Toxoplasma gondii Microneme Secretion Involves Intracellular Ca\(^{2+}\) Release from Inositol 1,4,5-Triphosphate (IP\(_3\))/Ryanodine-sensitive Stores*

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Calcium-mediated microneme secretion in Toxoplasma gondii is stimulated by contact with host cells, resulting in the discharge of adhesins that mediate attachment. The intracellular source of calcium and the signaling pathway(s) triggering release have not been characterized, prompting our search for mediators of calcium signaling and microneme secretion in T. gondii. We identified two stimuli of microneme secretion, ryanodine and caffeine, which enhanced release of calcium from parasite intracellular stores. Ethanol, a previously characterized trigger of microneme secretion, stimulated an increase in parasite inositol 1,4,5-triphosphate, implying that this second messenger may mediate intracellular calcium release. Consistent with this observation, xestospongin C, an inositol 1,4,5-triphosphate receptor antagonist, inhibited microneme secretion and blocked parasite attachment and invasion of host cells. Collectively, these results suggest that T. gondii possesses an intracellular calcium release channel with properties of the inositol 1,4,5-triphosphate/ryanodine receptor superfamily. Intracellular calcium channels, previously studied almost exclusively in multicellular animals, appear to also be critical to the control of parasite calcium during the initial steps of host cell entry.

Apicomplexans are unicellular parasites that must enter cells to replicate. Members of this phylum share a common vulnerability when the process of invasion is compromised, yet diseases caused by apicomplexans remain among the most difficult to cure. The invasion process is rapid and marked by the sequential secretion of parasite organelles. Micronemes, rhoptries, and dense granule contents are released by invading parasites and participate in attachment, vacuole formation, and intracellular survival (1). Understanding the mechanisms regulating parasite secretion could thus be useful in the design of new therapeutic strategies aimed at blocking invasion.

Micronemes are the first secretory organelles to be discharged during Toxoplasma gondii invasion. Microneme proteins possess a variety of adhesive domains and include soluble and transmembrane forms (2, 3). Their release appears highly regulated in that host cell contact triggers a burst of microneme release (1). Binding studies suggest that microneme proteins participate in attachment to host cell surfaces (4–7) and that the transmembrane form of the adhesin MIC2 may link parasite and host cell membranes during invasion.

Previous studies demonstrated that increases in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) mediate microneme secretion in T. gondii. For example, association of T. gondii tachyzoites with host cells results in increases in parasite [Ca\(^{2+}\)]\(_i\), (8) and chelation of T. gondii [Ca\(^{2+}\)]\(_i\), blocks microneme secretion and invasion (9). Moreover, reagents that raise T. gondii [Ca\(^{2+}\)]\(_i\), levels stimulate microneme discharge in the absence of host cells (10). These include Ca\(^{2+}\)-ionophores ionomycin and A23187, as well as the sarco-endoplasmic reticulum ATPase inhibitor thapsigargin (10). Identification of alcohols as potent artificial triggers of microneme secretion led to studies demonstrating that ethanol also raises parasite [Ca\(^{2+}\)]\(_i\), levels (11). T. gondii possesses several intracellular stores of Ca\(^{2+}\), including the acidic calciosomes (12), mitochondria, and the endoplasmic reticulum (13). Whether all or some of these stores participate in microneme secretion during host cell invasion is unknown. The mechanism of Ca\(^{2+}\)-mediated microneme release in both artificially triggered and natural microneme secretion during invasion remains largely undefined.

