Rapid Activation of Protein Tyrosine Kinase and Phospholipase C-γ2 and Increase in Cytosolic Free Calcium Are Required by *Ehrlichia chaffeensis* for Internalization and Growth in THP-1 Cells

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Ehrlichia chaffeensis*, a bacterium that cannot survive outside the eukaryotic cell, proliferates exclusively in human monocytes and macrophages. In this study, signaling events required for ehrlichial infection of human monocytic cell line THP-1 were characterized. Entry and proliferation of *E. chaffeensis* in THP-1 cells were significantly blocked by various inhibitors that can regulate calcium signaling, including 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate and 2-aminoethoxydiphenyl borate (intracellular calcium mobilization inhibitors), verapamil and 1-{β-[3-(4-methoxyphenyl)propyl]-4-methoxyphenethyl}-1H-imidazole (SKF-96365) (calcium channel inhibitors), neomycin and 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione (U-73122) (phospholipase C [PLC] inhibitors), monodansylcadaverine (a transglutaminase [TGase] inhibitor), and genistein (a protein tyrosine kinase [PTK] inhibitor). Addition of *E. chaffeensis* resulted in rapid increases in the level of inositol 1,4,5-trisphosphate (IP_3) and the level of cytosolic free calcium ($[Ca^{2+}]_i$) in THP-1 cells, which were prevented by pretreatment of THP-1 cells with inhibitors of TGase, PTK, and PLC. *E. chaffeensis* induced rapid tyrosine phosphorylation of PLC-γ2, and the presence of a PLC-γ2 antisense oligonucleotide in THP-1 cells significantly blocked ehrlichial infection. Furthermore, tyrosine-phosphorylated proteins and PLC-γ2 were colocalized with ehrlichial inclusions, as determined by double-immunofluorescence labeling. The heat-sensitive component of viable *E. chaffeensis* cells was essential for these signaling events. *E. chaffeensis*, therefore, can recruit interacting signal-transducing molecules and induce the following signaling events required for the establishment of infection in host cells: protein cross-linking by TGase, tyrosine phosphorylation, PLC-γ2 activation, IP_3 production, and an increase in $[Ca^{2+}]_i$.

Intracellular bacteria are known to usurp many preexisting host cell signaling and membrane sorting mechanisms for entry, survival, and proliferation. Recent findings concerning a variety of molecular mechanisms of intracellular parasitism, therefore, have been enlightening not only in terms of microbial pathogenesis but also in terms of host cell biology (14, 18, 21). *Ehrlichia chaffeensis* is a recently discovered bacterium that is found in the United States, and it targets and multiplies in only primary host defensive cells, the monocytes and macrophages. *E. chaffeensis* causes a Rocky Mountain spotted fever-like illness called human monocytic ehrlichiosis, which can be fatal in immunocompromised patients or patients who are treated inappropriately (42). Unlike facultatively intracellular bacteria, such as *Salmonella*, *Yersinia*, *Mycobacterium*, etc., *E. chaffeensis* cannot survive extracellularly. Therefore, upon binding to host cells, *E. chaffeensis* must trigger rapid and precise signals and spatially assemble host molecules for internalization in a specific intracellular compartment, an early endosome (7, 32) conducive to proliferation. The majority of bacteria and various bacterial components, such as lipopolysaccharides, peptidoglycans, heat shock proteins, and CpG-encoding DNA, activate monocytes and macrophages through their pathogen-associated molecular patterns. In order to survive inside monocytes and macrophages, ehrlichiae must not induce activation signals, but they must selectively induce ehrlichial entry signals which are independent from macrophage activation signals. Most obligately and facultatively intracellular bacteria, such as *Orientia tsutsugamushi* (formerly *Rickettsia tsutsugamushi* [38]), *Salmonella* (17), *Bartonella* (20), and *Listeria* (24), mobilize microfilaments and enter host cells by phagocytosis. However, we found previously that entry of *Neorickettsia risticii* (formerly *Ehrlichia risticii* [15]), a monocytic obligately intracellular bacterium that causes Potomac horse fever (the level of 16S rRNA gene sequence identity between *N. risticii* and *E. chaffeensis* is 85% [42]), into host cells is unusual, since it consistently exhibits greater sensitivity to monodansylcadaverine (MDC), which is an inhibitor of transglutaminase (TGase) related to receptor-mediated endocytosis (1, 11, 27), and to taxol or colchicines, which are inhibitors of microtubules, than to cytochalasins, which are inhibitors of microfilament assembly (31, 40). We recently found that the entry of *E. chaffeensis* and entry of the granulocytic obligately intracellular bacterium *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent; the level of 16S rRNA gene sequence identity between *A. phagocytophila* and *E. chaffeensis* is 92.5% [15, 42]) are also sensitive to MDC (8, 33, 55).

Furthermore, *N. risticii* entry into host cells is unique since it is very sensitive to Ca^{2+} channel blockers, calmodulin antag-

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onists (41), and protein tyrosine kinase (PTK) inhibitors (56). None of these inhibitors has direct inhibitory effects on generation of CO_2 from L-glutamine by host-cell-free ehrlichiae (40). Ca^{2+} is required for internalization of *Salmonella enterica* serovar Typhimurium (35). PTK activities are required for internalization of enteropathogenic *Escherichia coli* (EPEC) (43), *Listeria* (52), *Yersinia* (44), and *Campylobacter jejuni* (54). However, except for the EPEC proteins, the identities of the tyrosine-phosphorylated proteins are largely unknown. *N. risticii* is distinct from these bacteria since it consistently requires both Ca^{2+} and PTK signals for internalization.

Since previous studies of *N. risticii* were limited to the use of inhibitors and the role of phospholipase C (PLC) was not examined and to determine whether the calcium signals are also required for infection by *E. chaffeensis*, in the present study we examined the effects of inhibitors of PTK, PLC, intracellular calcium mobilization, and calcium channel blockers on internalization and proliferation of *E. chaffeensis* in THP-1 cells, a human monocytic cell line. Also, the production of inositol 1,4,5-trisphosphate (IP_3), the level of cytosolic free calcium ($[Ca^{2+}]_i$) in response to *E. chaffeensis*, and the effects of the inhibitors on IP_3 generation and $[Ca^{2+}]_i$ were determined. Finally, the isozyme of PLC involved in IP_3 generation, its role in ehrlichial internalization, and localization of PLC and tyrosine-phosphorylated proteins were examined.

MATERIALS AND METHODS

E. chaffeensis and THP-1 cells. *E. chaffeensis* Arkansas (12) was propagated in THP-1 cells (American Type Culture Collection, Rockville, Md.), a human monocytic leukemia cell line (50), in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% L-glutamine at 37°C in a 5% CO_2–95% air atmosphere. No antibiotics were used in this study. Host-cell-free *E. chaffeensis* was prepared by sonicating a preparation for 8 s at an output setting of 2 with an ultrasonic processor (W-380; Heat Systems, Farmington, N.Y.). After low-speed centrifugation to remove nuclei and unbroken cells, the supernatant was centrifuged at $10,000 \times g$ for 10 min, and the pellet enriched with host-cell-free organisms was used to infect THP-1 cells. Based on pilot experiments, a multiplicity of infection (MOI) (the ratio of the number of bacteria to the number of host cells) of 100 was used in this study, unless indicated otherwise. To prepare heat-treated *E. chaffeensis*, host-cell-free *E. chaffeensis* was incubated in a 42 or 60°C water bath for 15 min.

