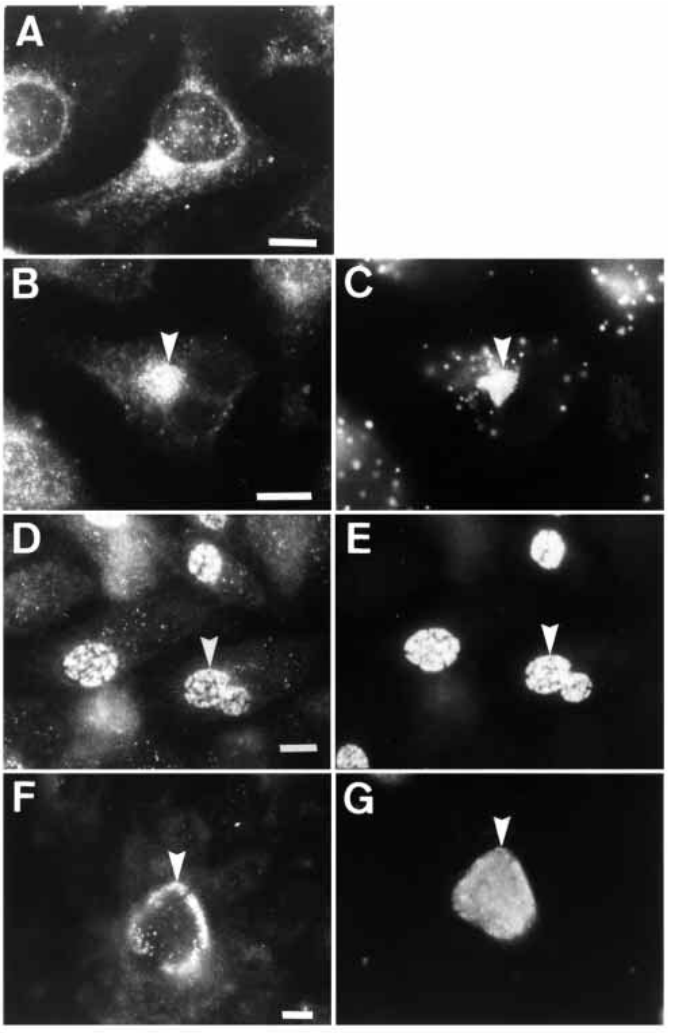

Fig. 3 Fluorescence micrographs of cells stained for *C. trachomatis* and SERCA2. (A) Uninfected cells, incubated for 2 hours at 4°C plus 3 hours at 37°C, stained with antibody to SERCA2. (B,C) HeLa cells infected with *C. trachomatis* at an MOI of 300 EBs per cell, incubated as in A and double fluorescence labelled with antibody to SERCA2 (B) and with a monoclonal antibody to MOMP (Washington Research Foundation) (C). (D,E) HeLa cells infected with *C. trachomatis* at an MOI of 2 EBs per cell and incubated for 1 hour at 4°C plus 24 hours at 37°C in Ca^{2+}-containing MEM, and double fluorescence labelled with antibodies to SERCA2 (D) and MOMP (E). (F,G) Cells incubated for 48 hours and double-stained for SERCA2 (F) and for MOMP (G). Aggregated EBs and localized SERCA2 are indicated by arrowheads. Bars, 10 μm.

Localization of Ca^{2+} regulating proteins around chlamydial aggregates and inclusions

Since intracellular Ca^{2+} is required for intracellular aggregation of EBs, we tested the hypothesis that the aggregation of EBs in infected fibroblasts is linked to mobilization of intracellular Ca^{2+} stores. We utilized polyclonal antibodies raised against the N-terminal sequence of the human non-muscle SERCA2, against the C-terminal part of type 1 IP3-R and against recombinant human calreticulin.

Immunofluorescence (IF) staining of SERCA2 in uninfected HeLa cells revealed a fine granular pattern distributed throughout the cytoplasm (Fig. 3A). When infected cells were incubated for 3-24 hours at 37°C the fluorescence pattern changed. At these time points, SERCA2 and chlamydiae fluorescence patterns were superimposed (Fig. 3B-E). At 48 hours after infection, however, the distribution of SERCA 2 was localized at the periphery of chlamydial inclusions (Fig. 3F,G).

