Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells

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Summary

Invasion of host cells by *Toxoplasma gondii* is accompanied by secretion of parasite proteins that occurs coincident with increases in intracellular calcium. The source of calcium mobilized by the parasite and the signals that promote calcium increase remain largely undefined. We demonstrate here that intracellular stores of calcium in the parasite were both necessary and sufficient to support microneme secretion, motility and invasion of host cells. In contrast, host cell calcium was largely unaltered during parasite entry and not essential for this process. During parasite motility, cytosolic calcium levels underwent dramatic and rapid fluxes as imaged using the calcium indicator fluo-4 and time-lapse microscopy. Surprisingly, intracellular calcium in the parasite cytosol was rapidly quenched during the initial stages of host cell invasion, suggesting that while it is needed to initiate motility, it is not required to complete entry. These studies indicate that intracellular calcium stores govern secretion and motility by *T. gondii* and that the essential role of calcium in these events explains its requirement for cell entry.

Key words: Calcium, Invasion, Parasite, Secretion, Motility, Intracellular, Signaling

Introduction

The protozoan parasite *Toxoplasma gondii* is responsible for widespread infections in animals and humans, and is the cause of toxoplasmosis. One of the distinguishing features of the phylum Apicomplexa to which *T. gondii* belongs is the presence of apical secretory organelles. Discharge of apical organelles occurs during invasion (Carruthers and Sibley, 1997), and their contents mediate attachment to host cells and formation of the specialized parasitophorous vacuole occupied by the parasite (Carruthers, 2002). The first of the specialized secretory organelles to be discharged are micronemes, which contain a variety of adhesive and transmembrane proteins (Soldati et al., 2001; Tomley and Soldati, 2001). The proposed function of microneme proteins is to bridge parasite and host cell membranes during invasion. Consistent with this, several micronemal proteins have been shown to bind to host cells or cell surface molecules including MIC1 (Fourmaux et al., 1996), MIC2 (Carruthers et al., 2000; Brossier et al., 2003), MIC3 (Garcia-Réguet et al., 2000; Cérede et al., 2002), AMA-1 (Donahue et al., 2000; Hehl et al., 2000) and MIC4 (Brecht et al., 2000). The diversity of cell adhesive motifs among various micronemal proteins may reflect the wide rage of host cell types that are recognized by apicomplexan parasites.

Artificially increasing intracellular calcium in the parasite through the use of calcium ionophores A23187 or ionomycin triggers parasite microneme secretion in the absence of host cells (Carruthers and Sibley, 1999). Conversely, chelation of intracellular calcium in the parasite blocks microneme secretion and invasion (Carruthers and Sibley, 1999). Studies showing that EGTA blocks invasion by *T. gondii* suggest that extracellular calcium might also play a role in the host-parasite interaction (Pezzella et al., 1997). Fluorescent imaging studies have revealed that parasites in association with host cells show elevated levels of cytoplasmic calcium (Vieira and Moreno, 2000). These studies have led to a model suggesting that contact with host cells triggers a rise in cytoplasmic calcium in the parasite, thus triggering exocytosis of adhesins that are needed for strengthening attachment and promoting cell entry.

T. gondii possess several stores of calcium, including the acidocalcisomes, mitochondria, and the endoplasmic reticulum (Moreno and Zhong, 1996). Whether all or some of these stores are released during invasion is unknown. Recent studies have identified IP_3 as a second messenger that mediates increases in intracellular calcium in *T. gondii* following artificial stimuli (Lovett et al., 2002). *T. gondii* also responds to caffeine and ryanodine, suggesting apicomplexans also contain a ryanodine-like response channel for regulating release of intracellular calcium (Lovett et al., 2002).

Although previous studies have indicated an important role for calcium during parasite invasion of host cells, the source of calcium and whether it acts within the parasite or the host cell remain unresolved. To resolve this important issue, we examined the contribution of intracellular and extracellular calcium sources during parasite motility and invasion into host cells. Additionally, we monitored dynamic calcium changes in parasites and host cells during invasion using the calcium sensitive indicator fluo-4.

Materials and Methods

Parasite culture

T. gondii strain RH (ATCC# 50838) was propagated as tachyzoites in

human foreskin fibroblast cells as previously described (Lovett et al., 2002). Freshly harvested parasites were purified by passage through a 23-gauge needle and filtration through three micron Nuclepore membranes (Whatman, Clifton NJ). All cultures tested free of *Mycoplasma* using the GenProbe™ mycoplasma detection system (GenProbe, San Diego, CA).

Chemicals and solutions

EGTA (Sigma, St Louis, MO) stock solutions (2×) were prepared in low calcium Ringer's (155 mM NaCl, 3 mM KCl, 5 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, 0.1 mM EGTA) and adjusted to pH 6.0 or 7.2. BAPTA-AM [1,2-*bis* (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, sodium salt] and BAPTA were purchased from Calbiochem (San Diego, CA), and fluo-4 AM was purchased from Molecular Probes (Eugene, OR). All other reagents were analytical grade. Stock solutions of BAPTA were prepared in distilled water, and BAPTA-AM solutions were made in DMSO.

SDS polyacrylamide gel electrophoresis and western blotting SDS-PAGE was performed in 7% minigels under reducing conditions and transferred to nitrocellulose as previously described (Carruthers and Sibley, 1999). Western blotting was performed with mouse anti-TgMIC2 monoclonal antibody 6D10 (ascites, 1:10,000) (Carruthers and Sibley, 1999) and rabbit polyclonal anti-TgACT1 actin antibody (1:10,000) (Dobrowolski et al., 1997). Blots were detected using enzyme conjugated secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA) combined with SuperSignal ECL (Pierce, Rockford, IL).

Gliding assay

Glass coverslips (13 mm round) were incubated overnight with complete media (Dulbecco's modified Eagle's medium, 10 mM HEPES, 44 μM sodium bicarbonate, 10% fetal bovine serum, 2 mM glutamine, 20 μg/ml gentamicin). Parasites were resuspended in low calcium Ringer's. BAPTA-AM was loaded into host cells or parasites for 10 minutes at 18°C, while BAPTA or EGTA were added to parasites immediately prior to use to minimize leaching of intracellular calcium stores. Parasites were added to coverslips previously washed with low calcium Ringer's and incubated for 15 minutes in a 37°C water bath. After removal from the water bath, coverslips were washed twice with warm low calcium Ringer's and fixed with 2.5% formalin for 20 minutes at 4°C. Coverslips were stained with Alexa Fluor® 488 (Molecular Probes) conjugated monoclonal antibody DG52 against SAG1 (1:1000) and observed using a Zeiss Axioskop (Carl Zeiss) equipped with epifluorescence and phase contrast optics. Images were captured using an ORCA-ER digital cooled CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) at 63× magnification controlled by Openlab v3.0.8 imaging software (Improvision, Lexington, MA).