Evaluation of the effects of various compounds on ehrlichial internalization or proliferation. The following compounds and concentrations were used to evaluate the effects of various compounds on ehrlichial internalization or proliferation: 100 μM genistein (Sigma, St. Louis, Mo.); 50 μM neomycin (BioMol, Plymouth Meeting, Pa.); 10 μM 1-(6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1H-pyrrole-2,5-dione (U-73122) (BioMol); 250 μM MDC (Sigma); 100 μM 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (BioMol); 75 μM 2-aminoethoxydiphenyl borate (2-APB) (Calbiochem, San Diego, Calif.); 60 μM 1-{β-[3-(4-methoxyphenyl)propyl]-4-methoxyphenethyl}-1H-imidazole (SKF-96365) (Calbiochem); and 100 μM verapamil (BioMol). To determine the effect on ehrlichial internalization, THP-1 cells (10^6 cells/well) were incubated in 12-well plates with various compounds for 1 h prior to the addition of host-cell-free *E. chaffeensis*. At 3 h postinfection (p.i.), the cells were centrifuged to remove extracellular ehrlichiae and treated with pronase E (2 mg/ml) for 3 min to remove bound but uninternalized *E. chaffeensis* as previously described (40). The THP-1 cells were cultured without inhibitors for an additional 3 days, and then infectivity was determined by counting the number of *E. chaffeensis* cells in 100 THP-1 cells in triplicate assay wells (41). To determine the effects of compounds on ehrlichial proliferation, the compounds were added at 3 h p.i. to mixtures of THP-1 cells and host-cell-free *E. chaffeensis* organisms. They were incubated for 3 days, and infectivities were determined.

IP_3 assay. IP_3 contents were determined by using the D-*myo*-IP_3-^3H assay system as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, N.J.). Briefly, THP-1 cells (1×10^7 cells/ml) were placed into 1.7-ml microcentrifuge tubes and incubated at 37°C for 5 min. Then 0.5-ml portions of prewarmed host-cell-free *E. chaffeensis* were added to the THP-1 cells at an MOI

of 100, and the contents were mixed rapidly by inverting the tubes several times. At the appropriate time, the cells were centrifuged at 2,000 rpm and 4°C for 1 min, and perchloric acid was added to each pellet. The times recorded were the times when the tubes were taken out of the incubator. IP_3 extracted in perchloric acid and ^3H-labeled IP_3 standards were coincubated with IP_3-binding protein for 15 min on ice. After unbound $[^3H]IP_3$ was washed, the amount of $[^3H]IP_3$ released from IP_3-binding protein by 0.15 N NaOH was determined with a scintillation counter.

Measurement of $[Ca^{2+}]_i$ with a fluorometer. THP-1 cells were loaded with the acetoxymethyl (AM) ester derivative of fluo-3 (fluo-3/AM) (Molecular Probes, Eugene, Oreg.) at a concentration of 2 μM at 37°C for 30 min on the rotating platform of a water bath. After the cells were washed twice, they were resuspended in RPMI 1640 medium (with 5% FBS but without phenol red) and incubated for 30 min to allow complete hydrolysis of intracellular AM esters. The cells were then stored on ice until they were assayed. A total of 1.5×10^6 fluo-3-preloaded cells were incubated with or without compounds for 1 h at room temperature. After it was warmed to 30°C by using a circulating water bath in the fluorometer, *E. chaffeensis* was added to the THP-1 cells, the preparation was mixed briefly, and emission at 525 nm after excitation at 488 nm was recorded every 5 s for 10 min at 30°C. The $[Ca^{2+}]_i$ was calculated using a calcium calibration buffer kit according to the manufacturer's instructions (Molecular Probes).

Measurement of $[Ca^{2+}]_i$ by ratiometric imaging. THP-1 cells were loaded with fura-2/AM (Molecular Probes) as described above for fluo-3/AM loading. A total of 5×10^5 preloaded cells were incubated with or without compounds in 1 ml of RPMI 1640 (with 5% FBS but without phenol red) for 1 h. The cells were then added to a poly-L-lysine (Sigma)-coated round coverslip in a chamber, allowed to settle for 10 min, and gently washed twice to remove unattached cells. Changes in $[Ca^{2+}]_i$ in individual cells were monitored at room temperature by fluorescence video microscopy by using a PTI ImageMaster system (Photon Technology International, Monmouth Junction, N.J.) attached to an Eclipse TE200 inverted microscope (Nikon, Melville, N.Y.). Alternating excitation wavelengths of 340 and 380 nm were provided by a DeltaRAM monochromator at 1-s intervals. Fields containing approximately 20 cells were selected, and images at an emission wavelength of 520 nm were recorded for at least 5 min. After images were recorded for 30 s to determine the basal level, an equal volume of a host-cell-free *E. chaffeensis* preparation at an MOI of 100:1 or at an MOI indicated below was added to the chamber slowly, and the contents were mixed by careful pipetting so that image recording was not disturbed. The ratios of fluorescence intensities (emission wavelength, 520 nm) obtained at excitation wavelengths of 340 and 380 nm for individual THP-1 cells were plotted against time.

Immunoprecipitation and Western blotting. A total of 1×10^7 THP-1 cells that had been pretreated with compounds for 1 h were incubated with host-cell-free *E. chaffeensis* as described above for the IP_3 assay. The cells were lysed in 1 ml of an ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) with freshly added protease and phosphatase inhibitors (1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM NaVO₃). After incubation on ice for 20 min, the cells were sonicated and centrifuged at $10,000 \times g$ for 10 min to collect the supernatants (cell lysates). For immunoprecipitation, cell lysates were incubated with anti-PLC-γ1 or -γ2 antibodies (0.2 μg/ml; Santa Cruz) overnight, and immunocomplexes were captured by using protein A-agarose beads (Santa Cruz). After the preparations were washed three times with phosphate-buffered saline, the protein A-agarose beads were resuspended in 1× sodium dodecyl sulfate sample buffer and boiled for 5 min to dissociate the immunocomplexes from the beads. Samples were subjected to Western blotting and reacted with anti-phosphotyrosine antibody, anti-PLC-γ1 and -γ2 antibodies, and horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with enhanced chemiluminescence by incubating the membrane with LumiGLO chemiluminescent reagent (Cell Signaling, Beverly, Mass.) and exposing it to X-ray film.

Double-immunofluorescence labeling. *E. chaffeensis*-infected THP-1 cells (2 days p.i.) were cytocentrifuged onto glass slides, and then the cells were fixed and permeabilized in Diff-Quik fixative containing methanol for 15 s (32). For antibody labeling, cells were sequentially incubated with primary antibodies (rabbit anti-PLC-γ1 and -γ2 antibodies, mouse anti-phosphotyrosine antibody, or dog anti-*E. chaffeensis* serum antibody preabsorbed with THP-1 cells) and secondary antibodies (lissamine rhodamine-conjugated anti-rabbit or -mouse antibody, fluorescein isothiocyanate-conjugated anti-dog antibody) in phosphate-buffered saline. The labeled cells were observed with a Nikon microscope coupled to an MRC-1024 confocal laser scanning unit (Bio-Rad, Hercules, Calif.).

TABLE 1. Effects of various inhibitors on internalization of *E. chaffeensis*[a]

Treatment[b]	No. of ehrlichiae per 100 cells	% Infected cells
None	$3,209 \pm 236$	83.5 ± 6.5
Genistein (100 μM)	0	0
Neomycin (10 μM)	18 ± 11	2.0 ± 0.5
Verapamil (100 μM)	0	0
MDC (250 μM)	132 ± 17	9.0 ± 3.3

[a] Reagents were added to THP-1 cells 1 h before infection. At 3 h p.i., the cells were centrifuged and treated with 2 mg of pronase E per ml for 3 min to remove bound uninternalized *E. chaffeensis*, and then the cells were continuously cultured. The numbers of internalized *E. chaffeensis* organisms were determined by counting the cells 3 days p.i. The values are means ± standard deviations ($n = 3$).

[b] Genistein is an inhibitor of PTK; neomycin is an inhibitor of PLC; verapamil is an inhibitor of calcium influx; and MDC is an inhibitor of TGase.