The distribution of calreticulin was similar to that of SERCA2 in uninfected HeLa cells (Fig. 4A). Upon infection with *C. trachomatis*, calreticulin redistributed around the EB aggregates (Fig. 4B,C). After 3 hours incubation at 37°C, 86% of infected cells showed this pattern of EB aggregates and calreticulin. The same redistribution was also seen after 24 hours (Fig. 4D,E) and 48 hours infection (data not shown).

In similar experiments, we analyzed the localization of anti-IP3-R. In uninfected cells the staining of IP3-R showed a fine granular pattern throughout the cell (Fig. 5A). During infection for 3 hours at 37°C, IP3-R translocated and colocalized with aggregated EBs (Fig. 5B,C), similar to the distribution of SERCA 2, but distinct from the distribution of calreticulin. IP3-R did not, however, colocalize to individually distributed EB-containing endosomes (Fig. 5B,C). Similar findings were also observed for SERCA2 (Fig. 3B,C) and calreticulin (Fig. 4B,C). 80% of infected cells showed colocalization of EB aggregates and IP3-R. Colocalization of chlamydial inclusions and IP3-R was also seen after 24 (Fig. 6) and 48 hours (data not shown).

Effect of heat inactivation on HeLa cell association and ingestion of *C. trachomatis*

Heat-inactivated EBs were internalized by HeLa cells and remained in individually distributed endosomes, and did not form a perinuclear aggregate (Fig. 7). With heat-inactivated *C. trachomatis*, the number of cells with chlamydial aggregates was reduced from 53±3% to 3±1%. To investigate if the significant reduction of EB aggregates was due to decreased adherence and/or entry of EBs into the host cells, ^{14}C-labelled *C. trachomatis* was used. Heat inactivation had no major effect on either the association or the ingestion of chlamydiae, since in cells infected with heat-inactivated EBs, 69% of the total cell-associated radioactivity was proteinase K-resistant compared with 71% in cells infected with viable chlamydiae (Table 1).

Redistribution of SERCA2 and IP3-R is attributed to Ca^{2+}-regulating proteins of host origin: effects of emetine and chloramphenicol

The pattern of SERCA2 and IP3-R distributions in HeLa cells infected with chlamydiae raised the question of whether ongoing protein synthesis in chlamydiae and/or host cells is required for redistribution of the Ca^{2+}-regulating proteins. Two protein inhibitors were therefore used: emetine, an inhibitor of the host cell protein synthesis, and chloramphenicol, which blocks protein synthesis of chlamydiae. In chloramphenicol-treated cells no typical chlamydial inclusion was detected; however, neither the translocation of SERCA2 nor of IP3-R