Regulated exocytosis is associated with fluxes in [Ca\(^{2+}\)]\(_i\), although the exact step requiring Ca\(^{2+}\) is unclear and may depend on the system being studied (14). Ca\(^{2+}\) signaling involves the mobilization of Ca\(^{2+}\) from two sources: intracellular stores and the extracellular medium. In mammalian cells, the sarco-endoplasmic reticulum is a commonly utilized source of readily accessible [Ca\(^{2+}\)]\(_i\), which can be rapidly mobilized under a variety of stimuli. Two types of sarco-endoplasmic reticulum Ca\(^{2+}\) release channels have been identified in multicellular animals: the IP\(_3\) receptor and the ryanodine receptor, which are structurally similar and are thought to be evolutionarily related (15). Binding of the second messenger IP\(_3\) to its receptor releases Ca\(^{2+}\) into the cytosol, whereas ryanodine receptors are modulated in some cells by cyclic ADP-ribose (16). This release of intracellular Ca\(^{2+}\) can in some manner signal the activation of Ca\(^{2+}\) entry, a process known as capacitative Ca\(^{2+}\) entry (17).

Neither IP\(_3\), ryanodine receptors, or capacitative Ca\(^{2+}\) entry
have been identified in unicellular organisms, including yeast and protozoa. However, evidence linking IP3 to [Ca2+]i flux has been described in several unicellular systems. Treatment of membrane vesicles from Candida albicans (18) or Plasmodium chabaudi (19) with IP3 results in Ca2+ release and is blocked by the IP3 receptor antagonist heparin. IP3 induces Ca2+ release from Euglena gracilis microsomes in a dose-dependent manner (20). Carbachol stimulation of Trypanosoma cruzi results in increased [Ca2+]i, and IP3 (21). In characean algae, introduction of IP3 produces action potentials, an event known to involve increases in cytoplasmic Ca2+ (22). Collectively, these studies indicate a potential role for IP3 in Ca2+-mediated signaling events among early branching unicellular eukaryotes.

We were interested in determining whether IP3 functions as a mediator of microneme secretion by increasing parasite [Ca2+]i. In this work, we present evidence that T. gondii may possess intracellular release channels of the IP3/ryanodine receptor superfamily.

**EXPERIMENTAL PROCEDURES**

Chemicals—Caffeine, EGTA, and ryanodine were purchased from Sigma. BAPTA-AM and xestospongin C were purchased from Calbiochem. The BiotrakTM [3H]IP3 assay system was purchased from Pharmacia Biotec. All other reagents were analytical grade. Drugs were resuspended as 100× stock solutions in either distilled water or Me2SO.

Parasite Culture—T. gondii strain RH and the lacZ-expressing clone 2F were maintained as tachyzoites in human foreskin fibroblasts as described (23). For Ca2+ measurement experiments, RH tachyzoites were maintained in bovine turbinate cells (ATCC CRL 1390) (12). All experiments used freshly lysed out parasites purified by passage through a 23-gauge needle and filtration through a 3 micron Nucleopore membrane. All cultures tested free of Mycoplasma using the GenProbeTM mycoplasma detection system.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-PAGE was performed in 7% mini-gels under reducing conditions and then equilibrated at 37 °C with complete media. This fixation protocol prevents the majority of parasites from invading while still allowing adhesion to occur. Parasites were resuspended at 107/ml in invasion medium and treated for 10 min at room temperature with stock solutions of drugs. Treated parasites were added in 0.2 ml volume to warm 24-well plates of human foreskin fibroblasts in quadruplicate and incubated for 20 min at 37 °C for both assays.

**RESULTS**

**Ryanodine Stimulates Ca2+-mediated Microneme Secretion**—Ryanodine has been shown to mediate increases in [Ca2+]i in a variety of multicellular organisms (26). To determine whether ryanodine stimulated Trypanosoma microneme secretion, we treated tachyzoites with ryanodine at concentrations ranging from 1 nM to 100 μM, removed the cells by centrifugation, and examined secreted proteins MIC2 and MIC4 present in supernatants by Western blotting. MIC2 is a convenient marker for microneme secretion because the secreted 95–100-kDa form (sMIC2) is released into supernatants, while the cell-associated 115-kDa form (cMIC2) remains in the parasite pellet (27). Similarly, the secreted form of MIC4 is 70-kDa, while cell-associated MIC4 is 72 kDa. Constitutive expression of β-galactosidase in the 2F strain was used to control for inadvertent lysis of the parasites during the experiment and was typically between 1–5%, as indicated by comparison with dilutions of a parasite cell standard (% cell stds).

