Calcium Signaling throughout the *Toxoplasma gondii* Lytic Cycle

A STUDY USING GENETICALLY ENCODED CALCIUM INDICATORS[*][S]

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Background: Ca^{2+} signaling is important for the lytic cycle of *T. gondii*.
Results: Genetically encoded Ca^{2+} indicators revealed cytosolic Ca^{2+} changes in real time.
Conclusion: New approach highlights important features of the lytic cycle.
Significance: Ca^{2+} influx leads to signaling that results in enhancement of important lytic cycle features.

Toxoplasma gondii is an obligate intracellular parasite that invades host cells, creating a parasitophorous vacuole where it communicates with the host cell cytosol through the parasitophorous vacuole membrane. The lytic cycle of the parasite starts with its exit from the host cell followed by gliding motility, conoid extrusion, attachment, and invasion of another host cell. Here, we report that Ca^{2+} oscillations occur in the cytosol of the parasite during egress, gliding, and invasion, which are critical steps of the lytic cycle. Extracellular Ca^{2+} enhances each one of these processes. We used tachyzoite clonal lines expressing genetically encoded calcium indicators combined with host cells expressing transiently expressed calcium indicators of different colors, and we measured Ca^{2+} changes in both parasites and host simultaneously during egress. We demonstrated a link between cytosolic Ca^{2+} oscillations in the host and in the parasite. Our approach also allowed us to measure two new features of motile parasites, which were enhanced by Ca^{2+} influx. This is the first study showing, in real time, Ca^{2+} signals preceding egress and their direct link with motility, an essential virulence trait.

Fluctuations of the cytosolic calcium ion (Ca^{2+}) concentration regulate a variety of cellular functions in all eukaryotes, such as secretion, contraction, cell division, and differentiation (1). Cells contain a sophisticated set of mechanisms to balance their cytosolic Ca^{2+} levels, and the signals that elevate cytosolic Ca^{2+} are compensated by mechanisms that reduce it. Ca^{2+} is sequestered into different organelles, where it is released via calcium release channels in response to specific signals (2). Ca^{2+} signaling is universal, and it is utilized by prokaryotic and eukaryotic unicellular organisms as well as by multicellular eukaryotes (3). Ca^{2+} signals decoded by a specific cell may lead to specific physiological responses mediated either through gene expression changes or independent of changes in gene expression (3, 4).

Apicomplexan parasites include a number of pathogens of medical and veterinary relevance, such as *Toxoplasma gondii*, the agent of toxoplasmosis, and *Plasmodium* spp., the agents of malaria. During its lytic cycle, *T. gondii* actively invades host cells, creating a parasitophorous vacuole (PV),[9] where it divides to finally exit in search of a new host cell. Parasite invasion is a highly coordinated and active process involving several discrete steps (5). In *Toxoplasma*, indirect evidence showed that Ca^{2+} signals are decoded to result in stimulation of gliding motility, microneme secretion, conoid extrusion, and invasion. However, information on the mechanisms and the molecules involved is fragmented or missing. Our previous studies have shown that a nifedipine-sensitive Ca^{2+} channel is involved in Ca^{2+} entry (6), although the molecular components involved have not been identified. We also demonstrated that Ca^{2+} influx enhances lytic cycle traits of *T. gondii*, such as microneme secretion, conoid extrusion, motility, and invasion (6). Our work highlighted the significance of Ca^{2+} entry, resulting in Ca^{2+} signals that affect virulence traits of an intracellular pathogen.

[9] The abbreviations used are: PV, parasitophorous vacuole; TG, thapsigargin; SOCE, store-operated Ca^{2+} entry; GECI, genetically encoded calcium indicator; IP$_3$, inositol 1,4,5-trisphosphate; EB, extracellular buffer; IB, intracellular buffer; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; hTERT, human telomerase reverse transcriptase.

Previous studies on the role of Ca^{2+} fluctuations in gliding motility (7), conoid extrusion (8), microneme secretion (9–11), host cell invasion (7, 12), and egress (13) were done using indirect methods, such as labeling extracellular parasites with fluorescent dyes and following Ca^{2+} changes during their gliding motility (7) and using Ca^{2+} ionophores and other exogenous agents to elevate Ca^{2+} in extracellular parasites stimulating conoid extrusion (8) or microneme secretion (9–11). Another indirect strategy was the use of intracellular or extracellular Ca^{2+} chelators to prevent host cell invasion (7, 12) or egress (13). These methods have serious limitations. Loading with fluorescent Ca^{2+} indicators is highly invasive and can be damaging to cells. These dyes can compartmentalize during extended incubations and are incompatible with prolonged *in vivo* measurements; additionally, dyes cannot be used for studies involving intracellular parasites because parasite-specific loading cannot be accomplished because host cells will also be loaded.

Of all the Apicomplexan parasites, *T. gondii* is the most genetically tractable, and in this study we took advantage of this property to use genetically encoded calcium indicators (GECIs) to investigate the role of Ca^{2+} in motility, invasion, and egress. GECIs are powerful tools, and recent efforts in protein engineering have significantly increased their performance (14). GECIs have the advantage that they enable noninvasive imaging of defined cells and compartments. State-of-the-art GECIs include the single-wavelength sensor GCaMPs, which are based on circularly permuted green fluorescent protein, calmodulin, and the Ca^{2+}/calmodulin-binding "M13" peptide (M13pep) (14). "GECO" sensors, created from GCaMP3 by random mutagenesis (15), are also available in a variety of colors. Our experiments using *T. gondii* tachyzoites expressing GCaMP3 or GCaMP6f combined with host cells expressing Red-GECO or Blue-GECO (B-GECO) allowed us to directly follow changes in real time in cytosolic Ca^{2+} in the parasites, while they are inside their host cell and simultaneously in both the parasites and host cells during invasion and egress. The use of these tools provided exciting new information about communication between the cytosolic Ca^{2+} in the host and the parasite. Our approach has also allowed the measurement of two new features of motile parasites, which were enhanced by Ca^{2+} influx. This is the first study to show directly that Ca^{2+} signals precede egress and to establish a direct correlation between Ca^{2+} signals and motility, an essential virulence trait, throughout the *T. gondii* lytic cycle.

Experimental Procedures

Cell Cultures—*T. gondii* tachyzoites (RH strain) were maintained as described (16) using Dulbecco's modified minimal essential media (DMEM) with 1% FBS. HeLa cells (ATCC) were maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine. hTERT fibroblasts (originally from BD Biosciences) were used as host cells for growth of parasites and also for the invasion experiments described below. These cells were maintained in high glucose DMEM with 10% FCS. *GCaMP3*-transfected tachyzoites were maintained in hTERT fibroblasts in the presence of 1 μM pyrimethamine. *GCaMP6f*-transfected tachyzoites were main-

tained under similar conditions but in the presence of 20 μM chloramphenicol. Parasites were purified as described (16).

Chemicals and Reagents—Fluo-4/AM and Lipofectamine were from Invitrogen (Life Technologies, Inc.). GCaMP3, GCaMP6f (fast version of GCaMP6), R-GECO, and B-GECO were obtained from Addgene, and the plasmids were used for HeLa cells transfections or the respective genes were cloned into *T. gondii* expression vectors for stable expression in tachyzoites. Thapsigargin, ionomycin, saponin, dithiothreitol (DTT), histamine, and all other chemicals were obtained from Sigma.

Preparation of GECI-expressing Tachyzoites and HeLa Cells—The *GCaMP3* gene (17) was amplified from the commercial plasmid and cloned into the *T. gondii* expression vector pDHFRTubGFP (18) using BglII and AvrII restriction sites and adding a stop codon in front of the GFP sequence. The primers used were forward 5′-AGGCGTGTACGGTGGGAGGTC-3′ and reverse 5′-CTTCCTAGGTTACTTCGCTGTCATCAT-TTG-3′. The plasmid pDTGCaMP3 was transfected into tachyzoites of the RH strain for pyrimethamine selection. We then isolated cells with low fluorescence by cell sorting to eliminate those highly fluorescent cells in which the GCaMP3 could be buffering Ca^{2+} and preventing visualization of physiological changes in Ca^{2+} levels. Sixty nine clones were isolated and analyzed for their response to ionomycin. We incubated these cells in either buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 50 mM Hepes, pH 7.2) or buffer A plus 1 μM ionomycin in the presence of 1 mM external $CaCl_2$. We selected the 10 subclones that gave maximal fluorescence response to the ionophore, and we used two of these subclones in our experiments.

Red GECO-coding sequence, cloned into a vector for protein expression into mammalian cells (pCMV) (15), was amplified with the forward primer 5′-CCCGGGTAATGGTCGACTCT-TCACGTCGTAAG-3′ and reverse primer 5′-TACGTACTA-CTTCGCTGTCATCATTTGTAC-3′. SmaI and SnaBI restriction sites were added to the forward and reverse primers, respectively (underlined). R-GECO was cloned in the vector ptub_SAG1-IEα-DsRed_dhfr_sag1CATsag1 (kindly provided by Vern B. Carruthers) between the SmaI (5′)/SnaBI (3′) restriction sites, substituting the DsRed gene, previously shown to label the PV (19). *T. gondii* parasites (RH strain) were transfected, and selection was done with 20 μM chloramphenicol. This PV version of Red GECO was also transfected in a *T. gondii* clone (RH strain) expressing the cytosolic GCaMP3 calcium sensor. These parasites were selected in the presence of both 1 μM pyrimethamine and 20 μM chloramphenicol.

Plasmids for expression of *GCaMP6* in *T. gondii* were kindly provided by Kevin Brown and David Sibley, Washington University.[10] The coding DNA sequence for *GCaMP6f* (Addgene) was amplified by PCR and cloned into a *T. gondii* vector for expression behind the tubulin promoter. Plasmids were electroporated into RH strain parasites and clones selected by chloramphenicol resistance.