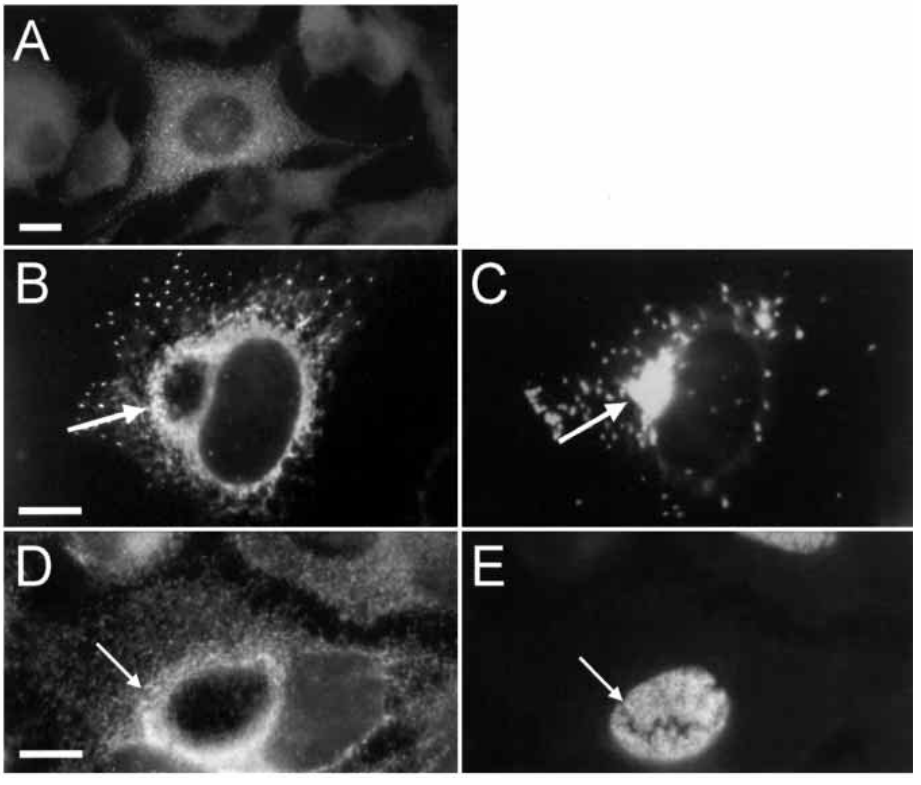

Fig. 4. HeLa cells stained for *C. trachomatis* and calreticulin. (A) Uninfected cells, incubated for 2 hours at 4°C plus 3 hours at 37°C, stained with antibody to calreticulin. (B,C) HeLa cells infected with *C. trachomatis* at an MOI of 300 EBs per cell, incubated as in A and double fluorescence labelled with antibody to calreticulin (B) and with a monoclonal antibody to MOMP (C). (D,E) HeLa cells infected with *C. trachomatis* at an MOI of 2 EBs per cell and incubated for 1 hour at 4°C plus 24 hours at 37°C in Ca^{2+}-containing MEM, and double fluorescence labelled with antibody to calreticulin (D) and MOMP (E). Chlamydiae and concentrated calreticulin are indicated by arrows. Bars, 10 μm.

was affected (data not shown). Similar results were seen when chloramphenicol was added 2-12 hours after addition of chlamydiae. Emetine inhibited neither SERCA2 nor IP3-R translocation. Thus, these observations demonstrate that the immunofluorescence patterns of SERCA2 and IP3-R are independent of protein synthesis, both in chlamydiae and host cells.

Host cell Ca^{2+} modulations

Recently we demonstrated that the $[Ca^{2+}]_i$ is important for the intracellular aggregation of chlamydiae (Majeed et al., 1993). The redistribution of calreticulin was therefore evaluated under reduced $[Ca^{2+}]_i$ conditions using either the Ca^{2+}-chelator MAPT/AM or thapsigargin (Tg), a drug that inhibits all SERCA enzymes (Thapstrup et al., 1990; Lytton et al., 1991). Infected and Tg-treated HeLa cells showed decreased numbers of cells with aggregated EBs and redistributed calreticulin with a concomitant increase of punctate distribution of calreticulin throughout the cytoplasm compared with untreated cells (Figs 4D, 8). In infected HeLa cells incubated for 3 hours in Ca^{2+}-containing MEM, 61±5% of the cells showed EB-aggregates, compared with 33±4% and 18±4% in Tg- and MAPT/AM-treated cells, respectively ($P \leq 0.001$) (Fig. 9). Concomitantly, colocalization between EB-aggregates and calreticulin in individual cells were 85±2% in untreated cells and 58±4% and 72±2% in Tg- or MAPT/AM-treated cells, respectively (Fig. 9).

To further confirm our observations on Ca^{2+} store markers, we analyzed the distribution of the ER-markers by confocal laser scanning microscopy. Confocal serial sections (0.2 μm apart) of EB-containing endosomes and inclusions verified that the fluorescence of SERCA2 (not shown in figure) and IP3-R (Fig. 6 shows one of these sections) superimposed that of EBs, whereas calreticulin was localized at the periphery of chlamydial endosomes and inclusions (not shown in figure).

ATP and Tg induce Ca^{2+} mobilization in infected HeLa cells

The results obtained with IF led us to test the hypothesis that the accumulation of intracellular Ca^{2+} stores provides a localized increase in $[Ca^{2+}]_i$ that is required for development of chlamydial inclusions. It was essential to ensure that our system accurately reflected release of Ca^{2+} from the intracellular stores, and not extracellular Ca^{2+} influx across the cell membrane. Therefore, during the $[Ca^{2+}]_i$ measurements, the infected cells were cultured in Ca^{2+}-free KRG containing 2 mM EGTA and 10 μM $La(NO_3)_3$, a Ca^{2+} channel blocker. Before addition of ATP to mobilize Ca^{2+}, the level of $[Ca^{2+}]_i$ in the proximity of chlamydial inclusion was 105±17 nM; n=7) compared to 50±13 nM in the rest of the cell cytoplasm (Fig. 10). Extracellularly applied ATP (50 μM) induced an immediate and transient rise in $[Ca^{2+}]_i$ in the vicinity of the chlamydial inclusion (295±50 nM) (Fig. 10). ATP also caused a similar rise of $[Ca^{2+}]_i$ (280±45 nM; n=7) throughout the cytoplasm of uninfected cells. Similar experiments were done using 100 nM Tg. The results showed that the gradient of $[Ca^{2+}]_i$ (140±20 nM; n=9; basal level) in the proximity of chlamydial inclusion was elevated to 350±30 nM after addition of Tg (Fig. 11B). In uninfected cells, the mean level of $[Ca^{2+}]_i$ in the cytoplasm was 72±20 nM; n=9; basal level). This level increased to 172±15 nM after addition of Tg (Fig. 11A).