Secretion of MIC2 and MIC4 was stimulated by ryanodine at doses from 1 nM to 10 μM but inhibited at 100 μM when compared with parasites treated with Me2SO (Fig. 1A). The inhibition of microneme secretion at 100 μM is consistent with previous studies indicating that high doses keep the ryanodine receptor in a closed conformation (26). To determine whether ryanodine stimulation of microneme secretion involved an increase of [Ca2+]i, from either intracellular stores or the extracellular medium, we pretreated parasites with the membrane-permeable calcium chelator BAPTA-AM to prevent [Ca2+]i increases (8) or added EGTA to the parasites immediately prior to addition of ryanodine to chelate extracellular Ca2+ and prevent Ca2+ entry (12). BAPTA-AM pretreatment inhibited microneme secretion upon stimulation with 100 nM ryanodine (Fig. 1A), indicating that ryanodine-mediated secretion required a rise in parasite [Ca2+]i. Additionally, EGTA also blocked ryanodine stimulation of microneme secretion, showing that extracellular Ca2+ was required by the parasite. Monitoring of [Ca2+]i, using fura-2-loaded parasites confirmed these results. The increase in [Ca2+]i, produced by ryanodine in the
absence of extracellular Ca\(^{2+}\) (with 1 mM EGTA added to the incubation medium; Fig. 1B, +EGTA) confirmed the hypothesis that ryanodine is able to release Ca\(^{2+}\) from intracellular stores of T. gondii. Under these conditions [Ca\(^{2+}\)], rapidly decreased, probably through Ca\(^{2+}\) efflux to the external medium or Ca\(^{2+}\) uptake into other intracellular stores. In the presence of extracellular Ca\(^{2+}\), a greater and sustained response was observed upon addition of ryanodine (Fig. 1B). Both the magnitude and duration of this elevated [Ca\(^{2+}\)] response were greater in the presence of extracellular Ca\(^{2+}\) (Fig. 1B). This sustained [Ca\(^{2+}\)], elevation in the presence of extracellular Ca\(^{2+}\) could be due either to capacitative Ca\(^{2+}\) entry (17) or to inhibition of Ca\(^{2+}\) efflux by ryanodine as has been postulated to occur in smooth muscle cells (28). Because treatment of tachyzoites with EGTA prevented microneme secretion (Fig. 1A) a sustained [Ca\(^{2+}\)] increase is apparently responsible for this process.

Caffeine Stimulates Ca\(^{2+}\)-mediated Microneme Secretion—Caffeine is a pharmacological agonist of ryanodine receptor Ca\(^{2+}\) release (26). Based on the above evidence that T. gondii has a ryanodine-sensitive Ca\(^{2+}\) response, we tested caffeine for its ability to stimulate microneme secretion. Tachyzoites were incubated with caffeine at concentrations ranging from 1 mM to 1 mM before collection of supernatants for analysis by Western blot. Caffeine stimulated microneme secretion at all doses, with 100 mM caffeine-stimulated dose-dependent secretion of MIC2 and MIC4. B, intracellular calcium measurements of fura-2/AM-loaded tachyzoites. Tachyzoites loaded with fura-2/AM as described under “Experimental Procedures,” were incubated in buffer A; traces were made in the presence of 1 mM EGTA or 1 mM CaCl\(_2\) (Ca\(^{2+}\)); the arrow indicates the point of addition of 1 mM caffeine (CAFF).


Calcium Release Pathways in T. gondii

25873

Fig. 3. Ethanol increases T. gondii IP$_3$. A, tachyzoite IP$_3$ levels were monitored by radioreceptor assay at intervals following the addition of 1% (171 mM) ethanol (+EtOH) or in control cells (−CTL) as described under “Experimental Procedures.” Ethanol caused a transient increase in parasite IP$_3$ after its addition. B, Western blot of parasite protein pellets obtained after IP$_3$ extraction. Secreted MIC2 (sMIC2) is visible after 1.5 min, while levels of the cellular form (cMIC2) are unchanged. Both kinetic in A and Western blot in B are representative of three independent experiments, which showed similar results.