Microneme secretion assay

Parasites were resuspended at 10^8/ml in low calcium Ringer's. BAPTA-AM was loaded for 10 minutes at 18°C, while BAPTA or EGTA were added immediately prior to stimulation to minimize leaching of intracellular Ca^{2+} stores. Secretion was stimulated by addition of ethanol to 1% and incubation at 37°C for 2 minutes (Carruthers et al., 1999b). An unstimulated sample, that was not raised to 37°C and to which ethanol was not added, served as a negative control for background. Parasite supernatants were separated from pellets by centrifugation at 4°C and run on 7% SDS-PAGE gels. Each gel contained standard dilutions of a parasite cell lysate corresponding to percentages of the total number of parasites used. Inadvertent lysis of parasites was monitored by the release of constitutively expressed actin, which is 98% globular in *T. gondii* (Wetzel et al., 2003).

Attachment and invasion assays

Glass-bottom 10 mm microwells (MatTek Corp, Ashland, MA) were seeded with host cells to a final density of 60 to 80% confluency. Parasites were resuspended at 10^8 per ml in low calcium Ringer's. BAPTA-AM was loaded into monolayers of host cells or parasites for 10 minutes at room temperature, while BAPTA or EGTA were added to parasites immediately prior placement on coverslips to minimize leaching of intracellular calcium stores. Microwells were washed with low calcium Ringer's before the addition of parasites and incubated for 15 minutes in a 37°C water bath. After removal from the water bath, coverslips were washed twice with warm low calcium Ringer's and fixed with 2.5% formalin for 20 minutes at 4°C. Coverslips were blocked for 30 minutes with PBS containing 5% (v/v) fetal bovine serum, and 5% (v/v) normal goat serum. Coverslips were sequentially stained with Alexa Fluor® 594 (Molecular Probes) conjugated monoclonal antibody DG52 against SAG1 (1:1000) to visualize extracellular parasites, permeabilized with 0.01% saponin, then stained with Alexa Fluor® 488 conjugated monoclonal antibody DG52 against SAG1 (1:1000) (Håkansson et al., 1999) to visualize intracellular parasites. Coverslips were observed with a Zeiss Axioskop microscope and a 63× objective. Results of three independent experiments were compiled. For each experimental condition, five fields with approximately 20 parasites each were scored for the number of parasites intracellular, extracellular but touching a fibroblast, or extracellular but not in contact with host cells.

Video microscopy

Freshly egressed parasites were loaded with 300 nM Fluo-4 for 5 minutes at 37°C, centrifuged at 400 ***g*** for 5 minutes at room temperature, and resuspended in 37°C Ringer's plus calcium (155 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose). Sub-confluent monolayers of host cells were loaded by resuspending in Ringer's plus calcium containing 2 μM fluo-4 and incubating at 37°C 15 minutes. After incubation, parasites or cells were washed and resuspended in Ringer's plus calcium. Parasites were added to the culture dishes containing host cells dishes and observed on a Zeiss Axiovert equipped with phase contrast, epifluorescence microcopy and a temperature-controlled stage (Medical Systems, Greenvale, NY) held at 37°C. Parasite motility was observed within minutes after placing the dish on the heated stage and images were recorded over a period of up to 15 minutes. Time-lapse images were collected at two frames per second under low-light illumination using an ORCA-ER digital cooled camera at 63× magnification and 640×480 pixels controlled by Openlab v3.0.8 imaging software (Improvision, Lexington, MA). Phase and fluorescent images were cropped, merged, and saved as Quicktime movies (v5.0).

Results

Extracellular calcium is not required for attachment or invasion

To determine whether depletion of extracellular calcium affected parasite invasion into host cells, we used BAPTA or EGTA to chelate residual calcium in Ringer's solution that lacked any direct source of calcium (low calcium Ringer's). Invasion assays were performed by incubating parasites with host cells grown on glass coverslips. Parasites were scored as attached to host cells versus intracellular on the basis of differential staining with antibodies before and after addition of detergent, respectively (see Materials and Methods). In order to examine the role of extracellular calcium, parasite attachment and invasion were evaluated in media containing 1 mM BAPTA, a diluent control, or Ringer's containing 5 mM

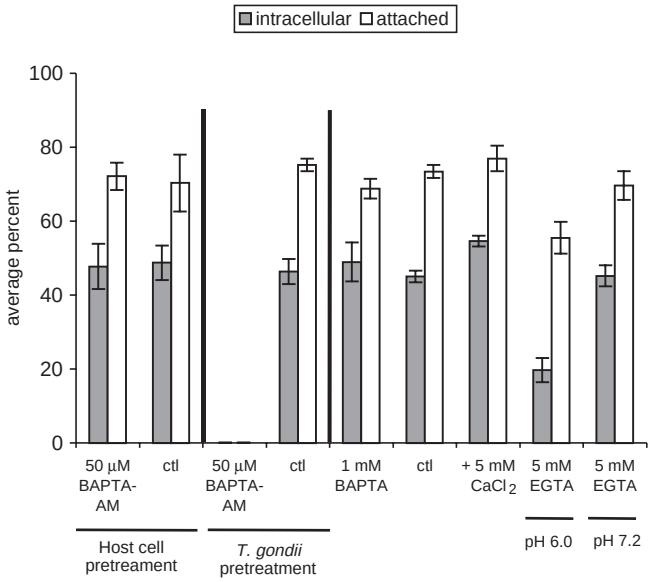

Fig. 1. Effect of extracellular calcium on attachment and invasion. Host cells were challenged with parasites in the presence of 1 mM BAPTA, a diluent control (ctl; refers to water or DMSO), 5 mM $CaCl_2$, or 5 mM EGTA in pH 6.0 or pH 7.2 low calcium Ringer's. To examine the role of intracellular calcium, parasites were pretreated with 50 μM BAPTA-AM (*T. gondii* pretreatment). In parallel, host cells were loaded with 50 μM BAPTA-AM (host cell pretreatment) or a diluent control (ctl) prior to contact with parasites. Invasion (solid bars) and attachment (open bars) were monitored as described in the methods and average percentages calculated from the results of three independent experiments (mean±s.e.).