Effect of PLC-γ2 antisense oligonucleotides on *E. chaffeensis* infection. Morpholino antisense oligonucleotides with complete resistance to nucleases, predictable targeting, and reliable activity in cells (49) were used to determine the effect of PLC-γ2 antisense oligonucleotides on *E. chaffeensis* infection. PLC-γ2 antisense morpholino oligonucleotides (5′-AATCTACATTGACCGTGGTGG ACAT-3′) were made based on the 5′ end 25-nucleotide sequences in the PLC-γ2 open reading frame (Gene Tools LLC, Corvallis, Oreg.). Standard control morpholino oligonucleotides (5′-CCTCTTACCTCAGTTACAATTTAT A-3′) having no target and no significant biological activity in THP-1 cells were supplied by Gene Tools. The morpholino oligonucleotides were mixed with ethoxylated polyethylenimine and added to 2×10^6 THP-1 cells in triplicate wells in a 12-well plate. The THP-1 cells were rinsed 3 h later and cultured in fresh medium for 3 days to reduce the level of previously synthesized protein. After the medium was changed, the cells were infected with host-cell-free *E. chaffeensis*. On 3 day p.i., infectivity was determined as described above, and a Western blot analysis was carried out to determine the protein levels of PLC-γ1 and PLC-γ2.

RESULTS

Effects of various compounds on *E. chaffeensis* internalization in THP-1 cells. It is difficult to visually count internalized ehrlichial organisms because they are so small and so few of them are internalized, so various methods, such as flow cytometry of immunofluorescence-labeled organisms and [^{35}S]methionine-labeled *N. risticii*, were devised previously to study internalization (31, 40, 41). Internalization of the majority of host-cell-free ehrlichiae takes place within 3 h during incubation with host cells, while extracellular ehrlichiae lose infectivity within 3 h (31, 36). In the present study, THP-1 cells were incubated with host-cell-free *E. chaffeensis* organisms for 3 h in the presence or in the absence of inhibitors. Bound but uninternalized *E. chaffeensis* organisms were removed by pronase E treatment. The cells were then incubated for 3 days in the absence of compounds, and the ehrlichiae in the THP-1 cells were counted. Since all of the compounds used are reversible inhibitors at the concentrations used and only *E. chaffeensis* organisms that had been internalized during the initial 3-h incubation period could proliferate, this method provided a simple way to determine the effects of compounds on internalization of *E. chaffeensis* by amplifying the signal (similar to colony counting for determining the number of viable bacteria). The concentrations of compounds used in this study were determined by dose-response experiments and had no direct toxic effects on host cells or ehrlichiae.

The TGase inhibitor MDC inhibits clustering and internalization of the ligand-receptor complexes in clathrin-coated vesicles (1, 11, 27) and thus prevents internalization of *N. risticii* in

P388D$_1$ cells (31, 40) and internalization of the human granulocytic ehrlichiosis agent in HL-60 cells (55) but not binding of these organisms to the host cells (31, 40). Internalization of *N. risticii* in P388D$_1$ cells was also inhibited by the PTK inhibitor genistein (56), the Ca^{2+} channel blocker verapamil, the calmodulin antagonist W-7, and an inhibitor of intracellular Ca^{2+} mobilization, TMB-8 (41). These inhibitors do not have direct ehrlichiacidal effects and are not toxic to the host cells at the concentrations used (40, 41). Like their effects on internalization of *N. risticii* in P388D$_1$ cells, MDC, genistein, and verapamil all prevented internalization of *E. chaffeensis* in THP-1 cells (Table 1). Therefore, internalization or initial establishment of an *E. chaffeensis* infection in THP-1 cells requires PTK and TGase activities and an increase in the $[Ca^{2+}]_i$. Our results also showed that an inhibitor of PLC, neomycin, prevents ehrlichial internalization (Table 1). Since preincubation of host-cell-free *E. chaffeensis* with neomycin for 30 min at 37°C did not reduce ehrlichial infectivity compared to the infectivity of *E. chaffeensis* preincubated with RPMI 1640 medium alone (negative control), neomycin does not seem to have an inhibitory effect directly on *E. chaffeensis* (data not shown).

Effects of various compounds on *E. chaffeensis* proliferation in THP-1 cells. Since most internalization of ehrlichiae takes place during the first 3 h of incubation at 37°C (31, 36), compounds were added at 3 h p.i. to assess their effects on ehrlichial proliferation independent of the inhibitory effects on the initial internalization. Proliferation of *N. risticii* in P388D$_1$ cells was inhibited by genistein (56), MDC, verapamil, and TMB-8 (41). These compounds also inhibited proliferation of *E. chaffeensis* in THP-1 cells (Table 2). Additional Ca^{2+} modulators used in the present study were 2-APB, a cell-permeable inhibitor of IP$_3$-induced Ca^{2+} release (28), and SKF-96365, an inhibitor of receptor-mediated Ca^{2+} entry (30). Each of these compounds significantly inhibited proliferation of *E. chaffeensis* at a concentration that was not toxic to THP-1 cells (Table 2). Two inhibitors of PLC, neomycin and U-73122 (48), also inhibited proliferation (Table 2). The effects of the PLC inhibitors depended on the dose and the time that the inhibitors were added (Fig. 1); the higher the concentration and the earlier the inhibitors were added, the greater the inhibitory

TABLE 2. Effects of various inhibitors on proliferation of *E. chaffeensis*[a]

Treatment[b]	No. of ehrlichiae per 100 cells	% Infected cells
None	$6,030 \pm 400$	94.1 ± 2.2
Genistein (100 μM)	90 ± 20	7.9 ± 18
Neomycin (10 μM)	75 ± 25	2.5 ± 0.9
U-73122 (10 μM)	485 ± 105	25.7 ± 3.6
TMB-8 (100 μM)	148 ± 30	18.8 ± 2.8
2-APB (75 μM)	475 ± 19	30.7 ± 4.6
Verapamil (100 μM)	12 ± 2	1.4 ± 0.4
SKF-96365 (60 μM)	195 ± 60	8.6 ± 0.5
MDC (250 μM)	0	0

[a] Reagents were added to THP-1 cells at 3 h p.i. and were present throughout the incubation period. The THP-1 cells were infected with host-cell-free *E. chaffeensis* at an MOI of 100, and infectivities were determined 3 days p.i. The values are means ± standard deviations ($n = 3$).

[b] Genistein is an inhibitor of PTK; neomycin and U-73122 are inhibitors of PLC; TMB-8 and 2-APB are inhibitors of intracellular calcium release; verapamil and SKF-96365 are inhibitors of calcium influx; and MDC is an inhibitor of TGase.
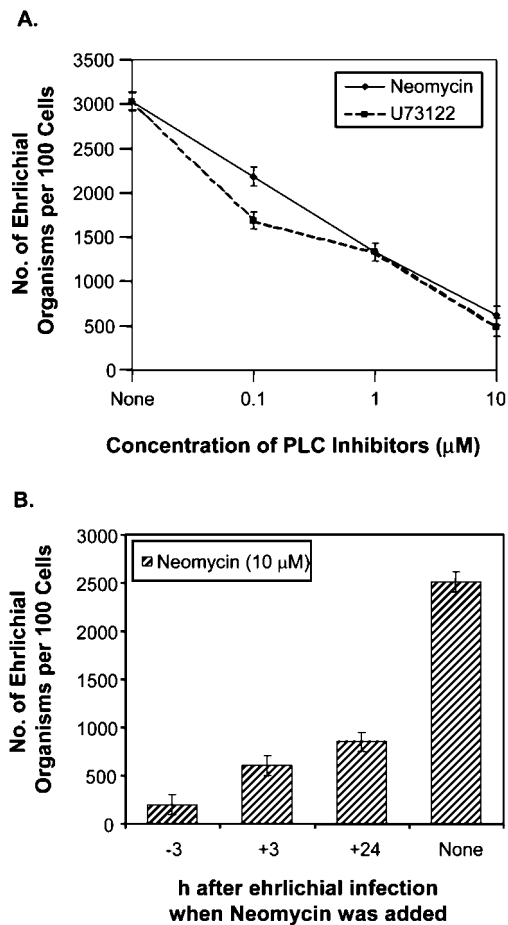
A.

B.