A

B

| pmol IP$_3$ | time (minutes) |
|------------|----------------|
| 0          | 0              |
| 0.2        | 1              |
| 0.4        | 2              |
| 0.6        | 3              |
| 0.8        | 4              |

- EtOH
- CTL

KDa: 106 - 77

Calcium Release Pathways in T. gondii

25873

DISCUSSION

Calcium-mediated exocytosis of T. gondii microneme proteins is a key step in the invasion of host cells. The control of intracellular Ca$^{2+}$ levels in T. gondii is not well characterized, prompting our search for modulators of secretion whose target was well studied in other Ca$^{2+}$ signaling systems. We identified ryanodine and caffeine, two ryanodine receptor agonists, as stimuli of Ca$^{2+}$-mediated microneme secretion. We also found that the second messenger IP$_3$ was induced upon ethanol stimulation of parasites. Finally, the IP$_3$ receptor antagonist xestospongin C inhibited the caffeine- and ethanol-induced increases in [Ca$^{2+}$], as well as preventing microneme secretion, attachment, and invasion. Together, these results strongly suggest that channels of the IP$_3$/ryanodine receptor superfamily in T. gondii mediate increases in [Ca$^{2+}$], which in turn promote microneme secretion.

Ryanodine is an active component of the botanical insecticide ryania, an alkaloid found in the shrub R尼亚ania spectora. Ryanodine has potent effects on a variety of multicellular organisms (25). Receptors that release calcium from intracellular stores in response to secretagogue treatment have been described in vertebrates, where they usually are expressed in specialized neurosecretory or muscle cells (15). Ryanodine receptors also exist in a variety of invertebrates including crustaceans, insects, and worms; however, they have not been described in unicellular organisms (32). At micromolar levels ryanodine stabilizes ryanodine receptors in the open form, allowing Ca$^{2+}$ liberation from the sarco-endoplasmic lumen (26). At higher concentra-

- J. L. Lovett and L. D. Sibley, unpublished data.
tions, ryanodine receptor channels in rabbit skeletal sarcoplasmic reticulum vesicles are persistently blocked (33). We observed a similar biphasic dose response in *T. gondii*, and ryanodine stimulation of microneme secretion was dependent on both intracellular and extracellular calcium. Ryanodine stimulation of \([\text{Ca}^{2+}]_{\text{i}}\) release has been shown to require extracellular Ca\(^{2+}\) as part of its \([\text{Ca}^{2+}]_{\text{i}}\)-induced Ca\(^{2+}\) release mechanism, suggesting it may activate flux through plasma membrane channels and capacitative entry (17, 26). The induction of calcium release in *T. gondii* treated with ryanodine parallels that in well-studied systems, suggesting the existence of ryanodine-responsive calcium channels in this early branching eukaryote.

Caffeine also induced microneme secretion by *T. gondii*, and was effective at doses that are 100-fold lower than its activity on mammalian cells. Unlike ryanodine, caffeine did not require extracellular Ca\(^{2+}\), a result similar to that reported for rat PC12 cells (35). In addition to acting on ryanodine-like calcium channels, caffeine and related alkylxanthines have been shown to inhibit cyclic nucleotide phosphodiesterases (25, 26, 33). The resulting increases in cyclic AMP and activation of protein kinase A can give rise to an increase in \([\text{Ca}^{2+}]_{\text{i}}\) in mammalian cells (36). Although isobutylmethylxanthine, a nonselective phosphodiesterase inhibitor, also stimulated microneme secretion by *T. gondii*, two drugs expected to directly increase cyclic AMP, forskolin and 8-bromo-cyclic AMP, had no affect on microneme secretion. Similarly, treatment of parasites with 8-bromo-cyclic GMP did not affect microneme secretion. Thus, while we cannot rule out possible inhibition of phosphodiesterases, the effect of caffeine in elevating \([\text{Ca}^{2+}]_{\text{i}}\) in *T. gondii* does not seem to be due to this mechanism. A third possible mode of action is that caffeine may inhibit the sarco-endoplasmic reticulum type ATPase involved in refilling calcium stores. This pump is not yet characterized in *T. gondii*, studies in the ciliate *Paramecium tetraurelia* have shown that the sarco-endoplasmic reticulum ATPase is exquisitely sensitive to caffeine inhibition (37). Ciliates are phylogenetically close relatives of the Apicomplexa.