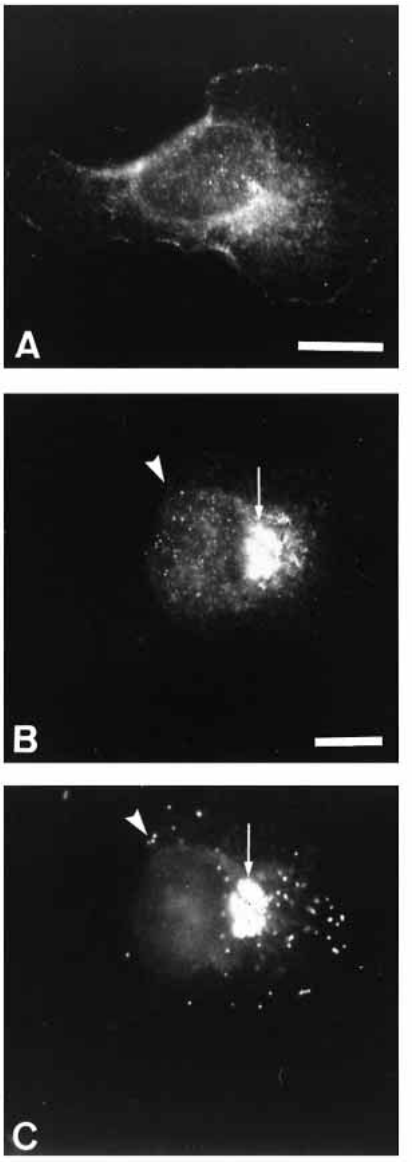

Fig. 5. HeLa cells were incubated for 2 hours at 4°C plus 3 hours at 37°C and stained for *C. trachomatis* and IP3-R. (A) Uninfected cells, cultured in Ca^{2+}-containing MEM, stained with antibody to IP3-R. (B,C) Infected HeLa cells double fluorescence stained for IP3-R (B) and for *C. trachomatis* (C). Individual endosomes are indicated by arrowheads. Arrows indicate the aggregations of IP3-R and EBs. Bars, 10 μm.

Since an elevation of $[Ca^{2+}]_i$ is required for phagolysosome formation in human neutrophils (Jaconi et al., 1990) and since heat-inactivated chlamydiae fuse with lysosomes in macrophages (Wyrick and Brownridge, 1978), we performed $[Ca^{2+}]_i$ measurements in the whole cell cytoplasm using heat-inactivated EBs. Heat-inactivated EBs did not cause any increase in the level of $[Ca^{2+}]_i$ (data not shown), which was similar to metabolically active chlamydiae (Majeed et al., 1993). Monitoring the total fluorescence before and after additions of digitonin and Triton X-100 showed that the majority of cytosolic fura-2 was released after digitonin treatment and a total release was observed after a subsequent

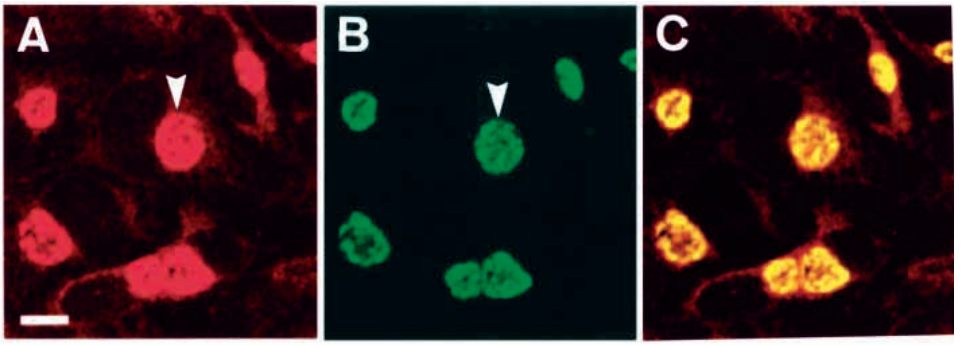

Fig. 6. Confocal images of IP3-R and chlamydial inclusions 24 hours post-infection. Cells labelled with anti-IP3-R antibody (A) and with anti-MOMP antibody (B). (C) Combination of A and B, giving simultaneous visualization of IP3-R and chlamydiae; yellow areas indicate colocalization of the proteins. Arrowheads indicate the localization of IP3-R and chlamydiae. Bar, 10 μm.