FIG. 1. Effects of PLC inhibitors on *E. chaffeensis* infection. The PLC inhibitors neomycin and U-73122 blocked *E. chaffeensis* infection in a dose-dependent manner (neomycin and U-73122 were added 1 h prior to ehrlichial infection) (A) or in a time-dependent manner (10 μM neomycin was added to THP-1 cells before or after infection) (B). Infectivity was determined 3 days p.i. as described in Materials and Methods. The values are means ± standard deviations ($n = 3$) and are representative of the results of more than three independent experiments.

effects on ehrlichial proliferation were. These results indicated that the TGase, PTK, and phosphatidylinositol-specific PLC activities and an increase in the $[Ca^{2+}]_i$ were required for proliferation of *E. chaffeensis* in THP-1 cells.

Effects of ehrlichiae and various compounds on production of IP_3 by THP-1 cells. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate and produces two well-characterized second messengers, IP_3 and diacylglycerol. Since ehrlichial entry and proliferation were prevented by PLC inhibitors, the levels of IP_3 before and after infection were measured to evaluate PLC activity. Diacylglycerol, an activator of protein kinase C, was not assayed, since our previous data had shown that protein kinase C activity is not required for *N. risticii* infection of $P388D_1$ cells (41). *E. chaffeensis* induced a rapid release in the IP_3 concentration in THP-1 cells, which reached a peak at 2 min p.i. and then decreased to almost the basal level by 5 min (Fig. 2A). Furthermore, inhibitors of PLC (neomycin and U-73122), PTK (genistein), and TGase (MDC) all blocked the increase in the IP_3 level induced by ehrlichiae (Fig. 2B), indi-

cating that IP_3 was produced by PLC in a TGase- and PTK-dependent manner. Since activation of PLC-γ isozymes requires tyrosine phosphorylation of the molecules by PTK (34, 46), these results suggest that *E. chaffeensis* induces the release of IP_3 by activating a PLC-γ isozyme through tyrosine phosphorylation.

Effects of ehrlichiae and various inhibitors on $[Ca^{2+}]_i$ in THP-1 cells. IP_3 binds to IP_3 receptors located on the endoplasmic reticulum membrane and causes the release of stored Ca^{2+}. Since Ca^{2+} channel blockers and inhibitors of intracellular Ca^{2+} mobilization blocked ehrlichial infection, $[Ca^{2+}]_i$ was determined in THP-1 cells infected with *E. chaffeensis*. In fluo-3-loaded THP-1 cells, a rapid increase in $[Ca^{2+}]_i$ to 300 nM was detected 30 s after host-cell-free *E. chaffeensis* was added, and the concentration reached a maximum by 1 min.
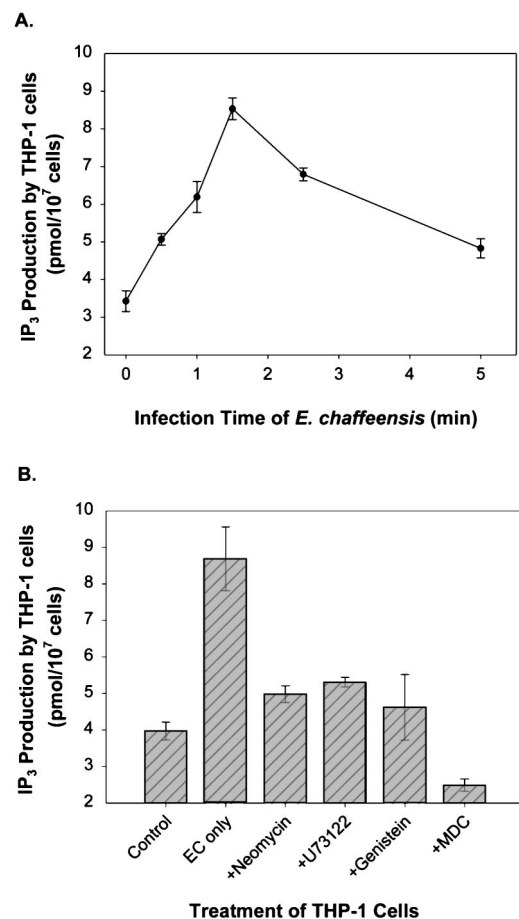
A.

B.

FIG. 2. Effects of ehrlichial infection and inhibitors on production of IP_3 in THP-1 cells. (A) Rapid release of IP_3 induced by ehrlichial infection. The release reached a peak (approximately threefold) at 1.5 min p.i. and decreased to the background level within 5 min. (B) Release of IP_3 induced by *E. chaffeensis* (EC) prevented by pretreatment with inhibitors of PTK, PLC, and TGase. Cells were pretreated with or without inhibitors 1 h before ehrlichial infection, and IP_3 samples were extracted with perchloric acid at different times (A) or 1.5 min p.i. (peak release of IP_3) (B). The release of IP_3 was determined by measuring competitive inhibition of binding of ^{3}H-labeled IP_3 to IP_3-binding protein. The values are means ± standard deviations ($n = 3$) and are representative of the results of three independent experiments.
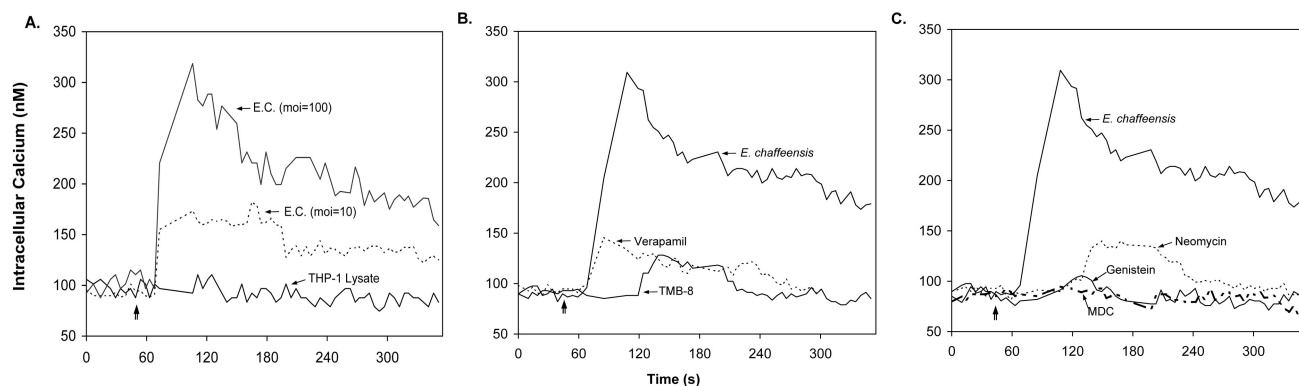
FIG. 3. Effects of ehrlichial infection and inhibitors on $[Ca^{2+}]_i$ in a population of cells. $[Ca^{2+}]_i$ increased rapidly and transiently in THP-1 cells infected with *E. chaffeensis* (E.C.) (A), but the increase was prevented by pretreatment with inhibitors of calcium mobilization (verapamil and TMB-8) (B) or inhibitors of PLC (neomycin), PTK (genistein), and TGase (MDC) (C). The intracellular calcium levels in fluo-3-loaded THP-1 cells were determined with a fluorometer as described in Materials and Methods. The inhibitors were added to cells 1 h before the assay, and host-cell-free *E. chaffeensis* was added to THP-1 cells at the time indicated by the vertical arrow (approximately 50 s p.i.). Assays were carried out at 30°C in a circulating water bath. Fluorescence was measured at 525 nm at 5-s intervals, and K_d was calibrated by using a calcium calibration kit from Molecular Probes. The values are representative of the results of at least four independent experiments. moi, multiplicity of infection.

The increase in $[Ca^{2+}]_i$ was greater with a higher MOI (Fig. 3A). As a control, uninfected THP-1 cell lysate did not induce an increase in $[Ca^{2+}]_i$.