HeLa cells (5×10^5) were grown on coverslips (high glucose DMEM with 10% FCS). After 24 h, cells were transfected with

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[10] K. Brown and D. Sibley, manuscript in preparation.

1.25 μg of plasmid DNA encoding R-GECO or B-GECO using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. 6–8 h later, HeLa cells were infected with 10^6 tachyzoites expressing *GCaMP3* or *GCaMP6f* and were grown for 15–20 h. Rosettes containing 4–8 parasites were used in all the experiments. Variations to the protocol are explained in the legends to the figures.

Cytosolic Ca^{2+} Measurements—Fluorometric measurements were done with *GCaMP3-* and *GCaMP6f*-expressing tachyzoites. The parasites were washed twice in Ringer buffer (155 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 10 mM Hepes, pH 7.3, and glucose 10 mM), resuspended in the same buffer to a final density of 1×10^9 cells/ml, and kept in ice. For fluorescence measurements, 50-μl portions of the cell suspension was diluted in 2.5 ml of Ringer (2×10^7 cells/ml final density) in a cuvette placed in a thermostatically controlled Hitachi 4500 spectrofluorometer. Excitation was at 485 nm and emission at 520 nm. Traces shown are representative of three independent experiments conducted on separate cell preparations. Calcium-defined conditions were determined by using EGTA or 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) and calcium chloride to arrive at calculated concentrations of free calcium. Calcium-EGTA combinations were determined using Maxchelator software. Fura-2/AM loading and measurements were done as described previously (6).

Invasion Assays—To monitor invasion, extracellular *GCaMP3-* or *GCaMP6f*-expressing parasites were collected and resuspended in Ringer buffer with 1 mM EGTA or 1 mM $CaCl_2$. The parasites were then added to a confluent monolayer of hTERT cells plated on MatTek microscopy dishes. The parasites were semi-synchronized by incubating them on ice for 20 min before imaging. The parasites were then imaged at 37 °C. Fluorescence images were captured using an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ charge-coupled device (CCD) camera driven by DeltaVision software (Applied Precision). Images were collected every 1 s for 10 min, totaling 603 frames, and were transformed in videos using Soft-Worx suite 2.0 software from Applied Precision. All experiments were done at 37 °C, and the results were obtained from at least three independent experiments.

Egress Assays—8–12 h after transfection, HeLa cells were infected with 1×10^6 *GCaMP6f*-expressing tachyzoites. Thirty hours after infection, parasitophorous vacuoles containing 4–8 parasites were observed by microscopy after washing them with Ringer buffer. Drugs were added in Ringer buffer at the concentrations indicated: ionomycin (1 μM), histamine (5 and 100 μM), thapsigargin (1 μM), DTT (5 mM), saponin (0.01%), nifedipine (10 μM). Ringer buffer was used as extracellular buffer (EB). $CaCl_2$ was omitted for experiments in the absence of extracellular calcium, and the media were supplemented with 100 μM or 1 mM EGTA. For experiments using intracellular conditions, an intracellular buffer was used considering the ionic composition of the cytosol as follows: potassium gluconate 140 mM, NaCl 10 mM, $MgSO_4$ 2.7 mM, ATP (sodium salt) 2 mM, glucose 1 mM, EGTA 200 μM, $CaCl_2$ 65 μM (90 nM free Ca^{2+}), and 10 mM Tris/Hepes, pH 7.3. Images were acquired as for the invasion assays (time-lapse mode with an acquisition rate of at least 2–3 s during 20–30 min).

Motility Assays—35-mm glass bottom cover dishes (MAT-TEK) were treated with 2 ml of 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS), pH 7.4, overnight at 4 °C. The following morning the excess FBS was washed with PBS. Freshly egressed tachyzoites expressing *GCaMP6f* were collected, purified, and resuspended in 1 ml of Ringer buffer without calcium. ~2.5 $\times 10^7$ parasites were loaded onto cover dishes using a cell culture cylinder and incubated for ~15 min on ice to allow for cells to adhere. After removing the cell culture cylinder, Ringer buffer was added to a 2-ml final volume. Cells were imaged using a Zeiss LSM 710 confocal microscope set at 37 °C. Before image acquisition, cells were allowed to equilibrate at 37 °C for 5 min. A 488-nm laser power was set at 4%, and cells were imaged for ~10 min. After establishment of a baseline for image acquisition, thapsigargin (2 μM final) and $CaCl_2$ solution (2 mM final) were added, and motility was recorded. Ca^{2+} was added 1 min after thapsigargin addition. For analysis of the data, Trackmate, a plugin available in Fiji, was used to measure fluorescence changes in moving cells (20).

Results

Ca^{2+} Signaling and Egress—It has been shown that calcium ionophores and other agents induce egress of tachyzoites from their host cells. However, it is difficult to study Ca^{2+} changes directly in the parasite because any perturbation caused by either Ca^{2+} ionophores, (21), dithiols (22), Ca^{2+} chelators (23), or permeabilizing agents such as saponin (24) will affect both the host and parasite. In addition, chemical Ca^{2+} indicators are not useful because they label both the host and parasite. To investigate whether there is a correlation between Ca^{2+} signals in the host cell and in the parasite during parasite egress, we created *T. gondii* tachyzoites expressing *GCaMP3* or *GCaMP6f* and monitored Ca^{2+} dynamics while they were intracellular. HeLa cells transiently transfected with the Ca^{2+} indicator, R-GECO, were infected with GECI-expressing tachyzoites and subjected to various treatments previously shown to stimulate egress.

We first tested the effect of ionomycin (1 μM) on HeLa cells transiently transfected with *R-GECO* and infected with *GCaMP3*-expressing tachyzoites. Parasite egress was preceded by a Ca^{2+} increase in the parasite cytosol and increased motility (Fig. 1*A* and supplemental Video S1). A dramatic Ca^{2+} increase always immediately preceded the increase in motility and active egress. The first parasites to leave the host cells were able to actively traverse their intact plasma membrane, as shown by the appearance of a moving junction (supplemental Video S1). Only after several parasites had left the host cell was the plasma membrane permeabilized and the remaining tachyzoites egressed passively. The effect of ionomycin is the result of the ionophore inserting in membranes of both host and parasite, leading to simultaneous Ca^{2+} increase in both compartments (Fig. 1*C*). This simultaneous increase in Ca^{2+} was followed by parasite egress (supplemental Video S1). It was not possible to discriminate whether host cell Ca^{2+} increase was necessary for parasite Ca^{2+} increase and egress.
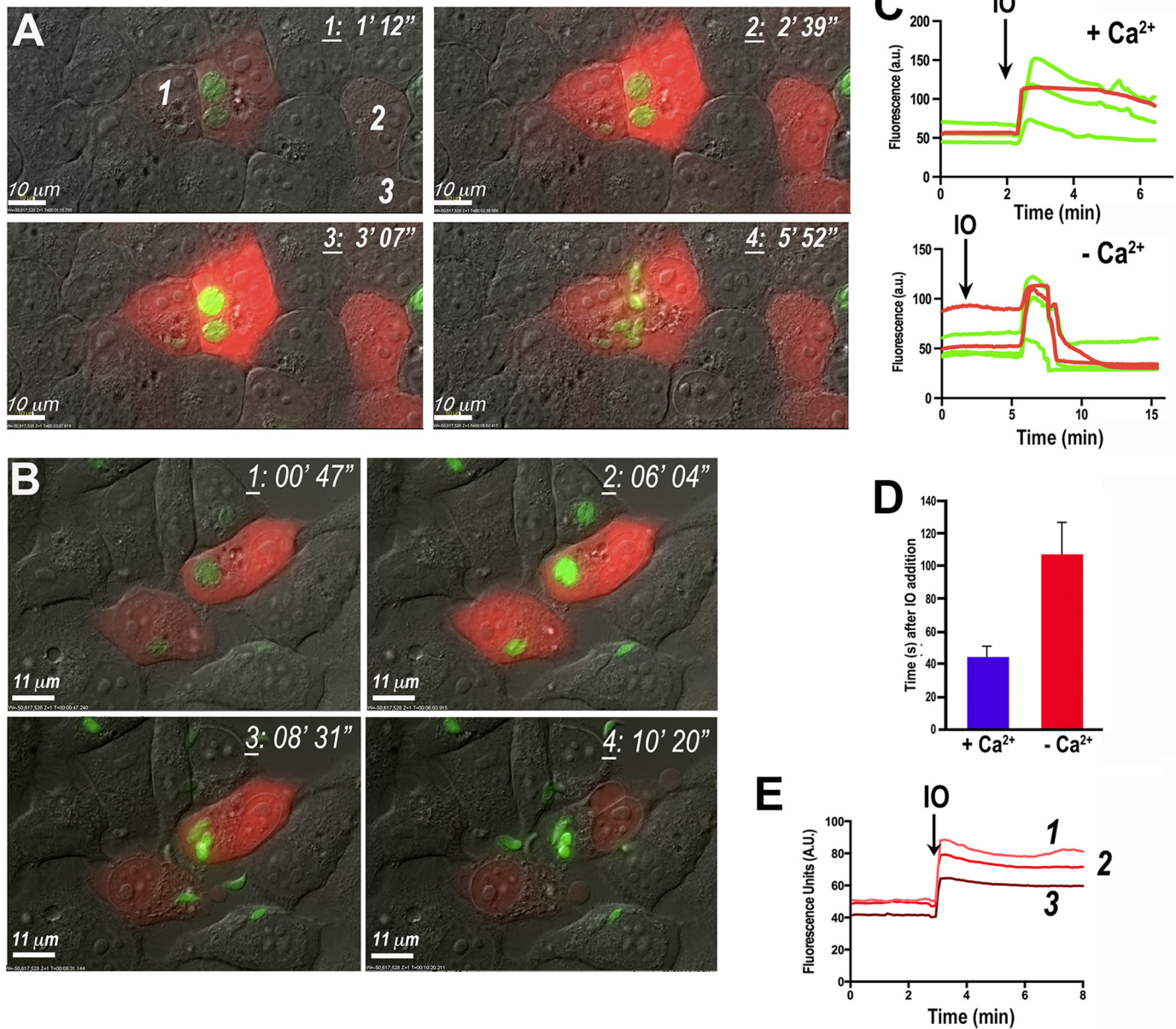
FIGURE 1. **Ionomycin stimulates parasite Ca^{2+} increase and egress in the presence and absence of extracellular Ca^{2+}.** Egress of *GCaMP3*-expressing tachyzoites from *R-GECO*-expressing HeLa cells in medium with or without extracellular Ca^{2+} is shown. *A*, time frames show increases in intracellular Ca^{2+} in both tachyzoites (*green*) and host cells (*red*) upon addition of 1 μM ionomycin (*frame 2*). Once tachyzoites egressed, the R-GECO fluorescence decreases due to cell permeabilization (*frame 4*). Frames were obtained from supplemental Video S1 at the indicated times. *B*, similar experiment to the one shown in *A*. Cells were incubated in Ringer buffer without $CaCl_2$ and supplemented with 1 mM EGTA. Addition of ionomycin is able to increase Ca^{2+} in both parasites (*green*) and host cells (*red*) (*frame 2*) and induce parasite egress (*frames 3* and *4*). Images were taken from a video not shown. *C*, changes in GCaMP3 (*green*) and R-GECO (*red*) fluorescence over time in medium with ($+ Ca^{2+}$) or without ($- Ca^{2+}$) extracellular Ca^{2+}. Ionomycin (*IO*) was added at 2 min (*arrow*) leading to abrupt increases in both parasites (*green*) and the host cell (*red*) intracellular Ca^{2+}, even in the absence of extracellular Ca^{2+}, but it was more sustained and faster in the presence of extracellular Ca^{2+}. Fluorescence change of a region of interest from supplemental Video S1 (plus Ca^{2+}) and not shown (no Ca^{2+}) was done using ImageJ. *D*, quantification and statistical analysis of a minimum of three independent experiments of stimulation of egress with 1 μM ionomycin in the presence of Ringer buffer with 2 mM Ca^{2+} (*blue bar*) *versus* the same buffer with 1 mM EGTA (*red bar*). Ionomycin was added 1 min after the start of video recording. The time recorded is the time that parasites started to exit and lysed the PV. HeLa cell growth was the same as for the other egress experiments, *i.e.* grown to confluence in ~24 h. *E*, fluorescence recording of non-infected host cells showing increase of Geco fluorescence in response to the addition of ionomycin. *A.U.*, arbitrary units. The *numbers* indicate the fluorescence of the cells labeled in *A*.