addition of 1% Triton X-100. This shows that fura-2 is not sequestered in granule compartments.

DISCUSSION

Activation of cells by different stimuli depends on internal signal systems in which $[Ca^{2+}]_i$ is pivotal to subsequent cell responses (Lew, 1989). In mammalian cells, $[Ca^{2+}]_i$ is maintained at a low level, 50-100 nM, by active processes that move Ca^{2+} ions out of the cell or concentrate them in intracellular storage organelles (Randriamampita et al., 1991; Meldolesi et al., 1990). However, the long-term control of $[Ca^{2+}]_i$ is governed by the equilibrium between influx and efflux of Ca^{2+} ions across the plasma membrane, whereas intracellular Ca^{2+} stores can play a role only as short-term regulators. Moreover, the influx of Ca^{2+} from the extracellular milieu and the initial release of Ca^{2+} from intracellular stores are linked. Stimulation of influx is triggered by depletion of Ca^{2+} from storage organelles (Putney, 1990).

In cells infected with *C. trachomatis*, the aggregation of chlamydia-containing endosomes and inclusion formation are dependent on a physiological concentration of $[Ca^{2+}]_i$. However, there is no significant difference in $[Ca^{2+}]_i$ between infected and uninfected cells (Majeed et al., 1993). These results support those of Tauber et al. (1989), showing that chlamydiae did not induce any $[Ca^{2+}]_i$ changes in neutrophils. Several studies have reported, however, a role for $[Ca^{2+}]_i$ in chlamydial infection. Ca^{2+} facilitated the attachment of *C. psittaci* to L cells (Hatch et al., 1981), and Sneddon and Wenman (1985), showed that the adhesion and internalization of *C. trachomatis* by HeLa cells are significantly reduced when extracellular Ca^{2+} is omitted. However, our recent results have demonstrated that adhesion and uptake of chlamydiae can proceed independently of Ca^{2+} in the growth medium and at low $[Ca^{2+}]_i$ (20≤nM) (Majeed et al., 1993).

The purpose of this study was to test the hypothesis that intracellular Ca^{2+} stores may accumulate at sites of Ca^{2+} action during chlamydial infection, thereby creating a Ca^{2+} gradient. Our findings show that three components of intracellular Ca^{2+} stores are mobilized during the formation of chlamydial aggregates and inclusions. In addition, there is a local ATP- and Tg-dependent $[Ca^{2+}]_i$ increase in the proximity of chlamydial inclusions. This implies that the Ca^{2+} stores accumulate and provide localized changes in $[Ca^{2+}]_i$ that are required for different steps of chlamydial growth. Calreticulin is a Ca^{2+}-binding luminal protein and SERCA2 and IP3-R are membrane-bound proteins that regulate accumulation and release of Ca^{2+} from specific Ca^{2+} stores or ER. Our finding that these proteins distribute differently in chlamydia-infected HeLa cells after 3 hours infection, i.e. calreticulin localizes to the periphery of chlamydial aggregates and inclusions, whereas SERCA2 and IP3-R colocalize with chlamydiae, indicates that the Ca^{2+} stores and/or Ca^{2+} storage portions of ER translocate to the EB aggregates as intact organelles and that SERCA2 and IP3-R subsequently interact and possibly fuse with the EB-containing endosomes. Another possibility is that the EB endosomes are processed in and derived from ER vesicles. The fact that neither SERCA2 or IP3-R colocalizes with individually distributed endosomes argues against this hypothesis. Indeed, Hackstadt et al. (1995, 1996) have recently shown that a lipid component from the Golgi apparatus fuses with the expanding chlamydial inclusion membrane and is already incorporated by intracellular chlamydiae within 4 hours post-infection. No inclusion membrane proteins derived from the plasma membrane or selected internal membrane proteins such as Golgi apparatus or ER have been identified (Taraska et al., 1996). However, these data were collected 18-