Increases in $[Ca^{2+}]_i$ may result from Ca^{2+} influx from extracellular space or from Ca^{2+} released from internal stores. In order to determine the relative contributions of influx and release, $[Ca^{2+}]_i$ was measured in THP-1 cells that were preincubated with the Ca^{2+} channel blocker verapamil, the intracellular calcium release inhibitor TMB-8, or the PLC inhibitor neomycin and stimulated with host-cell-free *E. chaffeensis*. As shown in Fig. 3B, both TMB-8 and verapamil significantly inhibited an ehrlichia-induced increase in $[Ca^{2+}]_i$. However, TMB-8 and verapamil had different effects on $[Ca^{2+}]_i$. It appeared that the increase in $[Ca^{2+}]_i$ occurred in two phases, a rapid elevation phase that occurred within 60 s p.i., followed by a sustained phase. Verapamil inhibited the initial increase and the sustained elevation phase similarly, indicating that Ca^{2+} influx contributed to both phases (Fig. 3B). In contrast, TMB-8 and neomycin completely blocked the rapid initial increase, but a delayed increase in $[Ca^{2+}]_i$, presumably representing Ca^{2+} influx, still occurred (Fig. 3B and C). Furthermore, PTK and TGase activities were required for the increase in $[Ca^{2+}]_i$, since genistein and MDC almost completely prevented an increase in $[Ca^{2+}]_i$ during *E. chaffeensis* infection (Fig. 3C). The complete inhibition of an increase in $[Ca^{2+}]_i$ by MDC or genistein suggests that these compounds inhibit not only the release of calcium from intracellular calcium stores but also the influx of extracellular Ca^{2+} through calcium channels.

Since $[Ca^{2+}]_i$ was measured with a fluorometer by using a population consisting of 10^6 cells, the kinetics of the changes in $[Ca^{2+}]_i$ shown in Fig. 3 reflect the kinetics of the cumulative bacterium-host cell interactions. Therefore, we studied the kinetics of changes in the $[Ca^{2+}]_i$ in individual cells by fluorescence microscopy video imaging. As shown in Fig. 4A, the calcium levels in individual cells rose within 20 s after addition of ehrlichia and dropped to the basal level within 4 min. Also, to determine whether active participation of *E. chaffeensis* is required for the increase in $[Ca^{2+}]_i$, *E. chaffeensis* was pretreated for 15 min at 42 or 60°C. The results showed that the

increase in $[Ca^{2+}]_i$ did not take place when *E. chaffeensis* was heat treated (Fig. 4A). The calcium channel blocker SKF-96365 inhibited the initial peak phase and the sustained phase like verapamil in the fluorometric assay, whereas the cell-permeable IP_3 receptor blocker 2-APB and the PLC inhibitor neomycin were more effective in inhibiting the initial rapid increase in $[Ca^{2+}]_i$. Genistein completely prevented an increase in $[Ca^{2+}]_i$ caused by *E. chaffeensis* (Fig. 4B and C). Therefore, the two methods used to measure $[Ca^{2+}]_i$ gave similar results. However, the data obtained with the two methods were slightly different. Figure 3A shows that after the increase caused by *E. chaffeensis* addition, the $[Ca^{2+}]_i$ remained higher than the basal level prior to the addition of ehrlichiae even at 6 min p.i. In contrast, Fig. 4A shows that the $[Ca^{2+}]_i$ increased more rapidly but decreased to the basal level within 4 min. Because *E. chaffeensis* may bind to individual host cells at different times, the rate of the initial increase in $[Ca^{2+}]_i$ in a population of cells may appear to be lower than that in a single cell due to averaging (Fig. 3A and 4A). Alternatively, an apparent sustained high $[Ca^{2+}]_i$ may be due to fluo-3 leakage. The video microscopic method is not affected by leakage of the dye because the region of interest selected for calculating the ratios contains the cell and very little surrounding solution.

Activation of PLC-γ2 by tyrosine phosphorylation was required for *E. chaffeensis* infection. To our knowledge, three PLC isozymes are involved in eukaryotic calcium signaling. Unlike PLC-δ, PLC-β and PLC-γ have been well characterized. The activity of PLC-β is regulated by heterotrimeric G proteins. PLC-γ contains two *src*-homologous domains, SH2 and SH3, and activation of PLC-γ is mediated by the interaction between the C-terminal SH2 domain and the cognate tyrosine-phosphorylated peptide. The binding of PLC-γ to tyrosine-phosphorylated proteins (or receptors) via the SH2 domain accounts for the initial translocation of the enzyme from cytosolic to particulate fractions and the rapid phosphorylation of PLC-γ by receptor tyrosine kinases or cellular tyrosine kinases coupled to activated receptors. Our results demonstrated that PTK were required for PLC activation by *E. chaffeensis*
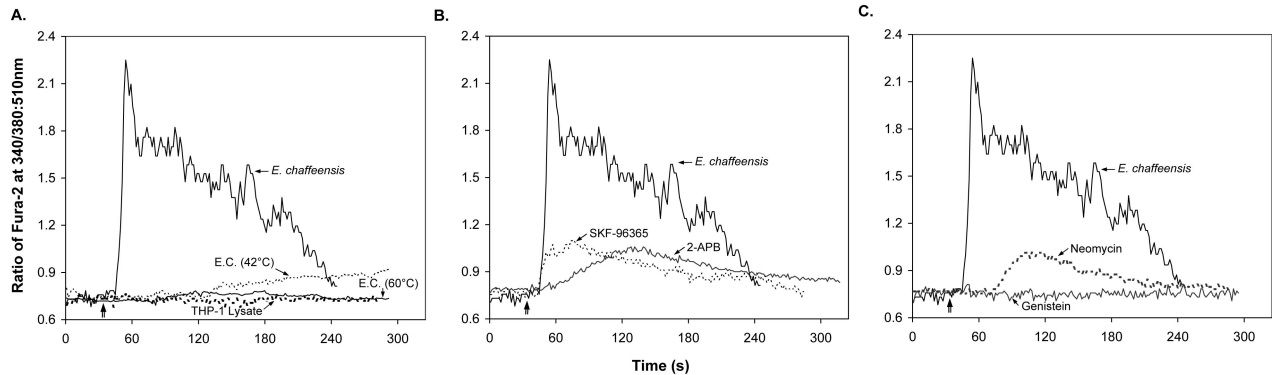
FIG. 4. Effects of ehrlichial infection and inhibitors on $[Ca^{2+}]_i$ in an individual cell. The rapid and transient increase in $[Ca^{2+}]_i$ (as shown by the ratio of emission by fura-2 at 520 nm under stimulation at 340 nm to emission by fura-2 at 520 nm under stimulation at 380 nm) was induced by *E. chaffeensis* (E.C.) infection in an individual THP-1 cell (A). The increase in $[Ca^{2+}]_i$ required active participation of live *E. chaffeensis* since heat-treated (42 or 60°C, 15 min) *E. chaffeensis* did not increase the $[Ca^{2+}]_i$. However, the increase in $[Ca^{2+}]_i$ induced by ehrlichial infection was prevented by the calcium mobilization inhibitors SKF-96365 and 2-APB (B) or by the PLC inhibitor neomycin and the PTK inhibitor genistein (C). THP-1 cells were loaded with fura-2/AM and treated with or without inhibitors for 1 h before measurements were obtained. Cells were added to poly-L-lysine-coated coverslip chambers, and images (emission at 520 nm after excitation at 340 and 380 nm) were recorded at 1-s intervals for at least 5 min. Host-cell-free *E. chaffeensis* was added to the chambers after measurements had been obtained for 30 s (vertical arrow). The values are representative of the results of at least three independent experiments, and similar results were obtained for more than 20 cells in each experiment.

infection; therefore, the role of PLC-γ during ehrlichial infection was examined.

Western blot analysis revealed that THP-1 cells express both PLC-γ1 and -γ2 isotypes, which have the same molecular mass, 145 kDa (data not shown). Western blot analysis in which anti-phosphotyrosine monoclonal antibody was used showed that a 145-kDa band was tyrosine phosphorylated in THP-1 cells as soon as 1 min after addition of *E. chaffeensis*, and the phosphorylation of this protein was inhibited by genistein and MDC (Fig. 5A), indicating that TGase is required for tyrosine phosphorylation of this protein. In addition, an approximately 200-kDa protein was also tyrosine phosphorylated during *E. chaffeensis* infection (data not shown).