Similar experiments with ionomycin in the absence of extracellular Ca^{2+} (Fig. 1*B*) led to an increase in cytosolic Ca^{2+} in both host cells and tachyzoites (Fig. 1*C*), albeit with a consistent time delay, and also resulted in parasite egress (Fig. 1*B*), suggesting that Ca^{2+} release from the parasite intracellular stores caused by ionomycin was sufficient to stimulate Ca^{2+} increase and parasite egress. We observed that parasites egressed sooner in the presence of extracellular Ca^{2+}, suggesting that Ca^{2+} influx enhances Ca^{2+} in the parasite and egress. Fig. 1*D* shows the quantification of at least three independent experiments for the time to egress after adding ionomycin. In all experiments Ca^{2+} oscillations preceded egress. Fig. 1*E* shows the response to ionomycin of non-infected HeLa cells expressing GECO.
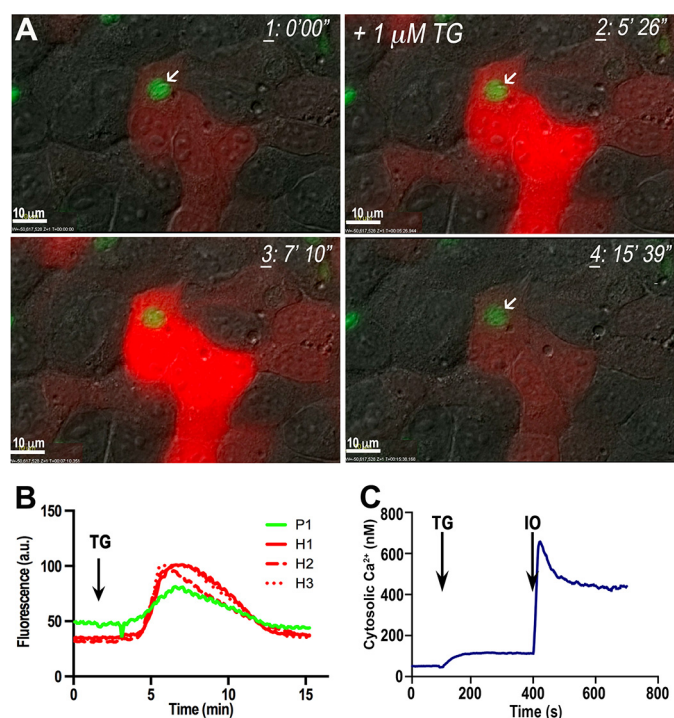
FIGURE 2. **Pharmacological increase of intracellular Ca^{2+} with TG is unable to trigger tachyzoite egress.** *A,* lack of egress of *GCaMP3*-expressing tachyzoites from *R-GECO*-expressing HeLa cells after addition of 1 μM thapsigargin (*frame 2*). TG increases host cell (*red*) and parasite (*green*) cytosolic Ca^{2+}, but the parasites do not egress. Frames were taken from supplemental Video S2 at the times indicated at the *top right corner*. *B,* changes in GCaMP3 (*green*) and R-GECO (*red*) fluorescence in one group of parasites (*P1*) and three HeLa cells (*H1, H2,* and *H3*) upon addition of thapsigargin (*arrow*). Ringer buffer was used containing 2 mM $CaCl_2$. More experimental conditions are indicated under "Experimental Procedures." Fluorescence graph represents the changes in host cells and the PV fluorescence. Analysis was done with ImageJ. *C,* cytosolic Ca^{2+} changes of Fura-2/AM-loaded tachyzoites upon addition of TG and ionomycin (*IO*), both at 1 μM. The effect of ionomycin is noticeably larger than the effect of TG. This is a representative tracing out of at least three identical experiments.

We next tested thapsigargin (TG), which inhibits the SERCA-type Ca^{2+}-ATPase at very low concentrations (25) resulting in an increase of cytosolic Ca^{2+} due to its continual leakage from the endoplasmic reticulum and blockage of its reuptake. Addition of TG in the presence of extracellular Ca^{2+} stimulates store-operated Ca^{2+} entry (SOCE) in mammalian cells (26, 27). We recently demonstrated that Ca^{2+} entry in *T. gondii* occurs through a pathway that does not involve SOCE (6). Addition of 1 μM TG to HeLa cells transiently transfected with *R-GECO* and infected with *GCaMP3*-expressing tachyzoites resulted in cytosolic Ca^{2+} increases in both the host cell and parasite without egress of the parasites during the time period examined (15 min) (Fig. 2, *A* and *B,* and supplemental Video S2). Higher concentrations of TG (10 μM) were toxic to HeLa cells, resulting in plasma membrane blebbing (data not shown). These results are in agreement with previous reports, which demonstrated the toxic effect of TG in eukaryotic cells at high concentrations (28) that can lead to apoptosis (29). However, parasites did not egress even under these conditions (data not shown) suggesting that Ca^{2+} changes in HeLa cells induced by SOCE are not sufficient to cause parasite egress. The amount of Ca^{2+} released by ionomycin into the cytoplasm of the cell was 5–6 times larger than the amount released by TG (Fig.

2*C*), which could explain why ionomycin is highly efficient at inducing Ca^{2+} increase and egress. These results suggest a threshold for the cytosolic Ca^{2+} concentration needed for stimulation of motility and egress.

It has been shown previously (22) that dithiothreitol (DTT) stimulates tachyzoite egress. Therefore, we investigated whether this effect was linked to cytosolic Ca^{2+} increase in the parasite. We tested 5 mM DTT on *R-GECO*-expressing HeLa cells infected with *GCaMP3*-expressing parasites. We first observed a Ca^{2+} increase in tachyzoites, followed by parasite egress, and finally a Ca^{2+} increase in the infected host cells (Fig. 3, *A* and *B*, and supplemental Video S3). Cytosolic Ca^{2+} in the parasite increased (Fig. 3*B*, *trace e*) before host cytosolic Ca^{2+} (Fig. 3*B*, *peak in red trace a,* followed by *b* and *c*). This is supported by control experiments showing that there was no effect of DTT when added directly to non-infected HeLa cells expressing R-GECO (Fig. 3*D*). It has been shown that DTT stimulates a secreted parasite nucleotidase (22), resulting in depletion of host cell ATP levels and exit of parasites (30). ATP is used by both the SERCA pump and the plasma membrane ATPase to pump Ca^{2+} out of the cytosol, and if it is not available both pumps would stop functioning resulting in an increase of host cytosolic Ca^{2+}. We also observed that the uninfected cells shown in the supplemental Video S3 do not respond to DTT (cells *b* and *c* in Fig. 3*A* and supplemental Video S3) until after tachyzoite egress, suggesting that this secondary Ca^{2+} increase is the consequence of Ca^{2+} waves from cell to cell probably through gap junctions (31). Interestingly, we did not observe a direct effect of DTT on the cytosolic Ca^{2+} of tachyzoites, as tested in Fura-2/AM-loaded extracellular tachyzoites (Fig. 3*C*), suggesting that the DTT effect on the cytosolic Ca^{2+} of tachyzoites is indirect and involves a mechanism that is functional or expressed only in intracellular parasites. The *traces* shown in Fig. 3*C* compare the response to DTT in the absence of extracellular Ca^{2+} (*red, EGTA*) and in its presence (*blue*). Addition of TG leads to an increase in cytosolic Ca^{2+} resulting from endoplasmic reticulum leakage under both conditions. Further addition of Ca^{2+} shows Ca^{2+} entry (Fig. 3*C*, *red trace*).