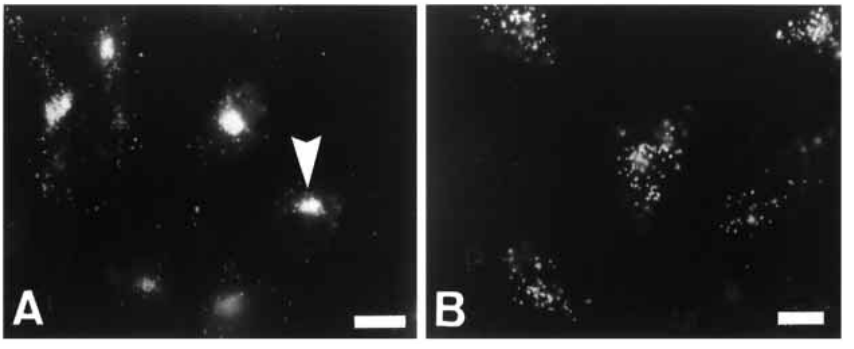

Fig. 7. Fluorescence micrographs of HeLa cells infected with *C. trachomatis* for 2 hours at 4°C plus 3 hours at 37°C. Cells were infected with viable Ebs (A) or with heat-inactivated EBs (B). Arrowhead indicates the aggregation of EBs. Bars, 15 μm.

Table 1. HeLa cell-associated fractions of *C. trachomatis* serovar L2[a]

Treatment of EBs	Cell-associated radioactivity (cpm) after[b]		
	Incubation for 2 hours at 4°C[c]	Incubation for 2 hours at 4°C plus 3 hours at 37°C[c]	Proteinase K treatment after incubation for 2 hours at 4°C plus 3 hours at 37°C[d]
Without heat inactivation	3846±200 (38%)	4359±120 (43%)	3100±88 (71%)
With heat inactivation for 3 minutes at 56°C	2854±114 (28%)	3299±95 (33%)	2268±100 (69%)

[a]Cells were washed twice in Ca^{2+}-free buffer, infected with *C. trachomatis* serovar L2, and incubated as indicated. Each value represents the mean ± s.d. of 8 dishes in 4 experiments.
[b]Cells were infected with 10,000 cpm corresponding to 5×10^7 ifu .
[c]Cpm (% of inculum associated with cells).
[d]Cpm (% proteinase K-resistant radioactivity/total cell-associated radioactivity).

24 hours post-infection and no results from early infectious stages have been previously published.

The observation that a higher amount of SERCA2 is associated with chlamydial inclusions after 24 hours at 37°C, than after 48 hours, suggests that this transient appearance of the SERCA2 may reflect a role for SERCA2 in regulating the timing and specificity of fusion events following entry of chlamydiae into the host cells.

Changes in $[Ca^{2+}]_i$ are essential for modulating a variety of cellular functions and are believed to be, in part, dependent on the generation of Ins(1,4,5)P3 (Berridge, 1993). We have previously shown that the intracellular aggregations of EBs, F-actin and certain annexins are inhibited when the $[Ca^{2+}]_i$ is reduced (20≤nM) (Majeed and Kihlström, 1991; Majeed et al., 1993, 1994). In Ca^{2+}-depleted cells, the redistribution of calreticulin is inhibited with a concomitant inhibition of chlamydial aggregation. Thus, a localized physiological $[Ca^{2+}]_i$ is probably a necessary signal for chlamydial aggregation.

Inhibition of protein synthesis of HeLa cells strongly suggests that de novo synthesis of SERCA2 or IP3-R is not required for their association with EB endosomes. Furthermore, the signal for translocation of the Ca^{2+}-regulating components is not dependent on protein synthesis in chlamydiae. Our observation that the redistibutions of calreticulin and EBs are inhibited under reduced $[Ca^{2+}]_i$ conditions suggests that these two events are linked and probably controlled by the same regulating mechanisms. However, we cannot exclude that small amounts of calreticulin translocate to individual EB-containing endosomes in Ca^{2+}-depleted cells because of the limitation in resolution of our microscopic techniques.