The 145-kDa tyrosine-phosphorylated protein was confirmed to be PLC-γ2 and not PLC-γ1 by immunoprecipitation with an anti-PLC-γ2 antibody and a Western blotting analysis performed with anti-phosphotyrosine antibody (Fig. 5B) or anti-PLC-γ2 antibody (Fig. 5C). The Western blot analysis with anti-phosphotyrosine antibody resulted in no bands from samples immunoprecipitated with anti-PLC-γ1 antibody (data not shown). PLC-γ2 remained tyrosine phosphorylated in *E. chaffeensis*-infected THP-1 cells even at 2 days p.i., as determined by Western blot analysis. When *E. chaffeensis* was preincubated at 42 or 60°C for 15 min, the levels of tyrosine phosphorylation of PLC-γ2 were reduced (Fig. 5D), suggesting that viable ehrlichiae or native ehrlichial proteins are required for activation of PLC-γ2.

Furthermore, confocal immunofluorescence microscopy revealed colocalization of tyrosine-phosphorylated proteins and PLC-γ2 but not PLC-γ1 with the inclusions of *E. chaffeensis* (Fig. 6). Tyrosine-phosphorylated proteins and PLC-γ2 were randomly distributed in the cytoplasm of uninfected THP-1 cells (data not shown). Like *N. risticii* inclusions, most tyrosine-phosphorylated proteins were concentrated in *E. chaffeensis* inclusions (56). It is likely that PLC-γ2 and other tyrosine-phosphorylated proteins during *E. chaffeensis* infection, as determined by Western blot analysis, are also concentrated in ehrlichial inclusions.

For further confirmation, the requirement for PLC-γ2 for *E. chaffeensis* infection was tested in THP-1 cells transfected with a PLC-γ2 antisense oligonucleotide. As shown in Fig. 7, delivery of PLC-γ2 antisense oligonucleotides into THP-1 cells sig-
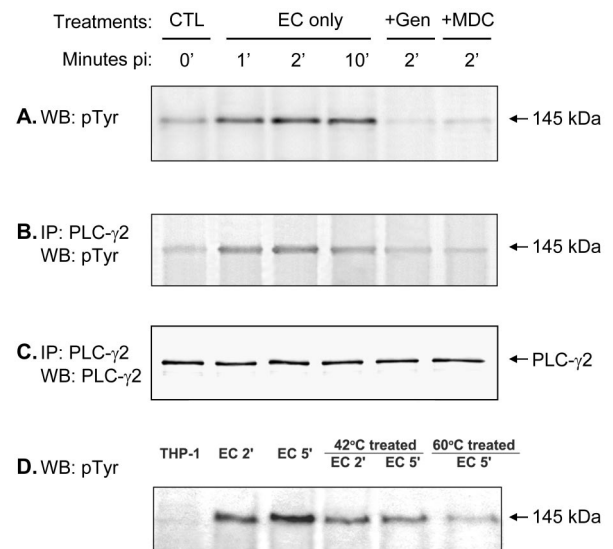

FIG. 5. Tyrosine phosphorylation of PLC-γ2 in ehrlichial infection. (A) A 145-kDa protein was rapidly tyrosine phosphorylated in THP-1 cells upon binding of *E. chaffeensis* (EC). This protein was confirmed to be PLC-γ2 by immunoprecipitation (IP) with anti-PLC-γ2 antibody and by Western blot (WB) analysis with detection with anti-phosphotyrosine (pTyr) antibody (B) or anti-PLC-γ2 antibody (C). Tyrosine phosphorylation of PLC-γ2 was reduced when *E. chaffeensis* was pretreated at 42 or 60°C for 15 min (D). THP-1 cells were pretreated with genistein (Gen) or MDC for 1 h prior to the addition of *E. chaffeensis*. At different times, cells were lysed in RIPA buffer. Whole-cell lysates were immunoprecipitated with anti-PLC-γ2 antibody, and the immunoprecipitates or whole-cell lysates were subjected to Western blotting by using anti-phosphotyrosine antibody or anti-PLC-γ2 antibody. The results are representative of the results of more than four independent experiments in which similar results were obtained. CTL, control.
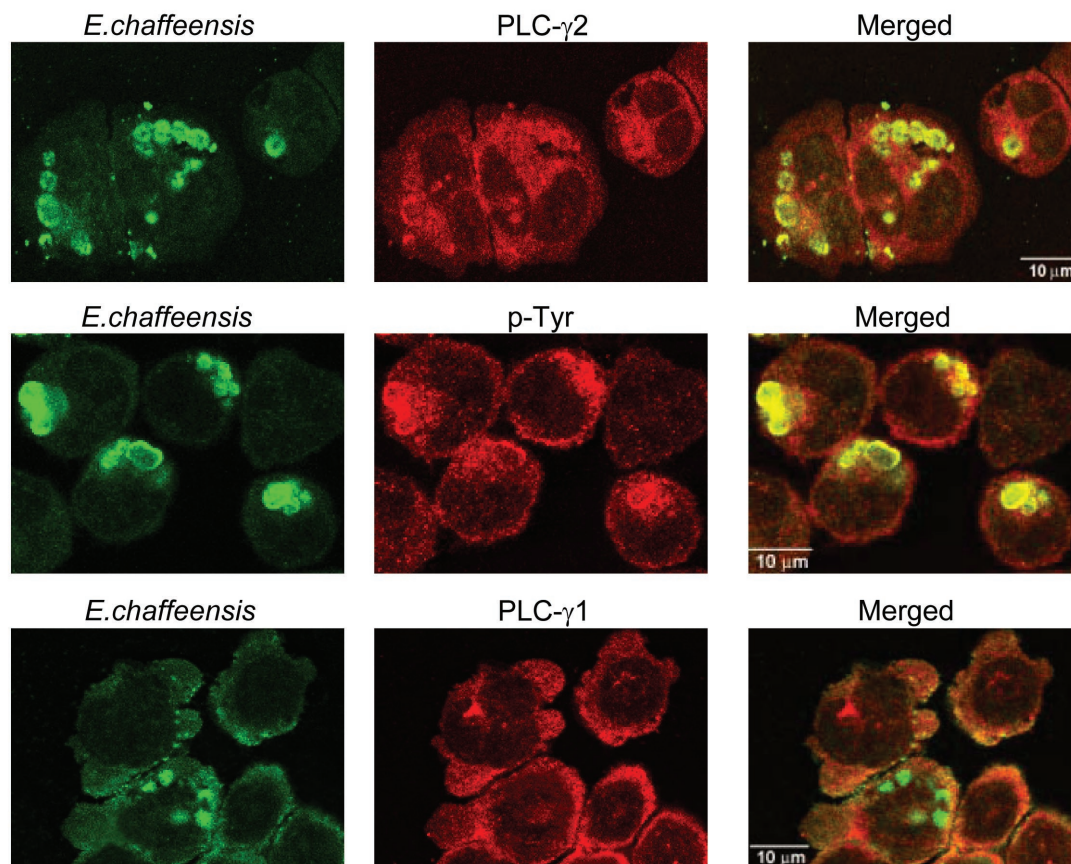
FIG. 6. Colocalization of *E. chaffeensis* inclusions with PLC-γ2 and tyrosine-phosphorylated proteins but not with PLC-γ1. *E. chaffeensis*-infected THP-1 cells (2 days p.i.) were double labeled as described in the text and observed by confocal microscopy. The following antibodies were labeled: *E. chaffeensis* (green, left panels) and PLC-γ1 or -γ2 or phosphotyrosine (red, middle panels). The panels on the right are superimposed images viewed with green and red filters. Note the colocalization of *E. chaffeensis* with PLC-γ2 and phosphotyrosine (yellow). The results are representative of the results of three independent labeling experiments.

nificantly decreased the protein level of PLC-γ2 but not the protein level of PLC-γ1 and blocked *E. chaffeensis* infection. The control oligonucleotides had no effect on the PLC-γ2 protein level or *E. chaffeensis* infection. These results demonstrate that *E. chaffeensis* induces the rapid tyrosine phosphorylation of PLC-γ2 required for internalization and proliferation of *E. chaffeensis* in a TGase-dependent manner. PLC-γ2 catalyzes the formation of IP_3 and mediates the increase in $[Ca^{2+}]_i$. Translocation and persistent colocalization of PLC-γ2 and other tyrosine-phosphorylated proteins in *E. chaffeensis* inclusions suggest that ehrlichial inclusions can actively retain these proteins, which play an important role in maintaining the integrity of inclusions and in facilitating ehrlichial proliferation.