**Proposed model for intracellular calcium signaling in *T. gondii***. Stimulation of a parasite signaling cascade during treatment with ethanol or contact with host cells leads to second messenger IP\(_3\) formation, increases in \([\text{Ca}^{2+}]_{\text{i}}\), via a calcium release channel, and microneme secretion. The releasable store of calcium was not sensitive to thapsigargin, indicating that it may comprise a unique intracellular pool. IP\(_3\), IP\(_3\) receptor.
Unlike paramaecium, the [Ca\textsuperscript{2+}] pool in T. gondii is sensitive to thapsigargin (12); however, ethanol stimulation of thapsigargin-pretreated parasites generates an additional increase in [Ca\textsuperscript{2+}], (11), implying that they also possess a thapsigargin-insensitive [Ca\textsuperscript{2+}] pool. Similarly, pretreatment with thapsigargin prior to stimulation with caffeine or ryanodine did not inhibit microneme secretion (data not shown), suggesting that this same thapsigargin-insensitive pool may be stimulated by caffeine or ryanodine.

Our studies demonstrate that IP\textsubscript{3} acts as a second messenger during the stimulation of tachyzoites with ethanol, a known Ca\textsuperscript{2+}-mediated trigger of microneme secretion (11). In higher eukaryotes, IP\textsubscript{3} induces calcium release from channels located in the sarco-endoplasmic reticulum in a variety of cell types (38). Typically, IP\textsubscript{3} is produced along with diacylglycerol after hydrolysis of phosphatidylinositol 4,5-bisphosphate by inositol phospholipid-specific phospholipase C (PLC). In higher eukaryotes, this process is mediated by PLC-\gamma signaling through tyrosine kinase receptors and PLC-\beta activated via heterotrimetric G-proteins (39). Comparisons of PLC isoforms \(\delta, \gamma, \) and \(\beta\) suggest that PLC-\(\delta\) was present in single-celled eukaryotes, while the \(\beta\) and \(\gamma\) forms arose after the development of multicellular organisms \(\sim 940\) million years ago (40). Consequently, parasites likely contain only the ancestral PLC-\(\delta\) isoform. Consistent with this idea, T. cruzi contains a PLC-\(\delta\) isoform (41) and PLC-\(\delta\) candidate sequences are present in the Plasmodium falciparum and Plasmodium vivax genomes (42). While not conventionally associated with Ca\textsuperscript{2+} signaling, PLC-\(\delta\) has recently been linked to Ca\textsuperscript{2+} transients associated with fertilization in mice (43). Thus, it is possible that PLC-\(\delta\) plays an important role in protozoan calcium regulation.

Previous evidence for IP\textsubscript{3}-responsive Ca\textsuperscript{2+} release channels in unicellular organisms has been indirect. Entamoeba histolytica crude membranes bind radiolabeled IP\textsubscript{3} (44), and a cross-reacting IP\textsubscript{3} receptor antibody detects an appropriately sized protein in the ciliate Blepharisma japonicum (45, 46). Combined with the present findings, these results indicate that single-celled eukaryotes also likely have intracellular calcium release channels that respond to IP\textsubscript{3}. However, our attempts to identify IP\textsubscript{3} or ryanodine receptor channels in T. gondii using a cross-reactive antibody (mouse monoclonal antibody 34C antichicken ryanodine receptor) (47) have not been successful. IP\textsubscript{3} and ryanodine receptors are \(\sim 40\%\) homologous between Caenorhabditis elegans and vertebrates, and they are likely to be significantly more divergent in protozoa due to their early branching phylogenetic position (48). We have analyzed the nearly complete genomes of P. falciparum (42) and Cryptosporidium parvum (www.parvum.mic.vcu.edu) and the Apicomplexan expressed sequence tag databases (parodb.cis.upenn.edu) and found that these organisms do not contain well recognized motifs for IP\textsubscript{3} or ryanodine receptors such as SPRY and MIR domains. Consequently, it is likely that protozoa such as T. gondii possess intracellular calcium release channels that are unconventional in sequence and/or function.