Differential Effects of Intracellular and Extracellular Ionic Conditions on Parasite Egress—Previous studies have used mild detergents to permeabilize the host cell plasma membrane allowing the intracellular parasites to become exposed to buffers with varying ionic compositions. Using this strategy, it was found that potassium-poor extracellular environment would lead to parasite egress (24). For our experiments, we infected HeLa cells expressing the Ca^{2+} indicator *R-GECO* with *GCaMP3*-expressing tachyzoites (see under "Experimental Procedures" for details) and permeabilized them with saponin (Fig. 4*A*). Fig. 4 shows Ca^{2+} oscillations in HeLa cells (Fig. 4*B*, *red trace*), followed by Ca^{2+} oscillations in the parasites (Fig. 4*B*, *light green trace*) and, minutes later, egress of the parasites (Fig. 4, *A* and *B*, and supplemental Video S4). The oscillations in the parasites are probably the result of Ca^{2+} entry from the host cytosol as saponin showed no direct effect on the parasite cytosolic Ca^{2+} (data not shown). However, it appears that the host plasma membrane is not initially permeabilized by this treatment as Ca^{2+} oscillations continue to occur, a phenomenon
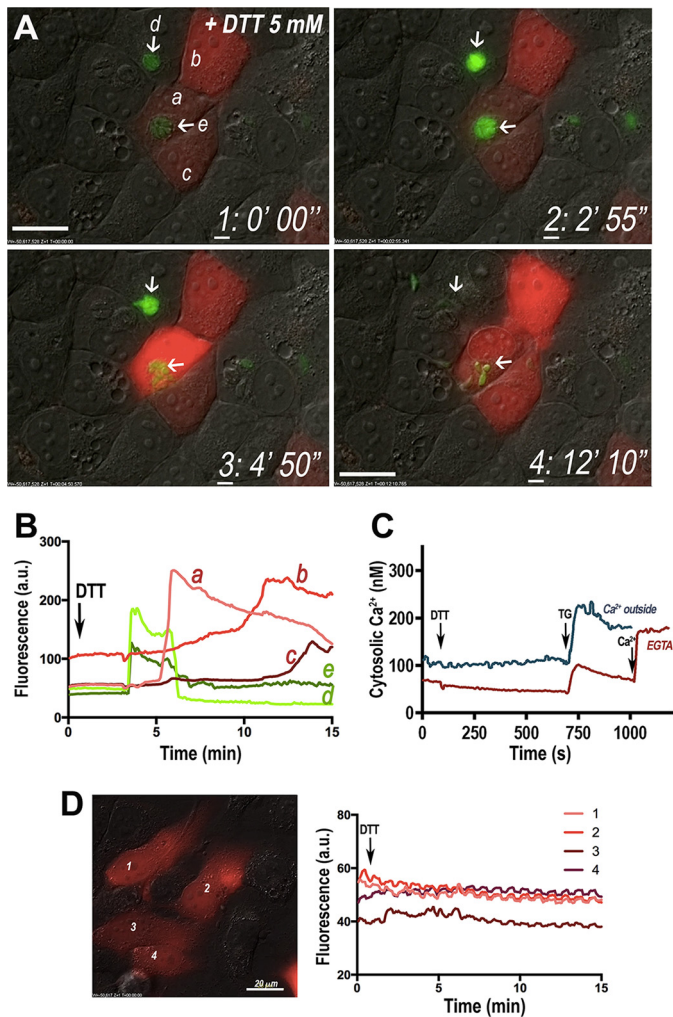
FIGURE 3. **DTT increases intracellular Ca^{2+} and tachyzoite egress.** *GCaMP3*-expressing tachyzoites egress from HeLa cells upon addition of DTT. *A*, frames from supplemental Video S3 at four different times after adding 5 mM DTT. The first frame shows cells before addition of DTT. Each frame was taken at the time indicated in the figure. Addition of 5 mM DTT increases intracellular Ca^{2+} of the tachyzoites (*arrows* show increase in *green fluorescence*). Tachyzoites egress, and this is followed by an increase in Ca^{2+} in the host cell (cell *a*). Cells *b* and *c* (uninfected) show a late increase in intracellular Ca^{2+}. Frames were taken from supplemental Video S3 at the times indicated in the *lower right corner*. *B*, graph shows the increase in Ca^{2+} in the two PVs with tachyzoites (*green tracings e* and *d*, which correspond to the areas indicated with *arrows* in *frames 1–4*), in an infected cell expressing *R-GECO* (*a*) and in uninfected cells (*b* and *c*) after addition of DTT (*arrow*). Two peaks of Ca^{2+} increase are recorded in *GCaMP3*-expressing tachyzoites (*d*), the second being accompanied by Ca^{2+} increase also in the *R-GECO*-expressing host cell (*a*). Fluorescence graph represents the changes in host cells and the PV fluorescence. Analysis was done with ImageJ. *C*, *T. gondii* tachyzoites were loaded with Fura-2/AM as described under "Experimental Procedures." 5 mM DTT was added where indicated with the *arrow* for both experiments (*blue* and *red tracings*). The experiment shown by the *red trace* was done in Ringer buffer without Ca^{2+} and with 100 μM EGTA from the beginning, and 1 mM Ca^{2+} was added at 1000 s. The experiment shown by the *blue trace* was performed in Ringer buffer with 2 mM Ca^{2+} from the beginning. In both cases, 1 μM TG was added at 750 s. This is representative from at least three experiments showing the lack of effect of 5 mM DTT in *T. gondii* intracellular Ca^{2+}. *D*, confluent HeLa cells were transfected with a plasmid containing the R-GECO Ca^{2+} indicator gene, and monolayers were stimulated with 5 mM DTT. The fluorescence was recorded at each one of the indicated positions (*1–4*). *Right panel* shows fluorescence changes at the areas of interest indicated in the image. *A.U.*, arbitrary units.

incompatible with permeabilization of the plasma membrane. Interestingly, it has been described that saponin treatment of rat myocytes (32) also resulted in Ca^{2+} oscillations suggesting

that saponin stimulates IP$_3$ release by activation of a phospholipase C (33).

Prolonged incubation with saponin could lead to permeabilization of the plasma membrane of the host, exposure of tachyzoites to the extracellular medium, and parasite egress. However, when these experiments were done in the same extracellular medium in the absence of extracellular Ca^{2+}, we observed a decrease in the number of parasites showing Ca^{2+} oscillations and Ca^{2+} spikes followed by egress (Fig. 4*C*, *green* and *purple bars*). Furthermore, when extracellular Ca^{2+} was absent, there was an increase in the number of parasites that did not change their intracellular Ca^{2+} levels or egress (Fig. 4*C*, *blue bars*).

We next investigated further the role of Ca^{2+} influx during egress, and for this, we used HeLa cells infected with *GCaMP6f*-expressing tachyzoites in buffers with extracellular or intracellular ionic compositions (EB and IB, see "Experimental Procedures" for a detailed description) (Fig. 5, *A–D*, and supplemental Video S5, *A and B*). Addition of saponin to infected HeLa cells in extracellular buffer in the presence of extracellular Ca^{2+} resulted in tachyzoite Ca^{2+} increases of high peak amplitude, followed by parasite egress, high motility of the extracellular parasites, and immediate invasion of neighboring cells (Fig. 5*A*). In the absence of Ca^{2+}, parasite Ca^{2+} increase was small; egress was delayed; parasites were less motile and were less likely to invade neighboring cells (Fig. 5*B*). We next tested an intracellular buffer containing high K$^+$, low Na$^+$, and also low Cl$^-$ simulating the intracellular composition of these ions. We find that Ca^{2+} influx still enhances motility and egress under these conditions (Fig. 5*C* and supplemental Video S5*A*). In the presence of 90 nM Ca^{2+} (Fig. 5*D*), Ca^{2+} oscillations are not evident; egress is delayed, and the parasites take longer to seek another cell to invade (Fig. 5*D* and supplemental Video S5 *B*). The graphics in the *right panels* of Fig. 5 show intracellular Ca^{2+} changes in intracellular tachyzoites exposed to these different conditions. Each fluorescence tracing tracks a single PV. These results highlight the enhancing effect of Ca^{2+} influx in tachyzoite motility and egress.

We have previously shown that Ca^{2+} influx in *T. gondii* tachyzoites occurs through a nifedipine-sensitive permeation pathway (6). We therefore tested the effect of nifedipine on parasite intracellular Ca^{2+} levels and egress under extracellular ionic conditions (Fig. 5*E*) with saponin-permeabilized host cells. The parasites showed a delayed Ca^{2+} increase and did not egress, even after a 20-min incubation (Fig. 5*E*, *right fluorescence panel*). This experiment supports the role of a Ca^{2+} channel involved in Ca^{2+} influx needed to trigger a signaling pathway leading to parasite egress. Quantification of egress in incubations in IB or EB with and without extracellular Ca^{2+} or with nifedipine is shown in Fig. 6. The number of ruptured PVs as a result of parasite egress is significantly lower in the absence of Ca^{2+}. As the host cells were permeabilized by prolonged incubations with saponin, these effects could not be due to changes in host cytosolic Ca^{2+}.