$CaCl_2$. Parasite attachment and invasion of host cells was similar under all three conditions (Fig. 1).

Previous reports indicate that chelation of calcium with high levels of EGTA decreased parasite invasion (Pezzella et al., 1997). However, our initial attempts to replicate this finding indicated that dilution of a 20× stock solution of EGTA (pH 8.0) into complete culture medium resulted in acidification. Nitrilo nitrogens in EGTA bind protons with pK_as of 8.96 and 9.58 (Martell and Smith, 1974), leading to pH-sensitive affinity for calcium. Consequently, the dilution of EGTA stock solutions into calcium-containing medium releases 2 mol H^+ for each mol Ca^{2+} bound, thus resulting in acidification. Previous studies have indicated that acidic pH is detrimental to parasite invasion (Sibley et al., 1985). To determine the relative contribution of acidification versus calcium chelation, we made 5 mM EGTA solutions in low calcium Ringer's and adjusted them to pH 6.0 or pH 7.2. These solutions were used for attachment and invasion assays shown in Fig. 1. Incubation of parasites in the presence of 5 mM EGTA at a medium pH of 7.2 did not affect attachment or invasion into host cells. In contrast, parasite invasion in the presence of 5 mM EGTA at a medium pH of 6.0 was substantially inhibited, although attachment appeared unchanged (Fig. 1). Collectively, these results indicate that although decreased pH reduces invasion, chelation of extracellular calcium has no effect.

We also examined the effect of chelating intracellular calcium on the attachment and entry of parasites into host cells. Loading of the cell-permeant chelator BAPTA-AM into host cells prior to invasion had no effect when compared to a diluent control (Fig. 1). Thus, host cell calcium does not appear necessary for invasion by *T. gondii*. Alternatively, preloading parasites with BAPTA-AM prior to invasion inhibited both attachment and invasion of host cells (Fig. 1) (Carruthers et al., 1999a).

Extracellular calcium is not required for trail formation

Parasite invasion into host cells is an active process that depends on parasite motility (Dobrowolski and Sibley, 1996). Parasites exhibit contact-dependent gliding motility that results in formation of membrane trails on the substratum (Håkansson et al., 1999). We examined whether the presence or absence of extracellular calcium affected motility as monitored by trail formation. Parasites were treated with BAPTA-AM, BAPTA, or EGTA before addition to coverslips where they were allowed to glide for 15 minutes. After washing and fixation, trails were visualized by staining of the parasite surface protein SAG1. BAPTA-AM pretreatment inhibited trail formation markedly versus a diluent control (Fig. 2). In contrast, BAPTA treatment had no apparent effect, and a similar density of trails was observed as in the diluent treated control (Fig. 2B,C). Addition of 5 mM calcium did not alter motility compared to the diluent control (Fig. 2D), yet trails appeared discontinuous and punctuated upon close examination. Interestingly, treatment of parasites with EGTA in either pH 6.0 or pH 7.2 media also did not affect trail formation (Fig. 2E,F).

Extracellular calcium is not required for microneme secretion

To determine the source of calcium important for influencing microneme secretion, we treated parasites with 50 μM BAPTA-AM to chelate intracellular calcium, or used 1 mM BAPTA or 5 mM EGTA to chelate extracellular calcium. Parasites were then placed at 37°C to stimulate secretion. Cells were removed by centrifugation and secretion was evaluated by detecting MIC2 in the supernatants by western blotting (Fig. 3). MIC2 is a convenient marker for microneme secretion because the secreted 95-100 kDa form (sMIC2) is released into the supernatant, whereas the cell-associated 115 kDa form (cMIC2) remains in the parasite pellet (Carruthers et al., 2000). Constitutive expression of actin was used to control for inadvertent lysis of the parasites during the experiment and was typically between 1% and 5%, as indicated by comparison with dilutions of parasite cell standards (cell stds) (Fig. 3).

Secretion of MIC2 was inhibited by pre-loading of parasites with BAPTA-AM in comparison with a DMSO diluent control, indicating an increase in intracellular calcium is necessary for secretion (Fig. 3). Treatment with non-permeant BAPTA did not affect MIC2 secretion, indicating that extracellular calcium is not required. Interestingly, addition of excess (5 mM) calcium did not provoke additional secretion, providing evidence that extracellular calcium does not increase the efficiency of secretion (Fig. 3). Secretion of MIC2 after treatment with EGTA at pH 7.2 did not compromise in the amount of protein released (Fig. 3). In contrast, treatment with EGTA at pH 6.0 led to a reduction

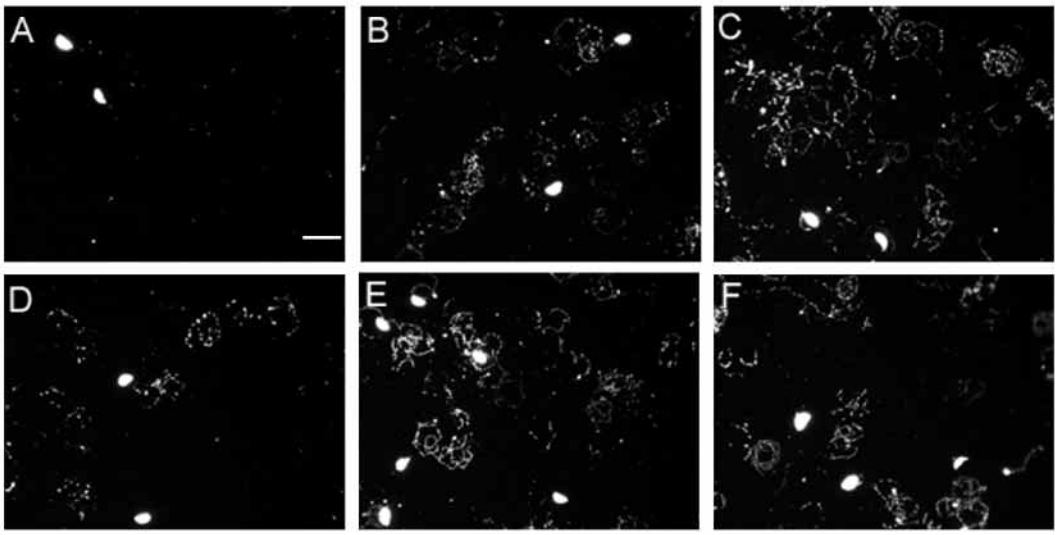

Fig. 2. Effect of calcium on trail formation by gliding parasites. Formation of gliding trails by parasites treated with (A) BAPTA-AM, (B) BAPTA, (C) a diluent control, (D) $CaCl_2$, (E) EGTA in pH 6.0 low calcium Ringer's or (F) EGTA in pH 7.2 low calcium Ringer's. Coverslips were stained with a fluorescently conjugated antibody against the plasma membrane marker SAG1 to visualize trails. Images shown are representative of three independent experiments showing similar results. Bar, 10 μm.

in the amount of MIC2 secreted when compared with the control (Fig. 3). Addition of 1% ethanol to stimulate secretion (Carruthers et al., 1999b) was used as a positive control, whereas unstimulated parasites that were not placed at 37°C were used as a negative control (Fig. 3). Collectively these results show that intracellular calcium in the parasite is essential for microneme secretion, whereas extracellular calcium plays little role in this process.