Our observations suggest that IP\textsubscript{3} catalyzes [Ca\textsuperscript{2+}]\textsubscript{i} release in the parasite through an intracellular channel related to the IP\textsubscript{3} ryanodine receptor superfamily during T. gondii invasion of host cells. To test whether production of IP\textsubscript{3} was necessary for microneme secretion and host cell attachment, we utilized xestospongin C, a compound that inhibits IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release from rat cerebellar microsomes (31) and Dictyostelium vesicles (34). Xestospongin C acts primarily as a competitive inhibitor of IP\textsubscript{3} to block calcium release from IP\textsubscript{3} receptors; however, it can also inhibit ryanodine receptors with somewhat lower potency (31). Xestospongin C treatment of T. gondii inhibited ethanol- and caffeine-mediated secretion of micronemes and prevented attachment and invasion of host cells. Together, these results imply that xestospongin C inhibits microneme secretion by modulating a T. gondii [Ca\textsuperscript{2+}]\textsubscript{i} release channel that responds to IP\textsubscript{3}. These findings suggest that the engagement of natural ligands during host cell invasion may also give rise to IP\textsubscript{3} to promote the observed increases in [Ca\textsuperscript{2+}]\textsubscript{i} in the parasite reported previously (10).

The results of our studies using Ca\textsuperscript{2+} release channel agonists and antagonists strongly suggest that the protozoan parasite T. gondii possess intracellular calcium release channels of the IP\textsubscript{3}/ryanodine superfamily. Relatively low concentrations of caffeine, ryanodine, and xestospongin C affected microneme release, indicating that protozoa may be unusually sensitive to these agents that affect calcium channels. While we cannot rule out secondary effects on other pathways, the primary response observed to all these compounds is consistent with their acting on a calcium release channel. A model integrating our findings with a hypothetical signaling cascade is presented in Fig. 5. We show that ethanol increases IP\textsubscript{3} production, a second messenger that could act to liberate the [Ca\textsuperscript{2+}]\textsubscript{i} pool. We also show that ryanodine increases [Ca\textsuperscript{2+}]\textsubscript{i}, suggesting it interacts directly with a putative [Ca\textsuperscript{2+}]\textsubscript{i}, release channel via a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism. The calcium-dependent stimulation of microneme secretion by ryanodine, caffeine, or ethanol was thapsigargin-insensitive, indicating that this unique pool may be responsible for calcium signaling in parasites. During contact with host cells, it is likely that a similar cascade is triggered to release Ca\textsuperscript{2+} from an intracellular store and stimulate microneme secretion. A role for a putative parasite PLC in the conversion of phosphatidylinositol bisphosphate to IP\textsubscript{3} and diacylglycerol is suggested by the available data but has not yet been directly demonstrated. The responsiveness to both IP\textsubscript{3} and ryanodine suggests that calcium release channels may exist as two separate entities. However, we have not excluded the intriguing possibility that protozoa possess a single ancient channel displaying hybrid properties of both. Analysis of calcium channels in protozoa will be illuminating to studies of the function and evolution of eukaryotic calcium signaling proteins.

Acknowledgments—We thank Drs. Steve Beverley, Vern Carruthers, Dan Goldberg, Andy Pekosz, Paul Schlessinger, and Tom Steinberg for advice and critical input and Jeff Diffenderfer for expert technical assistance.

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J. Biol. Chem. 2002, 277:25870-25876.
doi: 10.1074/jbc.M202553200 originally published online May 13, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202553200

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