The fact that heat-inactivated chlamydiae do not aggregate suggests that viable EBs are required for modulation of the chlamydia-containing endosomal membranes, thereby inducing subsequent signals necessary for membrane fusion. However, heat-inactivated and antibody-coated chlamydiae fuse with lysosomes in macrophages (Wyrick and Brownridge, 1978). The fact that heat-inactivated EBs do not induce changes in $[Ca^{2+}]_i$ implies that the phagolysosome formation (PLF) in cells infected with heat-inactivated chlamydiae may be a Ca^{2+}-

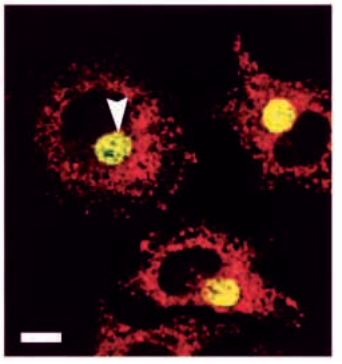

Fig. 8. Dual staining for MOMP and calreticulin of a confocal image showing the punctuate distribution of calreticulin. The cells were infected with chlamydiae treated with thapsigargin (100 nM) for 24 hours at 37 °C. Arrowhead indicates the chlamydial inclusion. Bar, 10 μm.

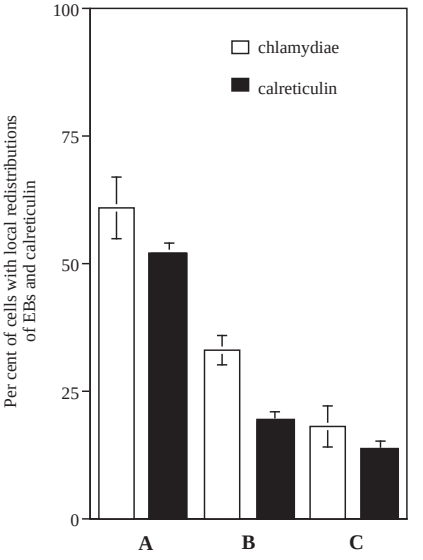

Fig. 9. Effect of thapsigargin, MAPT/AM and Ca^{2+} on the local redistributions of *C. trachomatis* serovar L2 and calreticulin in HeLa cells. Cells were incubated for 2 hours at 4°C with EBs at an MOI of 300 EBs per cell. Cells were reincubated for 3 hours at 37°C either in Ca^{2+}-containing MEM (A) or in Ca^{2+}-free MEM containing 100 nM thapsigargin (B) or in Ca^{2+}-free MEM plus 12.5 μM MAPT/AM (C). Cells were fixed in methanol and stained for EBs and calreticulin by indirect immunofluorescence (see Materials and methods), and the percentages of cells with local redistributions of EBs and calreticulin were determined microscopically. Values represent means ±s.d. of 9 experiments.

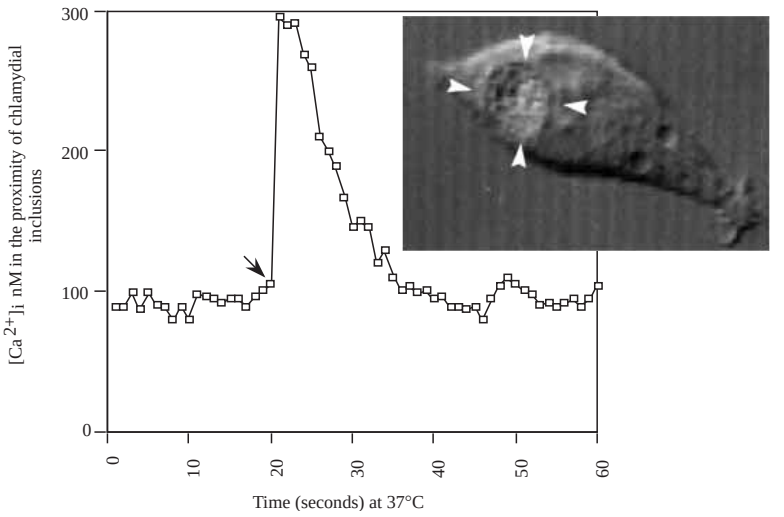

Fig. 10. Changes in $[Ca^{2+}]_i$ of infected HeLa cells after 24 hours incubation at 37°C. Arrow indicates the addition of ATP (50 μM). During the measurements, the cells were incubated in Ca^{2+}-free KRG containing 10 mM Hepes, 2 mM EGTA and 10 μM $La(NO_3)_3$. Inset shows a typical chlamydial inclusion. Arrowheads indicate area of Ca^{2+} measurements.

independent process similar to PLF in human and murine macrophages (Zimmerli et al., 1996).