Role of the Host Cytosolic Ca^{2+}—An interesting observation in our experiments with saponin was that the parasites inside HeLa cells without R-GECO appeared to egress sooner (Fig. 4*B*, *trace b*). Our interpretation was that R-GECO chelates Ca^{2+} in
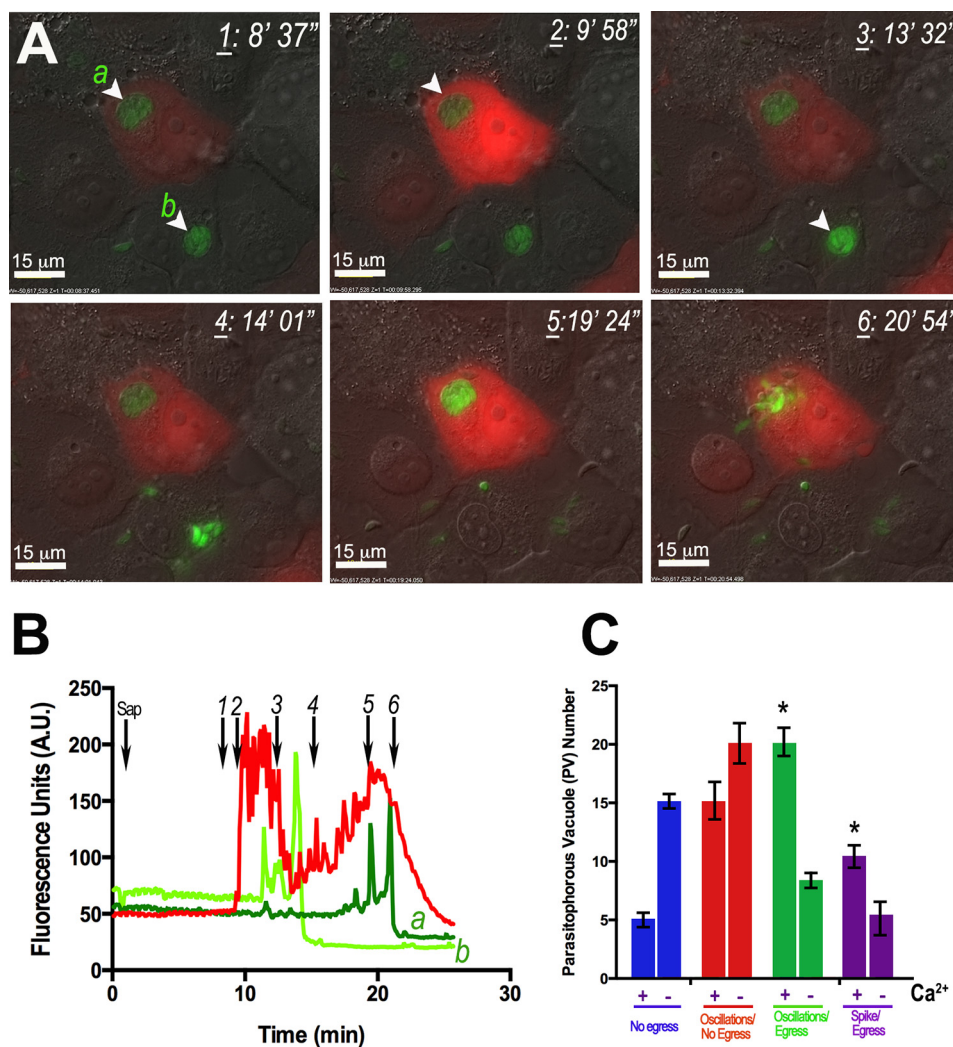
FIGURE 4. Saponin increases intracellular Ca^{2+} oscillations and tachyzoite egress. *GCaMP3*-expressing tachyzoites egress from *R-GECO*-expressing HeLa cells upon saponin addition. *A,* saponin (0.01%) addition resulted in Ca^{2+} oscillations in the host cell (*red*) and in the parasite (*green*). Ca^{2+} oscillations in the parasite led to egress and rupture of the PV and the host cell (*frame 6*). Frames were taken from supplemental Video S4 at the points indicated in *B* (*black arrows*). *B, graph* showing GCaMP3 (*green*) and R-GECO (*red*) fluorescence changes after addition of saponin. The *numbers* indicated are the times at which the frame was taken. Graph was obtained using ImageJ. *C,* quantification and statistical analysis of a minimum of three experiments of egress events in the presence of an extracellular buffer with (+*Ca^{2+}*) and without Ca^{2+} (−*Ca^{2+}*). There is an increase in the percentage of parasites that experience oscillations (*green bars*) and spikes (*purple bars*) in cytosolic Ca^{2+} and subsequently egress in the presence of Ca^{2+} (+*Ca^{2+}*). *Red bars* represent the parasites that oscillate and do not egress. There is a larger percentage of parasites that do not egress in the absence of Ca^{2+} (*blue*). *Bars* are means ± S.E. Results are from three independent experiments with at least 100 total parasites counted in the presence of each condition. * Student's *t* test *p* < 0.05. *A.U.,* arbitrary units.

the host cell and buffers the free Ca^{2+} available to parasites delaying oscillations and egress (Fig. 4*B*, compare *green tracing a* with *b*). We next investigated whether the host cytosolic Ca^{2+} was readily available to the parasites. It has been proposed that the PV membrane is a sieve that allows tachyzoites to freely exchange ions and small molecules with the host cell cytosol (34). We therefore investigated whether physiological Ca^{2+} signals could result in Ca^{2+} increases in the PV and intracellular parasites by infecting the HeLa cells transiently expressing *B-GECO* with tachyzoites expressing *GCaMP3* in their cytosol and *R-GECO* directed to the PV (19). We stimulated the cells with histamine, which activates the plasma membrane H1 receptors in HeLa cells leading to intracellular Ca^{2+} increase through the gating of the IP$_3$ receptor by IP$_3$ (15). Histamine stimulation of the IP$_3$ signaling pathway in HeLa cells led to Ca^{2+} increase in the host cytosol (Fig. 7, *A−D, blue*), and this led to a simultaneous Ca^{2+} increase in the PV (*red*) and later

increases in the parasites (*green*) (Fig. 7, *C* and *D,* and supplemental Video S6), but we could not detect parasite egress, suggesting that physiological increases in intracellular Ca^{2+} by histamine are not sufficient to stimulate parasite egress. Fig. 7*E* shows the average fluorescence changes for host cytosol (*blue*), parasite cytosol (*green*), and PV (*red*) of a minimum of three experiments after stimulation of the infected cells with 100 μM histamine. A similar concentration of histamine was tested against Fura-2/AM-loaded tachyzoites, but these cells were unresponsive, and no effect on cytosolic Ca^{2+} was observed (Fig. 7*F*) supporting our interpretation that Ca^{2+} oscillations in the parasite are the result of Ca^{2+} influx from the host and not a direct effect of histamine on the parasite.

Ca^{2+} Signaling and Motility and Invasion—T. gondii invasion of host cells is a highly coordinated process essential for the successful propagation and expansion of the parasite. Ca^{2+} signaling is essential for the stimulation and enhancement of this
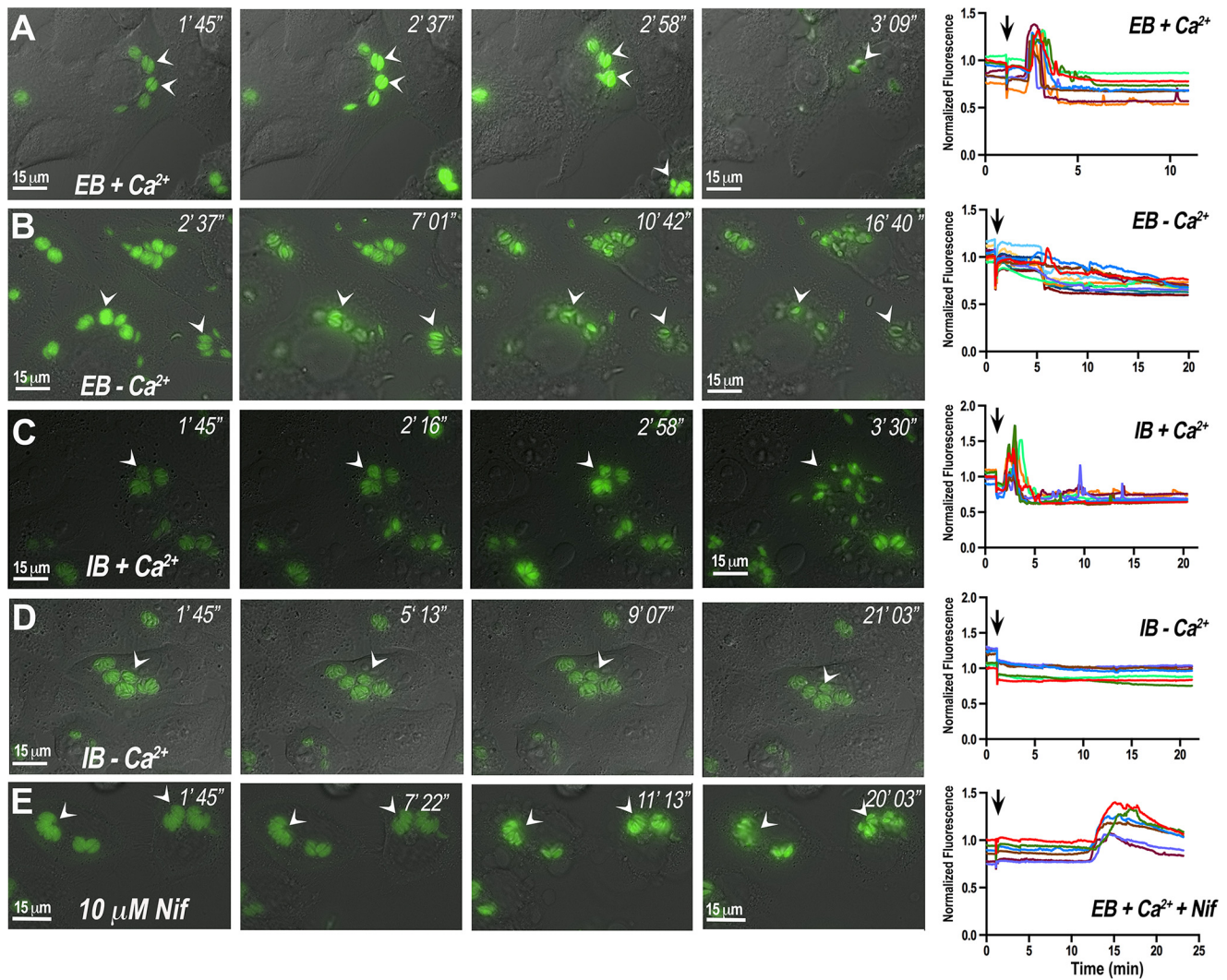
FIGURE 5. **Buffer composition and inhibitors of Ca^{2+} entry affect tachyzoite egress.** Frames from videos obtained during egress of *GCaMP6f*-expressing tachyzoites from infected HeLa cells. Saponin (0.01%) was added 1 min after video recording started under different conditions. *A,* EB plus 2 mM Ca^{2+}. Frames were taken from a video not shown. *B,* extracellular buffer plus 1 mM EGTA. Frames were taken from a video not shown. *C,* intracellular buffer plus 1 mM Ca^{2+}. Frames were taken from supplemental Video S5A. *D,* intracellular buffer plus 90 nM Ca^{2+}. Frames were taken from supplemental Video S5B. These are representative videos of at least three independent biological experiments. *E,* extracellular buffer plus 1 mM Ca^{2+} in the presence of 10 μM nifedipine. Frames were taken from a video not shown. The graphs in the *right panels* show Ca^{2+} changes associated with tachyzoites present in separate parasitophorous vacuoles. Each tracing in each graph represents the fluorescence change of one PV obtained with ImageJ.