DISCUSSION

In this study we demonstrated that *E. chaffeensis* induces a rapid and modest (300 nM, threefold) increase in $[Ca^{2+}]_i$. The rate of the increase in $[Ca^{2+}]_i$ and the maximum level reached may be important for internalization and proliferation of *E. chaffeensis* in THP-1 cells. In human monocytes incubated with opsonized zymosan particles, which induce superoxide generation in monocytes (37), $[Ca^{2+}]_i$ increases from a basal value of 75 ± 11 nM to 676 ± 78 nM by 34 ± 5 s (25). *Salmonella*

induces a slow increase in $[Ca^{2+}]_i$ from 100 to 800 nM in the intestinal epithelial cell line Henle-407 over a 30-min incubation period, and this increase is accompanied by membrane ruffling (35). Unlike ehrlichiae, salmonellae induce superoxide generation in monocytes (33, 51). Fc receptor-mediated phagocytosis, which induces rapid superoxide generation (2), is accompanied by an increase in $[Ca^{2+}]_i$ to around 700 nM in monocytes (16). The relatively modest increase in $[Ca^{2+}]_i$ caused by *E. chaffeensis* may prevent THP-1 cells from being activated to kill ehrlichiae. As a matter of fact, the Ca^{2+} ionophore A23187, which causes a substantial increase in $[Ca^{2+}]_i$, kills *N. risticii* and *E. chaffeensis* (41; Lin and Rikihisa, unpublished data), whereas *Salmonella* invades Henle-407 cells more efficiently when it is treated with A23187 (35).

The increase in $[Ca^{2+}]_i$ appears to result from both internal stores and the extracellular medium since inhibitors that block calcium mobilization from internal stores and inhibitors that block calcium mobilization from the extracellular space both prevented the increase in $[Ca^{2+}]_i$. Although an increase in $[Ca^{2+}]_i$ or a requirement for intracellular Ca^{2+} for establishment of infection has not been reported for any of the obligately intracellular bacteria, a few facultatively intracellular bacteria, such as EPEC and *S. enterica* serovar Typhimurium, are known to increase the $[Ca^{2+}]_i$ (5, 35). In the case of *S.*
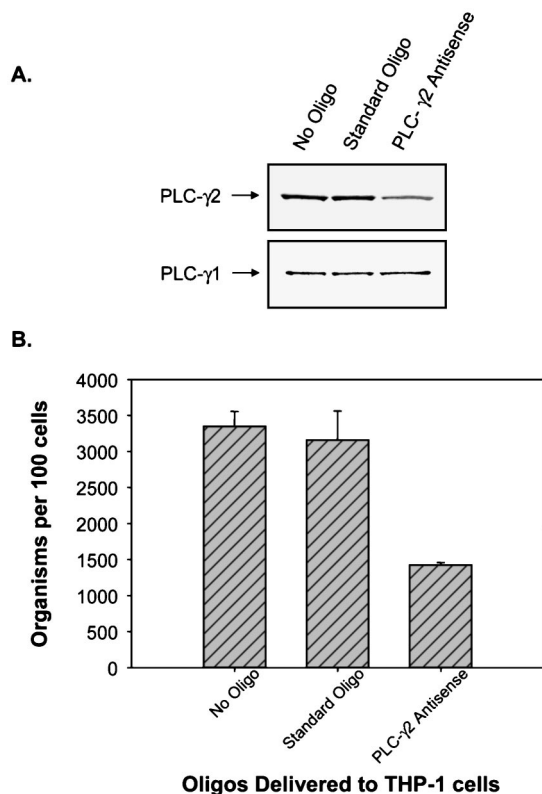
A.

PLC-γ2 →

PLC-γ1 →

B.

Oligos Delivered to THP-1 cells

FIG. 7. Effect of PLC-γ2 antisense oligonucleotides on *E. chaffeensis* infection. (A) Delivery of PLC-γ2 antisense oligonucleotides (Oligo) into THP-1 cells significantly reduced the level of PLC-γ2 but not the level of PLC-γ1 compared to the levels in untransfected cells or cells transfected with standard control oligonucleotides, as determined by Western blotting. (B) Delivery of PLC-γ2 antisense oligonucleotides into THP-1 cells also significantly reduced *E. chaffeensis* infection (>50%) ($P < 0.05$). THP-1 cells were transfected with anti-PLC-γ2 oligonucleotides or standard control oligonucleotides as described in Materials and Methods. Three days after the delivery of antisense oligonucleotides, the cells were infected with host-cell-free *E. chaffeensis*. On 3 day p.i., infectivity was determined, cells were extracted by using RIPA buffer, and a Western blot analysis was carried out to determine the levels of PLC-γ1 and PLC-γ2. The results are representative of the results of three independent experiments.

enterica serovar Typhimurium, an increase in $[Ca^{2+}]_i$ is required to induce membrane ruffling and internalization (35). However, internalization of *Shigella flexeneri* in HeLa cells occurs without an increase in $[Ca^{2+}]_i$ (10).

Using two different assays, we showed that infection with *E. chaffeensis* resulted in an increase in $[Ca^{2+}]_i$ in THP-1 cells. The PLC-induced increase in $[Ca^{2+}]_i$ was due to release of Ca^{2+} from the internal stores via IP_3 receptors and a subsequent influx of extracellular Ca^{2+} through receptor- or store-operated channels (9). The relative contributions of Ca^{2+} release and Ca^{2+} influx to the increase in $[Ca^{2+}]_i$ in THP-1 cells were examined by using specific inhibitors of these pathways. Two nonselective Ca^{2+} channel blockers, verapamil and SKF-96365, significantly inhibited the initial rapid phase and the sustained phase of the increase in $[Ca^{2+}]_i$, suggesting that Ca^{2+} influx is responsible for the bulk of the increase in $[Ca^{2+}]_i$ in response to the infection. These drugs are known to inhibit the activities of voltage-gated Ca^{2+} channels, as well as

receptor- or store-operated channels, including those formed by transient receptor potential proteins (58). Since monocytes do not express voltage-gated Ca^{2+} channels, it is likely that the influx is mediated by the receptor- or store-operated channels. Because store-operated channels are activated only after depletion of Ca^{2+} from internal stores, inhibition of intracellular Ca^{2+} release with TMB-8, 2-APB, and neomycin should also block their activities. Therefore, the delayed increase in $[Ca^{2+}]_i$ in the TMB-8-, 2-APB-, or neomycin-treated cells may represent the activity of receptor-operated channels that are not activated by store depletion.