Host cell calcium does not change during parasite invasion

Although our studies using BAPTA-AM indicated that host cell calcium was not required for parasite invasion, we were interested in determining whether host cell calcium changed during the process of parasite invasion. We monitored host cell calcium during parasite invasion using the indicator fluo-4, which is a qualitative indicator of intracellular calcium in the range of 0.1 to 1 μM (Gee et al., 2000). Monolayers of human fibroblasts were loaded with 2 μM fluo-4 AM and the excess dye washed away, leaving fluo-4 localized throughout the cytoplasm. Images were taken at ~0.5 second intervals, alternating between phase contrast and fluorescence modes. Unstimulated fibroblasts showed calcium increases lasting approximately six seconds, during which a wave of increased fluorescence spread across the cell (Movie 1, available at jcs.biologists.org/supplemental). The magnitude of these changes was dramatic, with increased signals of fluo-4 being up to 250% higher than background levels (data not shown, *n*=3 examples monitored over a 15-30 second period). These events were rare, occurring about once every 10 minutes (data not shown). Fibroblast calcium fluxes were likely to be associated with normal signaling processes, as the cells appeared healthy both before and after transients occurred.

To examine changes that occur during invasion, parasites were added to fluo-4-loaded fibroblasts, and phase and fluorescent images were acquired at 0.5 second intervals between each set of images. Host cell calcium levels remained relatively constant during invasion as judged by the fact that only small, localized increases in fluorescence were observed (Fig. 4B,C; Movie 2, available at jcs.biologists.org/supplemental). The magnitude of changes in the fluo-4 signal in host cells was ≤20% different from background (data not shown, *n*=3 separate examples monitored for 15-30 seconds), in contrast to the rather dramatic natural changes in host cell calcium that occasionally occurred in the absence of invasion (see Fig. 4A). These observations indicate that parasite invasion occurs without an active participation or response of calcium from the host cell.

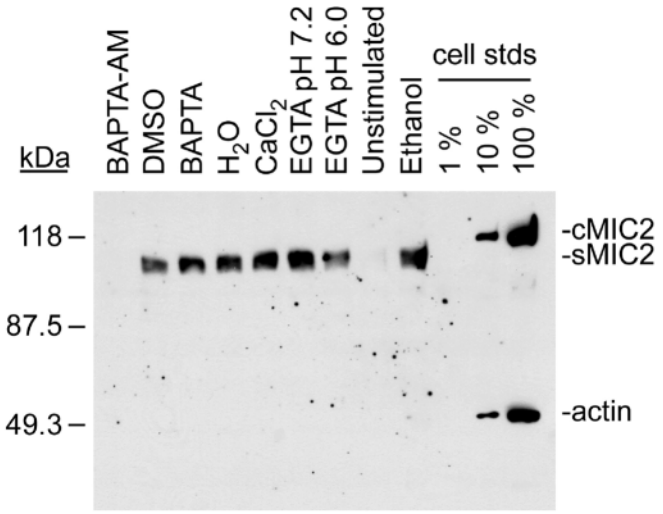

Fig. 3. Effect of calcium on microneme secretion by *T. gondii*. Western blot analysis of secretion by parasites treated with agents to chelate extracellular or intracellular calcium. Chelation of intracellular calcium with BAPTA-AM completely blocked secretion. Cellular MIC2 (cMIC2) forms present in dilutions of parasite pellets (cell stds) migrate at a larger size compared to the secreted form of MIC2 (sMIC2) found in supernatants. Ethanol combined with incubation at 37°C was used to stimulate secretion in all the samples. Unstimulated cells that were not treated with ethanol or raised to 37°C were used as a negative control. Unstimulated actin provided a control for inadvertent cell lysis in the experiment. Example shown is a representative blot from three independent experiments with similar results.