virulence trait. It was previously shown that tachyzoites labeled with the fluorescent calcium indicator Fluo-4 (7) underwent Ca^{2+} oscillations during motility and invasion. Extracellular Ca^{2+} enhances invasion events (6), and we therefore investigated whether this enhancement occurs at the single cell level.

By using *T. gondii* tachyzoites expressing *GCaMP6f*, we were able to demonstrate Ca^{2+} dynamics in the parasites during invasion. We carried out real time measurements of cytosolic Ca^{2+} fluctuations in *GCaMP6f*-expressing tachyzoites in the presence of hTERT cells and acquired images by time-lapse microscopy. To increase the probability of observing actively penetrating parasites, we allowed the parasites to settle onto host cells on ice, thereby synchronizing their invasion. After allowing the parasites to settle for 20 min, tissue culture dishes were transferred to 37 °C resulting in activation of motility and subsequent invasion over the next 10 min. A dramatic Ca^{2+} elevation was immediately followed by stimulation of motility and invasion of host cells (Fig. 8 and supplemental Video 7).

Quantification of these assays showed an enhancement in the number of cytosolic Ca^{2+} spikes associated with invasion events in the presence of extracellular Ca^{2+} (Fig. 8*B*, *green bars*). Interestingly, when we analyzed the average peak fluorescence for each of the groups (no oscillations/invasion, oscillations/invasion, and spike/invasion), we observed that spikes were larger with Ca^{2+} outside and that the parasites must fluoresce around 1.5-fold above background ($\sim$75 arbitrary units *versus* resting 50 arbitrary units) to invade (Fig. 8*C*). Once intracellular, tachyzoites ceased to show Ca^{2+} oscillations (Fig. 8*A*, *frame 6*, and supplemental Video S7). In the absence of extracellular Ca^{2+}, cytosolic Ca^{2+} oscillations and invasion events still occurred, but the presence of extracellular Ca^{2+} had an enhancing effect on the number of invasion events. The results from Fig. 8 and supplemental Video S7 show that there is a correlation between the increase in Ca^{2+} spikes and invasion in the presence of extracellular Ca^{2+}, indicating that Ca^{2+} influx increases invasion (Fig. 8*B*).

Calcium Signaling in T. gondii

The invasion experiments showed that a Ca^{2+} peak leads to parasites invading host cells, and extracellular Ca^{2+} enhanced these events. It has been shown that gliding movement is associated with Ca^{2+} oscillations and is stimulated by drugs that cause Ca^{2+} release (35).

We observed previously that Ca^{2+} influx enhances gliding motility (6). We wanted to investigate more in depth this phenomenon, and so we looked into the dynamics and the motility features that would be influenced by Ca^{2+} entry. *GCaMP6f*-expressing parasites provided optimal conditions for Ca^{2+} influx (addition of TG in the presence of extracellular Ca^{2+}) (supplemental Videos 8, *A* and *B*). We previously observed that cytosolic Ca^{2+} regulates Ca^{2+} entry, and TG pre-treatment leads to higher Ca^{2+} influx. We now show that under those conditions there is an increase in gliding motility (Fig. 9*A* and supplemental Video 8*B*). We also looked at Ca^{2+} entry after adding Ca^{2+} (supplemental Video 8*A*). We observed that under basal conditions parasites drifted and oscillated in a disorganized manner. However, upon Ca^{2+} addition, there was a very consistent increase in cytosolic Ca^{2+}, and a large number of parasites stopped drifting and then attached and started gliding. This suggests that the elicited increase in cytosolic Ca^{2+} leads to a significant change in parasite behavior, in which they become increasingly motile and invasive. We looked at two essential features associated with invasion and egress, the duration of motility, and the distance traveled (Fig. 9, *C* and *D*). Interestingly we observed that extracellular Ca^{2+} increases the peak fluorescence intensity and that there is a correlation
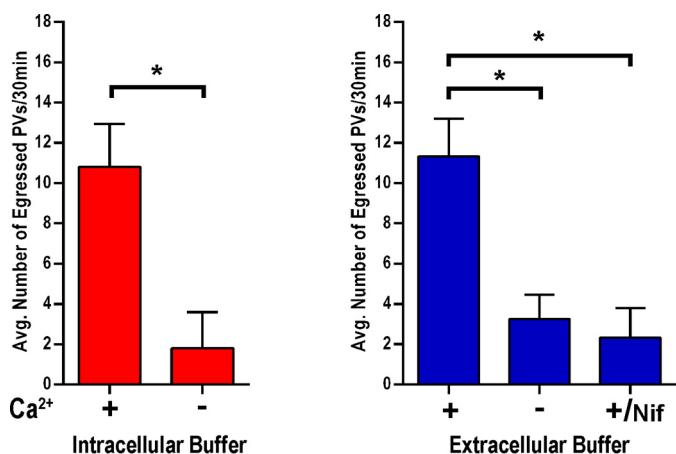

FIGURE 6. **Quantification and statistical analysis of egress events.** Egress experiments performed as for Fig. 5 were quantified by counting the number of PVs that fully egressed from host cells within 30 min. *Red bars* show the quantification of three independent experiments in IB conditions (described under "Experimental Procedures") with and without 1 mM Ca^{2+}. *Blue bars* show quantifications of three independent experiments in Ringer buffer with and without Ca^{2+}. $+Ca^{2+}$/Nif shows the data for egress in the presence of 10 μM nifedipine in the presence of Ca^{2+}. *Bars* are means $\pm$ S.E. Results are from three independent experiments with at least 20 total PVs counted for each condition. *Asterisks* indicate $p < 0.05$.
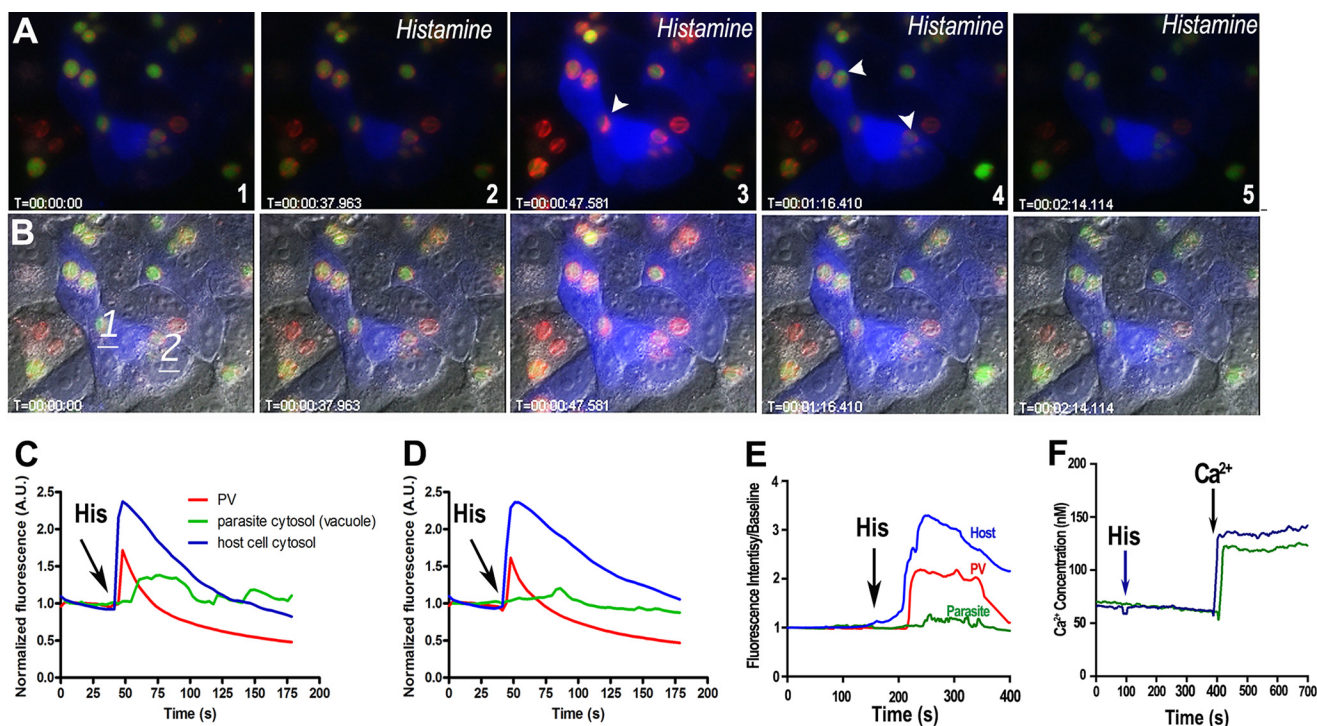

