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The Calcium Signaling Toolkit of the Apicomplexan Parasites *Toxoplasma gondii* and *Plasmodium spp*

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Abstract

Apicomplexan parasites have complex life cycles, frequently split between different hosts and reliant on rapid responses as the parasites react to changing environmental conditions. Calcium ion (Ca^{2+}) signaling is consequently essential for the cellular and developmental changes that support apicomplexan parasitism. Apicomplexan genomes reveal a rich repertoire of genes involved in calcium signaling, although many of the genes responsible for observed physiological changes remain unknown. There is evidence, for example, for the presence of a nifedipine-sensitive calcium entry mechanism in *Toxoplasma*, but the molecular components involved in Ca^{2+} entry in both *Toxoplasma* and *Plasmodium*, have not been identified. The major calcium stores are the endoplasmic reticulum (ER), the acidocalcisomes, and the plant-like vacuole in *Toxoplasma*, or the food vacuole in *Plasmodium* spp. Pharmacological evidence suggests that Ca^{2+} release from intracellular stores may be mediated by inositol 1,4,5-trisphosphate (IP_3) or cyclic ADP ribose (cADPR) although there is no molecular evidence for the presence of receptors for these second messengers in the parasites. Several Ca^{2+}-ATPases are present in apicomplexans and a putative mitochondrial Ca^{2+}/H^+ exchanger has been identified. Apicomplexan genomes contain numerous genes encoding Ca^{2+}-binding proteins, with the notable expansion of calcium-dependent protein kinases (CDPKs), whose study has revealed novel roles in gliding motility, microneme secretion, host cell invasion and egress, and parasite differentiation. Microneme secretion has also been shown to depend on the C2 domain containing protein DOC2 in both *Plasmodium spp.* and *Toxoplasma*, providing further evidence for the complex transduction of Ca^{2+} signals in these organisms. The characterization of these pathways could lead to the discovery of novel drug targets and to a better understanding of the role of Ca^{2+} in these parasites.

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1. Introduction

Apicomplexan parasites include a number of unicellular eukaryotes that infect humans and animals and cause diseases such as malaria (*Plasmodium* spp.), babesiosis (*Babesia* spp.), toxoplasmosis (*Toxoplasma gondii*), cryptosporidiosis (*Cryptosporidium parvum*), and cyclosporiasis (*Cyclospora cayetanensis*), among others. They are named Apicomplexan for the presence of a characteristic apical complex containing secretory organelles. The most studied of these organisms are those that cause malaria, one of the most devastating human infectious diseases, and toxoplasmosis, an important cause of congenital disease and infection in immunocompromised patients. We will limit our discussion to what is known about Ca^{2+} homeostasis and signaling in these organisms.

T. gondii and malaria parasites have intracellular stages and recent studies have revealed an important role for Ca^{2+} in parasite invasion and egress from their host cells. While *T. gondii* invade practically any cell containing a nucleus, the malaria parasites invade only hepatocytes and red blood cells of the mammalian hosts. Each life cycle stage of these parasites possesses specific characteristics concerning Ca^{2+} homeostasis and signaling and we will describe some of these differences.

2. The role of Ca^{2+} entry in Ca^{2+} homeostasis

Ca^{2+} homeostasis in *Plasmodium* spp. and *T. gondii* appears to differ significantly from that in mammalian cells. Intracellular calcium measurements in *T. gondii* tachyzoites performed using the fluorescent calcium indicator Fura-2/AM (Fura 2-acetoxymethylester) gave values of 70 ± 6 nM in the nominal absence of extracellular Ca^{2+} and 100 ± 9 nM in the presence of 1 mM extracellular Ca^{2+} [1]. Similar measurements in *Plasmodium chabaudi*, and *P. falciparum* also resulted in values in the nanomolar range as detected in single-cell imaging experiments [2] or in parasite suspensions [3]. Imaging *P. falciparum*-infected erythrocytes using a ratiometric calcium indicator with low pH sensitivity (Fura red) resulted in much higher values of intracellular Ca^{2+} $[Ca^{2+}]_i$ (289-352 nM) [4] and it was proposed that this high value could be the result of superposition of Ca^{2+} signals from the cytosol and the extensive ER compartment within these cells [4]. A $[Ca^{2+}]_i$ concentration of 100 nM was also found in *P. berghei* ookinetes [5]. In conclusion, $[Ca^{2+}]_i$ in both *T. gondii* and *Plasmodium* spp. is within the range observed in other eukaryotic cells (i.e. 90-100 nM).