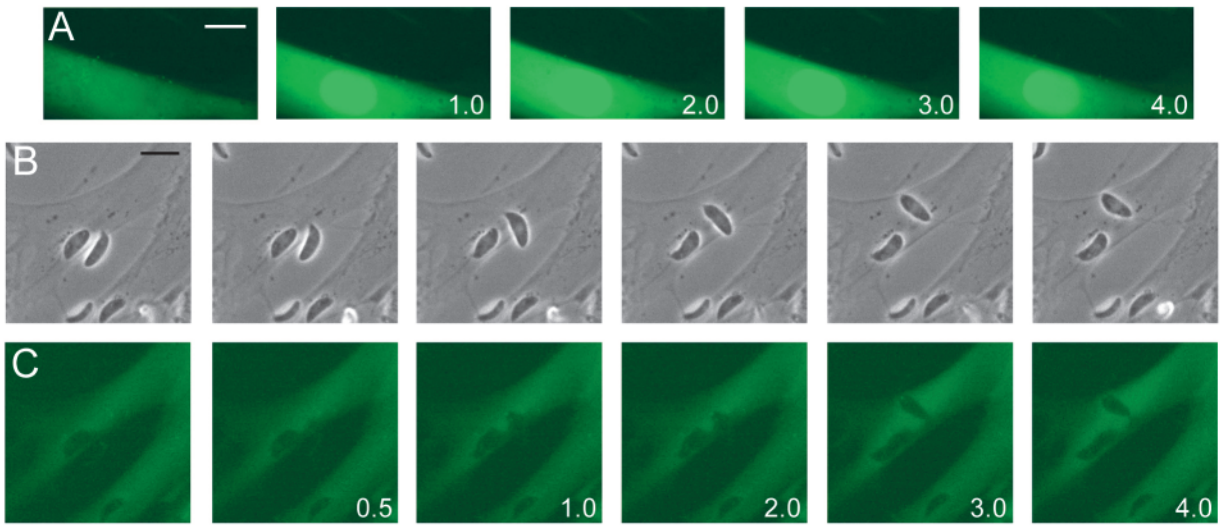

Fig. 4. Host calcium response during parasite invasion. (A) Fluo-4 calcium flux in untreated host cells. Fluo-4 AM loaded fibroblasts were observed by time-lapse fluorescent video microscopy with frames captured every 0.5 seconds. The bright object in the center is the nucleus of the cell. Shown are selected frames with the time elapsed between frames in seconds indicated. Bar, 10 μm. (B) Phase images of *T. gondii* parasites invading fluo-4 AM loaded fibroblasts and recorded by time-lapse video. Phase and fluorescent images were acquired with 0.5 seconds between each pair. Bar, 5 μm. (C) Fluorescent images corresponding to the panels in (B) are shown, with the elapsed time between frames indicated. Also see Movie 2 at jcs.biologists.org/supplemental.

Calcium fluxes occur during parasite gliding but are rapidly down regulated during invasion

Previous studies have suggested that increases in parasite intracellular calcium occur during contact with the host cell (Vieira and Moreno, 2000); however, the kinetics of calcium increase were not examined. We predicted that parasites entering cells would show increases in calcium during attachment and invasion. We used fluo-4 AM loaded tachyzoites to visualize qualitative changes in parasite calcium during gliding motility and host cell invasion.

Gliding was associated with brightly fluorescent parasites that underwent periodic cycles of increase followed by dampening of the signal (Fig. 5A; Movie 3, available at jcs.biologists.org/supplemental). Calcium fluxes correlated with parasites that were actively motile and often preceded a burst of motility; whereas dimly stained cells remained immotile. Kinetic changes in fluo-4 fluorescence were plotted over time by taking the average pixel intensity of the parasite from successive frames of the video recording (Fig. 6A). The cycles of increased fluorescence were monitored from independent recordings to determine the average time between successive cycles and the relative length of the cycles (Table 1). Typically, individual cycles lasted ~30 seconds and were closely spaced in sequence. The initial cycle was the brightest with successive cycles becoming dimmer. The drop in intensity correlated with the gradual loss of motility. Irrespective of whether they are loaded with fluo-4, parasites that are examined by phase contrast or epifluorescence illumination typically maintain motility for only one to two minutes. It is uncertain whether this decrease is because of photodamage or loss of essential factors such as energy stores that are necessary to maintain activity.

We also followed the calcium response of fluo-4-labeled parasites while they invaded fibroblasts. Surprisingly, the bright fluorescence exhibited by motile parasites rapidly diminished during the initial steps of invasion (Fig. 5B,C; Movie 4, available at jcs.biologists.org/supplemental). The drop in signal intensity following invasion occurred more rapidly than had been observed with fluxes occurring during gliding motility (Table 1 and Fig. 6B). The decrease in signal was not due to photobleaching, as other brightly labeled parasites in the same field did not display similar decreases in intensity (data not shown). Intracellular parasites remained dim even when analyzed for extended periods of time (up to five minutes, data not shown).

Discussion

Calcium is a widespread intracellular second messenger that is known to play a critical role in the invasion of mammalian cells by a number of microorganisms. Several intracellular pathogens modify host cell calcium signaling during invasion. Examples include *Trypanosoma cruzi* modulation of fibroblast signaling (Rodriguez et al., 1995), activation of calcium-dependent interleukin-8 release from endothelial cells by *Salmonella typhimurium* (Geritz et al., 2000), and inhibition of

Table 1. Duration of calcium transients in gliding and invading parasites

Event	Duration*	Peak to low‡	Time between§
Gliding	28.4±10.0	20.7±6.3	27.3±6.8
Invasion	NA	11.7±1.7	NA

Averages ± s.d. in seconds; *n*=6 for gliding, *n*=5 for invasion. NA, not applicable.

*Duration represents the average time between the beginnings of successive cycles.

‡Peak to low indicates the time from the highest signal to the lowest signal in a given cycle.

§Time between indicates the average time between successive peaks.