FIGURE 7. **Histamine increases intracellular Ca^{2+} in host cells, PV, and tachyzoites, without egress.** Changes in intracellular Ca^{2+} in tachyzoites expressing cytosolic *GCaMP3* and parasitophorous vacuole-targeted *R-GECO* in *B-GECO*-expressing HeLa cells. *A*, stimulation of an IP_3 signaling pathway in HeLa cells with histamine (*frame 2*) led to host cytosolic Ca^{2+} increase (*blue, frames 3–4*), and immediate increase in Ca^{2+} in the PV (*red, arrowhead* in *frame 3*), followed by a cytosolic Ca^{2+} increase in tachyzoites (*green, arrowheads* in *frame 4*). Ca^{2+} increase in tachyzoites (*green*) appears to be a response to the Ca^{2+} increase in the PV. *B*, differential interference contrast merged with fluorescence of the same frames shown in *A*. *C* and *D*, graphics show fluorescence changes at two different points (*1* and *2* in *B*, respectively). *Blue* indicates host cytosolic Ca^{2+}; *red* indicates PV Ca^{2+} fluctuations, and *green* indicates Ca^{2+} increase in the parasite cytosol. Frames were taken from supplemental Video S6. *E*, average histamine response in incubations of *B-GECO*-expressing HeLa cells infected with *GCaMP3*-expressing tachyzoites. Changes in Ca^{2+} in the host cell cytosol (*blue*), tachyzoite cytosol (*green*), and PV (*red*) after addition of histamine (*arrow*) are shown. At least three experiments were used to calculate the average response of 20 cells for each experiment. *F*, Fura-2/AM-loaded tachyzoites show no response to 100 μM histamine. Histamine was added where indicated (*arrow*), and $CaCl_2$ was added at 400 s (*arrow*). The *green tracing* show control cells without any previous addition, and the *blue tracing* shows the response to Ca^{2+} after adding histamine. This experiment was repeated at least three times with similar results. *A.U.*, arbitrary units.
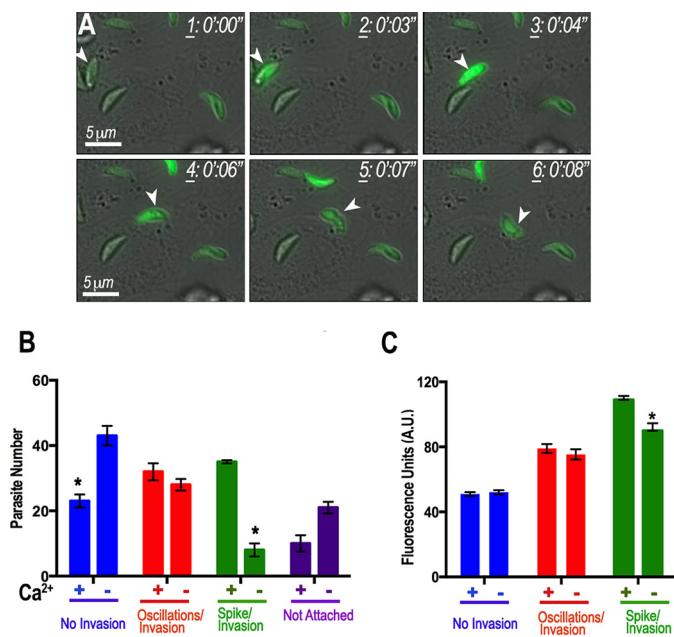
FIGURE 8. **Extracellular calcium enhances invasion.** *A,* GCaMP6f-expressing tachyzoite is highlighted with an *arrowhead* before (*frames 1–3*), during (*frames 4 and 5*), and after (*frame 6*) invasion. A Ca^{2+} peak (*frame 2*) precedes the initiation of motility (*frame 3*) and invasion (*frames 4 and 5*). The *arrowhead* in *frame 5* denotes the moving junction of the parasite during invasion. Ca^{2+} decreases and remains low after invasion (*frame 6*). Frames were taken from supplemental Video S7. *B,* quantification and statistical analysis of invasion events and Ca^{2+} peaks and oscillations. There is an increase in the percentage of parasites that experience a spike in cytosolic Ca^{2+} and subsequently invade host cells (hTERT) in the presence of extracellular Ca^{2+} (+Ca^{2+}, *green*). The percentage of parasites that oscillate and do not invade is higher in buffer without Ca^{2+} and with 1 mM EGTA (−Ca^{2+}, *blue*). There is no difference in parasites that oscillate and eventually invade (*red*). There is a larger percentage of parasites that do not attach or invade in a buffer without Ca^{2+} and with 1 mM EGTA (*purple*). *C,* quantification and statistical analysis of peak fluorescence values associated with invasion events. There is an increase in peak fluorescence of parasites that exhibit a spike in cytosolic Ca^{2+} and invade in the presence of extracellular Ca^{2+} (*green*). There is no difference in average peak fluorescence values calculated for both parasites that oscillate but do not invade (*blue*) and parasites that oscillate and eventually invade (*red*). *Bars* are means ± S.E. Results are from three independent experiments with at least 100 total parasites counted under each condition. *, Student's *t* test $p < 0.05$. *A.U.,* arbitrary units.

between Ca^{2+} influx and both duration of motility and distance traveled. These two features are essential for a successful invasion.

Discussion

We measured Ca^{2+} oscillations in intracellular tachyzoites and found that an increase in cytosolic Ca^{2+} precedes the increase in motility needed for egress. We utilized different types of GECIs so that we could simultaneously monitor calcium fluxes in both the host and the parasite during egress. We demonstrated that there is significant communication between the host and the parasite, although not always resulting in parasite egress. Additionally, we demonstrate that Ca^{2+} entry plays a relevant role in parasite egress, and this entry occurs through a Ca^{2+} channel that is sensitive to nifedipine. Physiological (histamine) or pharmacological (thapsigargin) agents, known to increase cytosolic Ca^{2+} in mammalian cells, were able to stimulate Ca^{2+} increase in the infected cells and only weakly in the parasites, but this increase did not lead to parasite

egress, suggesting that the parasite is able to buffer these physiological or pharmacological changes in host cell Ca^{2+}.

T. gondii has been reported to grow within vacuoles surrounded by host mitochondria (36), but the biological consequences of this association have remained enigmatic. It has been demonstrated that in some cells, such as pancreatic acinar cells, mitochondria could form a belt that acts as a "firewall" confining Ca^{2+} signals to the secretory pole (37). Ca^{2+} sequestration by mitochondria is also crucial in defining cellular subdomains in neurons, acting as Ca^{2+} buffers (38, 39). It is tempting to speculate that host mitochondria could also form a firewall close to the parasitophorous vacuole protecting tachyzoites from physiological or pharmacological cytosolic Ca^{2+} increases in the host cell thus preventing their premature release during the normal Ca^{2+} signaling activity of the host cell. Disruption of the structure of the PV during a natural egress event would expose the parasite to Ca^{2+} fluctuations or ionic changes surrounding the PV.

We also tested the effect of other agents such as ionomycin, DTT, and saponin. Ionomycin increases cytosolic Ca^{2+} in both parasite and host cell leading to egress, and this effect occurs with and without adding Ca^{2+} to the buffer, suggesting that Ca^{2+} release from the parasite or host intracellular stores is sufficient to account for the increased motility and egress of the parasites. Ionomycin releases Ca^{2+} from all neutral stores and results in a much higher level of intracellular Ca^{2+} than those obtained by adding other ionophores or inhibitors like thapsigargin (40), thereby explaining its powerful effect on egress.

The information on intracellular Ca^{2+} release pathways in Apicomplexan parasites is fragmented and poorly defined. There is pharmacological evidence for the presence of channels responsive to IP_3 (7, 41, 42) and cyclic ADP-ribose (43), although there are no orthologous genes to the mammalian IP_3 receptor or ryanodine receptor (in any of the Apicomplexan genomes). This is despite evidence for the presence of enzymes involved in the generation of some of these second messengers, such as a phosphoinositide phospholipase C (44) and cyclic ADP-ribose cyclase and hydrolase activities (43).