In the present study we showed that the increase in $[Ca^{2+}]_i$ was mediated by protein cross-linking by TGase, translocation of PLC-γ2 to the ehrlichial inclusions, activation of PLC-γ2 by tyrosine phosphorylation, and generation of IP_3. Parts of these signals are somewhat similar to signals induced by EPEC, *Yersinia* sp., and *Salmonella* sp., which internalize by inducing focal assembly of microfilaments at the site of attachment. EPEC inoculates its Tir (translocated intimin receptor) into a host cell by a type III secretion mechanism, and Tir is tyrosine phosphorylated upon insertion into the host cell membrane. Tir is the receptor for an outer membrane protein (intimin) and the focal site of actin polymerization and induces additional signals which subsequently activate PLC-γ1. The cytoskeletal rearrangement by EPEC does not involve the small GTP-binding proteins Rho, Rac, and Cdc42 (13). The effects of EPEC on $[Ca^{2+}]_i$ are, however, inconclusive. In a recent report Bain et al. showed that an increase in $[Ca^{2+}]_i$ was not required for the lesion caused by EPEC (4). In the case of *Yersinia pseudotuberculosis*, tyrosine phosphorylation of the focal adhesion protein CAS, subsequent formation of functional CAS-Crk complexes, and activation of the small GTP-binding protein Rac1 led to actin cytoskeleton remodeling and yersinial uptake (53). Tir-mediated uptake of EPEC (43) and invasin-promoted uptake of *Yersinia* sp. (44), but not *S. enterica* serovar Typhimurium uptake by HeLa cells, are blocked by PTK inhibitors (45). *S. enterica* serovar Typhimurium microfilament assembly is directed by a type III secretion system. The proteins delivered include SopE, which induces Rho GTPase activation, and SipA, an actin-binding protein (22, 57), neither of which requires tyrosine phosphorylation. In contrast, an increase in $[Ca^{2+}]_i$ and protein tyrosine phosphorylation signals do not lead to polymerization of microfilaments in ehrlichial infections; rather, microfilaments are disassembled during *N. risticii* infection (39), suggesting that the mechanism is distinct from those described above. Genes homologous to type III secretion machinery genes have not been found in ehrlichiae or in the genome sequence of a related bacterium, *Rickettsia prowazekii* (3). The identities of ehrlichial components required for induction of these signaling events leading to ehrlichial internalization are not known. However, the present study revealed that viable ehrlichiae or ehrlichial heat-sensitive components (most likely proteins) are required for induction of tyrosine phosphorylation of PLC-γ2 and increases in $[Ca^{2+}]_i$.

It is not clear why Ca^{2+} channel blockers or inhibitors of intracellular Ca^{2+} release inhibit not only ehrlichial internalization but also proliferation, since whole-cell $[Ca^{2+}]_i$ was not continuously elevated during an ehrlichial infection. The paraphagosomal $[Ca^{2+}]_i$ has been reported to be higher than

the surrounding cytosol $[Ca^{2+}]_i$ in human monocytes, although the whole-cell $[Ca^{2+}]_i$ is not higher than the control monocyte $[Ca^{2+}]_i$ (25). The local $[Ca^{2+}]_i$ around ehrlichial inclusions may be higher than the $[Ca^{2+}]_i$ in the remaining cytosol, and this local higher $[Ca^{2+}]_i$ may be required for ehrlichial proliferation.

The reasons why an increase in $[Ca^{2+}]_i$ is required for ehrlichial internalization and proliferation are not known. Perhaps one reason is that ehrlichial internalization is dependent on a calcium-dependent enzyme, TGase, and microtubules (40). A second reason is that to accommodate proliferating ehrlichial organisms, new membranes should be added and iron and other nutrients should be supplied by fusion of ehrlichial inclusions with transferrin receptor endosomes (7), which are Ca^{2+}-calmodulin- and TGase-dependent processes (19, 23).

A striking finding of the present study was that PLC-γ2 and tyrosine-phosphorylated proteins were rapidly recruited and retained with ehrlichial inclusions even 2 days p.i. or probably during the entire intracellular life span. *E. chaffeensis* proliferates within membrane-bound vacuoles that display early endosomal markers (7, 32) and do not fuse with lysosomes. The continued colocalization of PLC-γ2 and tyrosine-phosphorylated proteins (the latter are also observed with *N. risticii* inclusions [56]) may be required to maintain the status of an inclusion. This may explain why inhibitors of PTK and PLC inhibit both ehrlichial entry and proliferation in host cells. Moreover, the spread of *E. chaffeensis* requires exocytosis of *E. chaffeensis* or lysis of the host cell and subsequent endocytosis by another cell, which also requires intracellular Ca^{2+} mobilization and tyrosine phosphorylation; thus, the spread is inhibited by these inhibitors.

The inhibition by MDC of all of the signaling pathways, as well as internalization and proliferation of *E. chaffeensis*, indicates that activation of TGase may be the most upstream event known and is triggered by the binding of *E. chaffeensis*, although the mechanism of TGase activation is unclear. In our study, THP-1 cells showed constitutive TGase activity, and the in situ activity rapidly increased as soon as 30 s p.i. when *E. chaffeensis* was added (Lin and Rikihisa, unpublished data). TGase is required for ligand-receptor complex clustering and endocytosis of various ligands, such as viruses, low-density lipoprotein, α_2-macroglobulin, epidermal growth factor, transferrin, and polypeptide hormones like insulin (1, 11, 27, 29, 47). Our results suggest that TGase has a more extensive role in *E. chaffeensis* in transducing signals for entry, as well as in sustaining proliferation. Which proteins are transglutaminated and how these proteins induce tyrosine phosphorylation of PLC-γ2 remain to be studied. *E. chaffeensis* inclusions are part of the transferrin and transferrin receptor membrane recycling pathway, as indicated by the uptake of fluorescein isothiocyanate-conjugated iron-saturated transferrin by the inclusions (6). Since the internalization and recycling of transferrin and the transferrin receptor are dependent on TGase (26), this is another site at which MDC can block ehrlichial proliferation in host cells after entry. Transferrin receptors can deliver iron to the cytoplasm through a continual cycle that shuttles the ligand transferrin between the endosomal compartments and the plasma membrane. Iron is essential for ehrlichial survival, since ehrlichiae are extremely sensitive to the intracytoplasmic iron chelator deferoxamine (7).

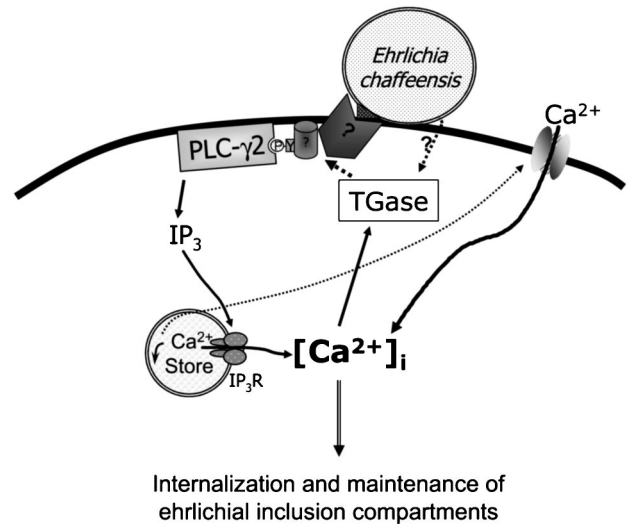Internalization and maintenance of ehrlichial inclusion compartments

FIG. 8. Model of signaling pathway induced by *E. chaffeensis* infection. Binding of *E. chaffeensis* to its receptor(s) on host monocytes triggers protein cross-linking by TGase, which activates the receptor or nonreceptor tyrosine kinase. PTK activation mediates the translocation and tyrosine phosphorylation of PLC-γ2, which produces IP_3. IP_3 in turn induces Ca^{2+} release from internal stores. The depletion of Ca^{2+} from internal stores activates store-operated calcium channels on the plasma membrane, and Ca^{2+} influx from external spaces contributes to the bulk of the increase in $[Ca^{2+}]_i$ in response to the infection. Activation of TGase by Ca^{2+} constitutes a positive feedback loop, and this may facilitate entry of ehrlichiae into host cells or ehrlichial infection. The dotted lines indicate suggested pathways, and the solid lines indicate proven pathways.

TGase is a calcium-dependent enzyme, and our data showed that it is also able to indirectly regulate $[Ca^{2+}]_i$ by activation of a tyrosine kinase–PLC-γ2 pathway. This implies that a positive feedback loop consisting of Ca^{2+} and TGase may facilitate ehrlichial entry and infection. As shown in Fig. 8, the results of the present study demonstrated that *E. chaffeensis* activates the following signaling sequences required for internalization: activation of TGase, protein tyrosine phosphorylation, PLC-γ2 activation, production of IP_3, and increase in $[Ca^{2+}]_i$.

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