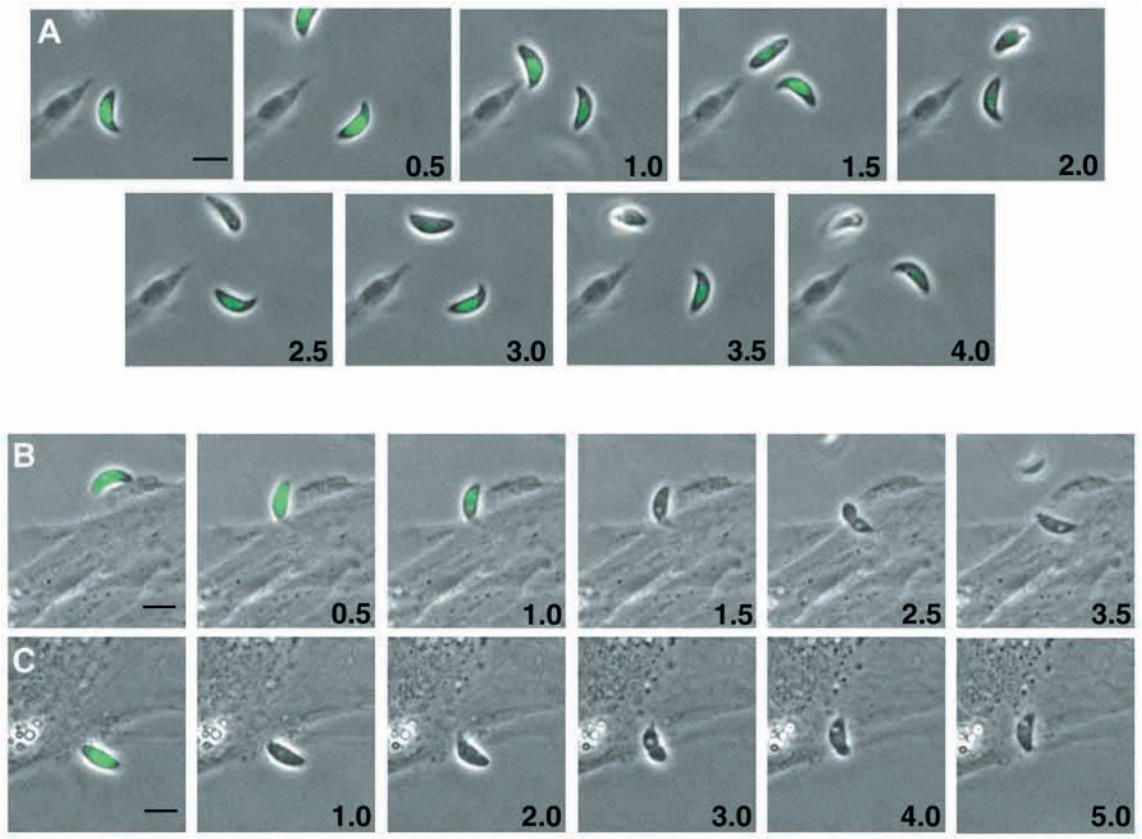

Fig. 5. Calcium responses in *T. gondii* during gliding and cell invasion. (A) Calcium fluxes in gliding parasites. Fluo-4 AM loaded parasites were observed by time-lapse fluorescent video microscopy, with both phase and fluorescent images acquired with 0.5 seconds between each pair. Shown are selected images that were merged, with the elapsed time between frames indicated. Scale bar, 5 μm. (B,C) Merged images of fluo-4-labeled parasites during invasion into fibroblasts. Shown are selected merged pairs of images, with the elapsed time between subsequent frames indicated. Scale bar, 5 μm. Also see Movie 4 at jcs.biologists.org/supplemental.

macrophage calcium signaling by *Mycobacterium tuberculosis* (Malik et al., 2000). These examples from a wide variety of pathogens led us to consider whether changes in host cell calcium contribute to invasion of *T. gondii*.

We addressed the role of host cell calcium by using the intracellular chelator BAPTA-AM. Chelation of host cell calcium using BAPTA-AM had no effect on parasite attachment or invasion. The concentration of BAPTA-AM used should have effectively chelated intracellular calcium in the fibroblasts based on other studies using lower concentrations of this compound on fibroblasts (Scheidegger et al., 1999; Shahrestanifar et al., 1999), and the observation that the cell monolayer began to detach from the substrate if loading times were prolonged (data not shown). Thus, unlike the entry of *T. cruzi* into mammalian cells (Rodriguez et al., 1995), changes in host cell calcium do not appear to be required for efficient entry of *T. gondii*.

Consistent with the inhibitor studies, direct monitoring of intracellular calcium in host cells indicated that only weak local calcium transients occur during parasite invasion. Such calcium transients may be associated with membrane perturbation by the parasite that was previously observed as a spike in capacitance of patch-clamped host cells (Suss-Toby et al., 1996). Alternatively, it could indicate activation of local host signaling pathways required for membrane repair. In either case, the very low response observed in the host cell parallels our finding that host cell calcium was not required for parasite invasion. These findings support previous observations that indicate a relatively passive role of the host cell during parasite invasion. For example, unlike phagocytosis or entry of many bacterial pathogens, invasion of mammalian cells by *T. gondii* does not evoke changes in the host cell cytoskeleton, tyrosine phosphorylation or membrane ruffling (Morisaki et al., 1995; Håkansson et al., 1999).

In contrast to the lack of an essential role for host cell calcium, chelation of intracellular calcium in the parasite using BAPTA-AM treatment prevented microneme secretion and dramatically reduced both motility and invasion into host cells. Since levels of calcium in the parasite appeared critical to the process of invasion, we sought to identify whether extracellular calcium was necessary for parasite invasion into host cells. A previous report that chelation of extracellular calcium by EGTA inhibited *T. gondii* invasion hypothesized that a flux of calcium from outside to inside the parasite was required during invasion (Pezzella et al., 1997). However, as we show here, this effect was likely to be as a result of the acidifying affects of EGTA on the medium. When EGTA containing medium was adjusted to pH 7.2, or when the non-acidifying chelator BAPTA was used, no effect of chelating extracellular calcium was observed. Furthermore, adding excess calcium had no

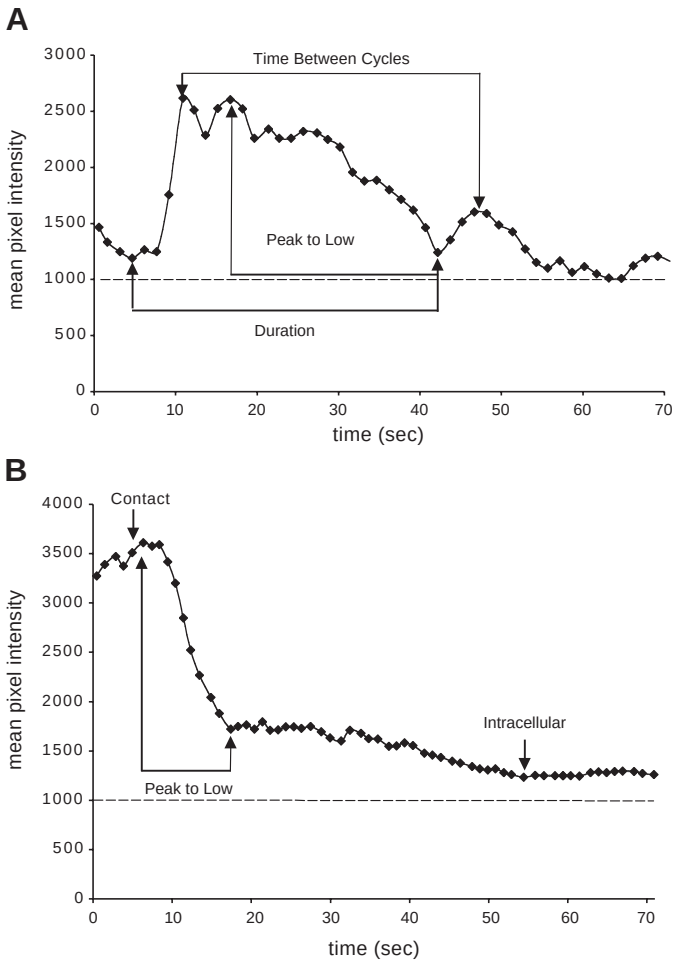

Fig. 6. Parasite calcium responses during gliding and invasion. (A) Frame-by-frame quantification of calcium changes in a parasite gliding on serum-coated glass. The parameters monitored for data in Table 1 are indicated. (B) Frame-by-frame analysis of calcium changes in a parasite during host cell invasion. Arrows indicate first contact with the host cell (contact), as well as when the parasite is fully inside the host cell (intracellular). Background is indicated by the dashed line.

influence on parasite invasion into host cells. We therefore conclude that extracellular calcium is not required for invasion of cells by *T. gondii*. These results indicate that a calcium-induced-calcium-entry pathway, where initial increases in cytosolic calcium result in influx of extracellular calcium from outside the cell (Berridge et al., 2000a; Berridge et al., 2000b), is not essential for parasite invasion of host cells.