Addition of DTT led to a Ca^{2+} increase and motility in the parasite, resulting in egress. These results revealed that the increase in cytosolic Ca^{2+} levels in the host cells is secondary to parasite egress, supporting previous proposals (22) that this compound acts through the activation of a parasite nucleotidase that depletes the host cell of ATP (30). Interestingly, adjacent uninfected cells also show Ca^{2+} increases after the release of parasites from infected cells, which could be the result of intercellular Ca^{2+} wave propagation (31). Intercellular Ca^{2+} waves could result from the diffusion of second messengers, such as IP_3, through gap junctions between adjacent cells. In addition or alternatively, cell rupture due to parasite egress could release agonists, which could activate the production of IP_3, in the adjacent cells generating a Ca^{2+} signal. Each mechanism can occur in isolation or synergistically (31). Interestingly, DTT did not have a direct effect on cytosolic Ca^{2+} of Fura-2/AM-loaded isolated parasites or on cytosolic Ca^{2+} of non-infected cells. The dramatic increase of cytosolic Ca^{2+}, which only occurs when the parasite is intracellular, is intriguing and likely involves either 1) other unknown factors of host
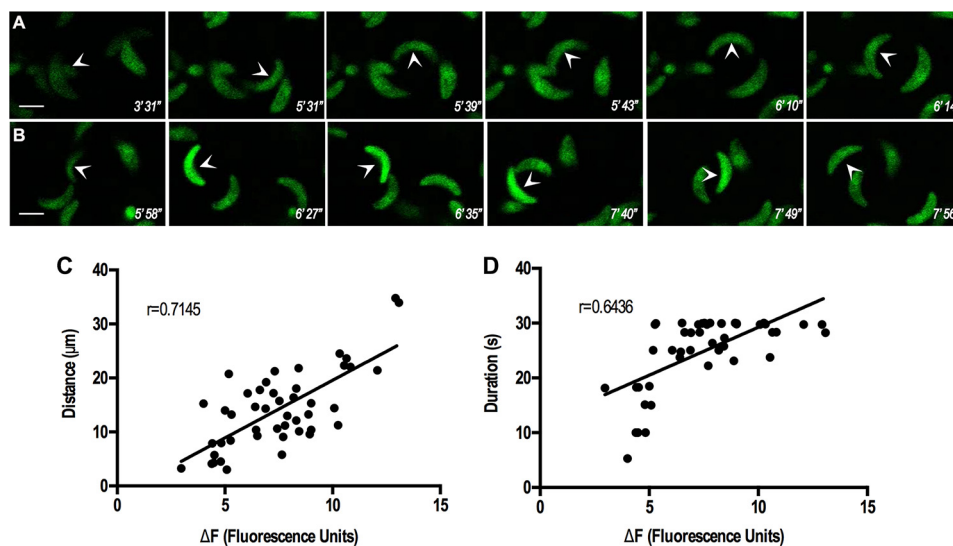
FIGURE 9. **Extracellular Ca²⁺ enhances motility.** *A*, GCaMP6f-expressing tachyzoites (*arrowhead*) show stimulation of gliding after adding Ca²⁺. Notice that parasites drift and oscillate, but after addition of Ca²⁺ to the buffer (from supplemental Video S8*A*), fluorescence increases, parasites halt, attach, and start gliding. Ca²⁺ was added at 3 min and 39 s after starting the video. *B*, GCaMP6f-expressing tachyzoite (*arrow*) shows stimulation of motility after addition of thapsigargin (3 min and 4 s) and Ca²⁺ (4 min and 43 s) (from supplemental Video S8*B*). *C*, relationship between average peak fluorescence and distance traveled by extracellular tachyzoites was plotted and showed significant correlation by Pearson correlation coefficient ($r = 0.7145$). *D*, relationship between average peak fluorescence and duration of motility was significant (Pearson correlation coefficient, $r = 0.6436$). Each *dot* indicates a single parasite that was analyzed from at least four different videos.

origin or 2) that the parasite nucleotidase in question is only expressed during intracellular stages of development.

Previous experiments using saponin suggested that the ionic composition of the buffer used, and in particular low potassium levels, was important to facilitate parasite egress by this permeabilizing agent (24). We then designed an intracellular buffer with low sodium and chloride and high potassium to compare with extracellular buffer conditions (high sodium and chloride and low potassium). Parasites were triggered to egress with saponin, and in both cases, egress was faster and more efficient in the presence of Ca²⁺, highlighting the role of Ca²⁺ entry in the stimulation of motility and lessening the role of other ions under these conditions. We did not observe a significant difference in egress between intracellular and extracellular ionic conditions when Ca²⁺ was present. Previous data from our laboratory supported the participation of a nifedipine-sensitive permeation pathway in Ca²⁺ entry in *T. gondii* (6). Our intracellular buffer ionic composition is high in potassium but also low in chloride (10 mM), resembling the ionic composition of the intracellular milieu. Previous observations have shown that *T. gondii* becomes depolarized in low chloride (45), a condition that would favor Ca²⁺ entry.

Our results using R-GECO targeted to the parasitophorous vacuole demonstrated that Ca²⁺ is freely exchanged between the host cytosol and the PV, supporting the suggestions that the PV membrane acts as a sieve for small ions and molecules.

Several studies have proposed a role for Ca²⁺ signaling during the lytic cycle of *T. gondii*. Using Ca²⁺ ionophores, Ca²⁺-chelating agents, and ethanol, a link between Ca²⁺ and conoid extrusion was postulated (8, 46), although no direct parallel Ca²⁺ measurements were reported. The effect of Ca²⁺ on gliding motility was studied on trypsin-permeabilized tachyzoites (47) or by measuring Ca²⁺ oscillations in extracellular tachyzoites loaded with the Ca²⁺ dye Fluo-4/AM (7, 35). A role

for Ca²⁺ signaling in invasion was postulated on the basis of the analysis of changes in Ca²⁺ occurring in parasites loaded with Fura-2/AM upon their attachment to host cells (12) or by detecting the cessation of Ca²⁺ oscillations in extracellular tachyzoites loaded with Fluo-4/AM (7, 48). These and more recent studies have provided indirect evidence that Ca²⁺ signaling is part of the pathways that result in the stimulation of conoid extrusion, gliding motility, microneme secretion, and invasion. Ca²⁺ signaling was also proposed to be involved in the pathway leading to parasite egress from the host cells based on the effect of Ca²⁺ ionophores, which stimulate egress, although it was never shown that cytosolic Ca²⁺ increased in tachyzoites before egress (13, 21). Most of the studies on Ca²⁺ signaling were done with extracellular tachyzoites (conoid extrusion, gliding motility, microneme secretion, and invasion) measuring indirectly the involvement of Ca²⁺ (using Ca²⁺ chelators or ionophores) or detecting Ca²⁺ changes with fluorescent dyes independently of the phenomena examined. We describe the use of tools to measure directly cytosolic Ca²⁺ changes during these processes. In this study we were able to perform real time measurements of Ca²⁺ changes in live intracellular and extracellular parasites. The use of GECIs provided the means to demonstrate that the parasite cytosolic Ca²⁺ increases and oscillates preceding egress. GECIs allowed measurements of Ca²⁺ separately and simultaneously in the parasite cytosol, PV, and host cytosol. These tools will prove to be very useful in future genetic studies for the phenotypic characterization of mutants lacking putative virulence-related genes.

Analysis of gliding motility and the fluorescence of the genetic Ca²⁺ indicator showed that Ca²⁺ influx correlated with the peak intensity, which correlated with the distance traveled by the parasites and the time that they are in motion. These two features are essential for a successful completion of the lytic cycle and agree with our results showing that invasion
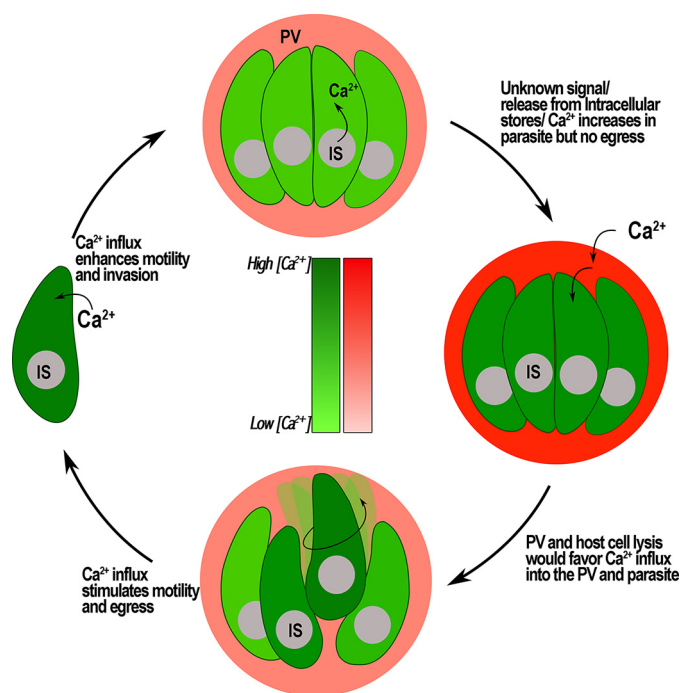
FIGURE 10. **Proposed model for the role of Ca^{2+} during egress.** Ca^{2+} enters the PV and the parasite when cells are treated with different agents. Intracellular Ca^{2+} stores (*IS*) of the parasite release Ca^{2+} into the cytosol, but no egress is triggered. However, this increase in Ca^{2+} could stimulate secretion of microneme proteins like perforin-like protein causing lysis of cell and Ca^{2+} influx from the extracellular media. Influx of Ca^{2+} leads to an activation of motility and egress. Parasites that egress seek other host cells to invade to continue with its lytic cycle. Ca^{2+} influx in extracellular parasites stimulates gliding motility and results in an increase of invasion events.

and egress events are enhanced under conditions that favored Ca^{2+} influx. The link between these two features to Ca^{2+} oscillations and peak intensity was possible because of the use of a clonal cell line expressing GCaAMP6, which is uniformly expressed in the cytosol. Chemical indicators would not have worked because of uneven loading, toxicity, and organellar compartmentalization.

In our model shown in Fig. 10, Ca^{2+} influx would stimulate the intensity of Ca^{2+} oscillations leading to initiation of gliding motility and exit from the PV shortly thereafter. Intracellular stores are likely very important for releasing Ca^{2+} into the cytosol, but they would not necessarily lead to activation of motility and egress. Ca^{2+} influx through a nifedipine-sensitive pathway will further stimulate cytosolic Ca^{2+} increase, motility, and egress of the parasite (6). Careful analysis of the nifedipine experiments showed that the PV still ruptured, but parasites did not show high motility. A perforin-like protein has been shown to be involved in the initial rupture of the PV (19), and the release of Ca^{2+} from intracellular stores could stimulate this release in the natural egress process. Our model proposes that this rupture could result in a downstream stimulation of Ca^{2+} influx, which would lead to stimulation of gliding motility and egress. We believe that the tools described here will help in the dissection of the factors/signaling molecules involved in the interaction of host and parasite resulting in egress, an essential part of the lytic cycle of *T. gondii*. In addition, the phylogenetic position of these eukaryotic unicellular microbes will advance our understanding of the evolution of these complex pathways.

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