We thus conclude that the regulation of localized $[Ca^{2+}]_i$ changes by mobilization of Ca^{2+} stores at the site of chlamydial aggregations and inclusions may be a way for a chlamydia-infected cell to regulate its Ca^{2+} requirement through a relatively low number of Ca^{2+} organelles rather than through an excess of uniformly distributed Ca^{2+} stores. The role of Ca^{2+} store redistribution during infection of the host cells with chlamydiae might be to generate subcellular $[Ca^{2+}]_i$ gradients

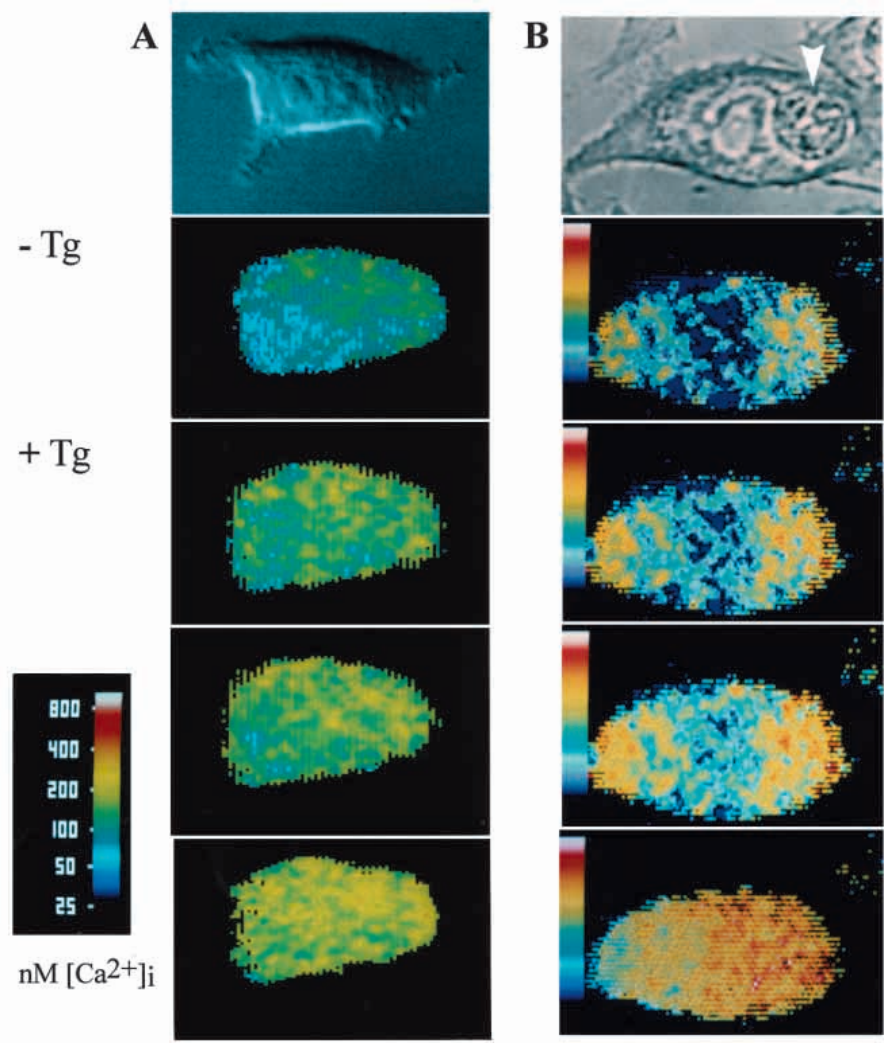

Fig. 11. Digital ratio images (128×128) showing the level of $[Ca^{2+}]_i$ before and after addition of 100 nM Tg, in uninfected (A) or infected (B) HeLa cells after 24 hours at 37°C. The cells were handled as in Fig. 10. The ratio images in A and B indicate changes in the level of $[Ca^{2+}]_i$ and the upper images in A and B show the corresponding low-resolution brightfield images. Images are separated by 2-second time intervals. Arrowhead indicates a chlamydial inclusion.

needed to control aggregation and fusion of EB-containing vesicles and inclusions. The precise mechanisms whereby the translocated SERCA2 and IP3-R interact to regulate Ca^{2+} around the chlamydial aggregates and inclusions require further investigations.

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