To explore the role of calcium on *T. gondii* secretion and motility, we used a similar approach to look at the role of extracellular versus intracellular calcium sources in mediating these events. Chelation of intracellular calcium prevented microneme release, consistent with a previous report (Carruthers and Sibley, 1999), whereas chelation of extracellular calcium had no effect. Likewise, although chelation of intracellular calcium with BAPTA-AM prevented motility, chelation of extracellular calcium had little effect on motility. Collectively, these studies indicate that intracellular calcium in the parasite controls microneme secretion and motility.

Monitoring of intracellular calcium in the parasite revealed oscillating fluxes in motile parasites, either immediately preceding or during gliding motility. Previous models suggested that calcium increases would correlate with contact with the host cells immediately prior to invasion. Instead, our observations indicate that oscillating increases in parasite intracellular calcium occur during gliding, regardless of contact with host cells. These changes in calcium may reflect an important role for this second messenger in regulating motility and/or release of adhesins that are necessary for attachment to the substratum. Calcium transients that were observed during motility halted abruptly and the fluorescent calcium signal dramatically decreased during invasion of the parasite into host cells. This finding was unexpected, since previous studies have shown increased cytoplasmic calcium in parasites that were associated with host cells (Vieira and Moreno, 2000). These previous findings may have reflected parasites moving while on top of host cells, which could explain their increased calcium levels. Collectively our findings suggest a more complex model wherein increases in intracellular calcium are needed for motility, while cell entry does not require continued elevation of intracellular calcium in the parasite. Furthermore, the rapid shut off of intracellular calcium in invading parasites may be important for downregulating cellular processes, such as microneme secretion and motility, once invasion is complete.

Despite the central importance of calcium in parasite secretion and motility, relatively little is known about the intracellular pools of calcium or how the release of calcium is regulated. The oscillating patterns of calcium in gliding parasites suggest that intracellular release channels and re-uptake mechanisms are highly active in the parasite. In most cells, the major pool of mobilizable calcium is the endoplasmic reticulum (ER) where release is mediated by IP_3 or ryanodine-type channels (Berridge et al., 2000a; Berridge et al., 2000b). *T. gondii* responds to agonists/antagonists of both IP_3-type release channels and ryanodine-type channels, suggesting both pathways could operate in the parasite (Lovett et al., 2002). Typically, ER stores are refilled by calcium transporters known as SERCA-type ATPases, which can be inhibited by thapsigargin (Thastrup et al., 1989). Protozoan parasites, including *T. gondii*, are sensitive to this inhibitor, and yet they also have thapsigargin-insensitive calcium pools (Carruthers et al., 1999). SERCA-type ATPases have not been described in *T. gondii*, although several putative orthologs are present in the genome databases for *T. gondii* (http://ToxoDB.org/ToxoDB.shtml). *T. gondii* also contains plasma membrane type Ca-ATPases, one of which has recently been shown to localize both to the plasma membrane and a unique organelle called the acidocalcisome (Luo et al., 2001). These acidic, calcium-rich organelles appear to play an important role in polyphosphate metabolism and serve as a storage site for calcium in protozoan cells (Docampo and Moreno, 2001; Rodrigues et al., 2002).

Our investigations demonstrate that intracellular calcium plays a crucial role in controlling protein secretion and motility in the parasite *T. gondii*. The complete reliance of the parasite on its own intracellular calcium stores for motility presents a potential target for intervention. Further studies might reveal sufficient differences between parasite and host calcium homeostasis mechanisms that can be used to disrupt calcium-regulated secretion and/or motility, and thereby prevent infection.

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References

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000a). Signal transduction. The calcium entry pas de deux. *Science* **287**, 1604-1605.

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000b). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11-21.

Brecht, S., Carruthers, V. B., Ferguson, D. J., Giddings, O. K., Wang, G., Jaekle, U., Harper, J. M., Sibley, L. D. and Soldati, D. (2000). The Toxoplasma micronemal protein MIC4 is an adhesin composed of six conserved apple domains. *J. Biol. Chem.* **276**, 4119-4127.

Brossier, F., Jewett, T. J., Lovett, J. L. and Sibley, L. D. (2003). C-terminal processing of the Toxoplasma protein MIC2 is essential for invasion into host cells. *J. Biol. Chem.* **278**, 6229-6234.

Carruthers, V. B. (2002). Host cell invasion by the opportunistic pathogen *Toxoplasma gondii*. *Acta. Trop.* **81**, 111-122.

Carruthers, V. B., Giddings, O. K. and Sibley, L. D. (1999a). Secretion of micronemal proteins is associated with Toxoplasma invasion of host cells. *Cell. Microbiol.* **1**, 225-236.

Carruthers, V. B., Moreno, S. N. J. and Sibley, L. D. (1999b). Ethanol and acetaldehyde elevate intracellular [Ca^{2+}] calcium and stimulate microneme discharge in *Toxoplasma gondii*. *Biochem. J.* **342**, 379-386.

Carruthers, V. B., Sherman, G. D. and Sibley, L. D. (2000). The Toxoplasma adhesive protein MIC2 is proteolytically processed at multiple sites by two parasite-derived proteases. *J. Biol. Chem.* **275**, 14346-14353.

Carruthers, V. B. and Sibley, L. D. (1997). Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *Eur. J. Cell Biol.* **73**, 114-123.

Carruthers, V. B. and Sibley, L. D. (1999). Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol. Microbiol.* **31**, 421-428.

Cérede, O., Dubremetz, J. F., Bout, D. and Lebrun, M. (2002). The *Toxoplasma gondii* protein MIC3 requires pro-peptide cleavage and dimerization to function as an adhesin. *EMBO J.* **21**, 2526-2536.

Dobrowolski, J. M., Niesman, I. R. and Sibley, L. D. (1997). Actin in *Toxoplasma gondii* is encoded by a single-copy gene, *ACT1* and exists primarily in a globular form. *Cell Motility Cytoskeleton* **37**, 253-262.

Dobrowolski, J. M. and Sibley, L. D. (1996). Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**, 933-939.

Docampo, R. and Moreno, S. N. J. (2001). The acidocalcisome. *Mol. Biochem. Parasitol.* **33**, 151-159.

Donahue, C. G., Carruthers, V. B., Gilk, S. D. and Ward, G. E. (2000). The Toxoplasma homolog of Plasmodium apical membrane antigen-1 (AMA-1) is a microneme protein secreted in response to elevated calcium levels. *Mol. Biochem. Parasitol.* **111**, 15-30.

Fourmaux, M. N., Achbarou, A., Mercereau-Puijalon, O., Bderre, C., Brache, I., Loyens, A., Odberg-Ferragut, C., Camus, D. and Dubremetz, J. F. (1996). The MIC1 microneme protein of *Toxoplasma gondii* contains a duplicated receptor-like domain and binds to host cell surface. *Mol. Biochem. Parasitol.* **83**, 201-210.

Garcia-Réguet, N., Lebrun, M., Fourmaux, N., Mercereau-Puijalon, O., Mann, T., Beckers, C. J. M., Samyn, B., van Beeumen, J., Bout, D. and Dubremetz, J. (2000). The microneme protein MIC3 of *Toxoplasma gondii* is a secretory adhesin that binds to both the surface of the host cells and the surface of the parasite. *Cell. Microbiol.* **2**, 353-364.

Gee, K. R., Brown, K. A., Chen, W. N., Bishop-Stewart, J., Gray, D. and Johnson, I. (2000). Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium* **27**, 97-106.

Geritz, A. T., Rao, A. S., Simon, P. O., Merlin, D., Carnes, D., Madara, J. L. and Neish, A. S. (2000). *Salmonella typhimurium* induces epithelial IL-8 expression via Ca(+2)-medicated activation of the NF-kappaB pathway. *J. Clin. Invest.* **105**, 79-92.

Håkansson, S., Morisaki, H., Heuser, J. E. and Sibley, L. D. (1999). Time-lapse video microscopy of gliding motility in *Toxoplasma gondii* reveals a novel, biphasic mechanism of cell locomotion. *Mol. Biol. Cell* **10**, 3539-3547.

Hehl, A. B., Lekutis, C., Grigg, M. E., Bradley, P. J., Dubremetz, J. F., Ortega-Barria, E. and Boothroyd, J. C. (2000). *Toxoplasma gondii* homologue of Plasmodium apical membrane antigen 1 is involved in invasion of host cell. *Infect. Immun.* **68**, 7078-7086.

Lovett, J. L., Marchesini, N., Moreno, S. N. and Sibley, L. D. (2002). *Toxoplasma gondii* microneme secretion involves intracellular Ca^{2+} release from IP3/ryanodine sensitive stores. *J. Biol. Chem.* **277**, 25870-25876.

Luo, S., Vieira, M., Graves, J., Zhong, L. and Moreno, S. N. (2001). A plasma membrane-type Ca(2+)-ATPase co-localizes with a vacuolar H(+)-pyrophosphatase to acidocalcisomes of *Toxoplasma gondii*. *EMBO J.* **20**, 55-64.

Malik, Z. A., Denning, G. M. and Kusner, D. J. (2000). Inhibition of Ca(2+) signaling by *Mycobacterium tuberculosis* is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. *J. Exp. Med.* **191**, 287-302.

Martell, A. E. and Smith, R. M. (1974). *Critical stability constants*. New York: Plenum Press.

Moreno, S. N. J. and Zhong, L. (1996). Acidocalcisomes in *Toxoplasma gondii* tachyzoites. *Biochem. J.* **313**, 655-659.

Morisaki, J. H., Heuser, J. E. and Sibley, L. D. (1995) Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J. Cell Sci.* **108**, 2457-2464.

Pezzella, N., Bouchot, A., Bonhomme, A., Pingret, L., Klein, C., Burlet, H., Balossier, G., Bonhomme, P. and Pinon, J. P. (1997). Involvement of calcium and calmodulin in *Toxoplasma gondii* tachyzoite invasion. *Eur. J. Cell Biol.* **74**, 92-101.

Rodrigues, C. O., Ruiz, F. A., Rohloff, P., Scott, D. A. and Moreno, S. N. J. (2002). Characterization of isolated acidocalcisomes from *Toxoplasma gondii* tachyzoites reveals a novel pool of hydrolyzable polyphosphate. *J. Biol. Chem.* **277**, 48650-48656.

Rodriguez, A., Rioult, M. G., Ora, A. and Andrews, N. W. (1995). A Trypanosome-soluble factor induces IP_3 formation, intracellular Ca^{2+} mobilization and microfilament rearrangement in host cells. *J. Cell Biol.* **129**, 1263-1273.

Scheidegger, K. J., Du, J. and Delafontaine, P. (1999). Distinct and common pathways in the regulation of insulin-like growth factor-1 receptor gene expression by angiotensin II and basic fibroblast growth factor. *J. Biol. Chem.* **274**, 3522-3530.

Shahrestanifar, M., Fan, X. and Manning, D. R. (1999). Lysophosphatidic acid activates NF-kappaB in fibroblasts. A requirement for multiple inputs. *J. Biol. Chem.* **274**, 3828-3833.

Sibley, L. D., Weidner, E. and Krahenbuhl, J. L. (1985). Phagosome acidification blocked by intracellular *Toxoplasma gondii*. *Nature* **315**, 416-419.

Soldati, D., Dubremetz, J. F. and Lebrun, M. (2001). Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int. J. Parasitol.* **31**, 1293-1302.

Suss-Toby, E., Zimmerberg, J. and Ward, G. E. (1996). *Toxoplasma* invasion: The parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fusion pore. *Proc. Natl. Acad. Sci. USA* **93**, 8413-8418.

Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B. and Hanley, M. R. (1989). Thapsigargin, a novel molecular probe for studying calcium release and storage. *Agents Actions* **27**, 17-23.

Tomley, F. M. and Soldati, D. S. (2001). Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. *Trends Parasitol.* **17**, 81-88.

Vieira, M. C. F. and Moreno, S. N. J. (2000). Mobilization of intracellular calcium upon attachment of *Toxoplasma gondii* tachyzoites to human fibroblasts is required for invasion. *Mol. Biochem. Parasitol.* **106**, 157-162.

Wetzel, D. M., Håkansson, S., Hu, K., Roos, D. S. and Sibley, L. D. (2003). Gliding motility by Apicomplexans is regulated by actin filament assembly. *Mol. Biol. Cell* **14**, 396-406.