Ca^{2+} entry plays an important role in replenishing intracellular organelles and in activating signaling pathways that respond to elevated cytosolic Ca^{2+} [6]. However, there is no molecular evidence for the presence of store-operated channels (ORAI), which are linked to the endoplasmic reticulum sensor protein stromal interaction molecule (STIM), ligand-operated channels [6], or second messenger-operated channels in Apicomplexan parasites. Sequences with general similarity to a voltage dependent Ca^{2+} channel were found only in *T. gondii* [7]. The demonstration that this gene product is functional as a Ca^{2+} channel awaits further electrophysiological work but evidence has been presented of the presence of a nifedipine-sensitive Ca^{2+} entry pathway that supports the function of a voltage-gated Ca^{2+} channel [8]. Sequences with similarity to transient receptor potential (TRP) channels were

found in both the *T. gondii* and the malaria genomes [7], and a Ca^{2+} channel is inserted in the plasma membrane of erythrocytes infected with *P. falciparum* [9].

As is common to other unicellular eukaryotes there are no gene orthologues to Na^{+}/Ca^{2+} exchangers involved in Ca^{2+} efflux in these organisms. There are several reports of plasma membrane type Ca^{2+}-ATPases (PMCA) in apicomplexan parasites [10] and one of these pumps (TgA1) has been studied in more detail in *T. gondii* [11]. The protein localizes to both the plasma membrane and acidocalcisomes and the gene is able to complement *Saccharomyces cerevisiae* mutants deficient in the *PMC1* gene providing genetic evidence of its role as a Ca^{2+} pump [11]. Mutants deficient in this pump have reduced virulence in vitro and in vivo [12]. Biochemical evidence for calmodulin (CaM) stimulation of the plasma membrane Ca^{2+} pump of *T. gondii* has been reported [13] although TgA1 appears to lack a typical CaM-binding domain. This might suggest the presence of a different domain able to bind CaM. A gene encoding for a second putative PMCA has been found in the *T. gondii* genome (TGME29_033770) [10]. The deduced amino acid sequence (1200 aa) shows 45% identity with *Tg*A1 [11]. No orthologues to these genes are found in the *Plasmodium* genomes.

3. Ca^{2+} release and leakage from intracellular sources

Apicomplexan parasites possess the typical calcium stores found in other eukaryotic cells such as the endoplasmic reticulum (ER), Golgi apparatus and mitochondria, and some additional compartments that could contribute to calcium signaling and include the acidocalcisomes, and food vacuoles in *Plasmodium* spp. and the plant-like vacuole in *T. gondii*. Ca^{2+} mobilizing signals are generated by the stimulation of the phosphoinositide-signaling pathway through binding of a ligand to a surface receptor, like a G-protein linked receptor (GPCR). Interaction via G proteins leads to the generation of inositol 1,4,5-trisphosphate (IP_3) through the activity of a phosphatidylinositol phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol 4,5 diphosphate (PIP_2) to form IP_3 and DAG. IP_3 receptors are intracellular Ca^{2+} channels usually present in the membrane of the endoplasmic reticulum, which open in response to binding of IP_3 resulting in Ca^{2+} release into the cytosol [14]. Through this mechanism, Ca^{2+} signals are generated that result in the stimulation of a diversity of cellular pathways [14]. Cyclic ADP ribose (cADPR) is also able to release intracellular Ca^{2+} acting through ryanodine receptors usually located in the endoplasmic reticulum [15]. The genome of apicomplexan parasites supports the presence of genes that predict for proteins with 7 transmembrane domains and two of those are annotated as GPCR (TGME49_233760 and TGME49_286490).

The enzymes that catalyze the formation of IP_3, the phosphoinositide phospholipases C (PI-PLCs) are present in both *T. gondii* (TgPI-PLC) [16] and malaria parasites [17] but there is no genomic support for the presence of IP_3 receptors in any apicomplexan parasite. However, it has been found that IP_3 stimulates Ca^{2+} release from intracellular stores of *T. gondii* [18, 19] and malaria parasites [20, 21]. Photolysis of caged IP_3 or melatonin-induced IP_3 formation also increases cytosolic Ca^{2+} in *P. falciparum*-infected red blood cells [21]. Cyclic ADP ribose is also able to release Ca^{2+} from intracellular stores of *T. gondii*, presumably by stimulating a ryanodine-type receptor [19]. *T. gondii* possesses ADP ribosyl

cyclase and hydrolase [19] but there is no molecular evidence of a ryanodine receptor. Recent work in the malaria parasite using chemical and genetic approaches found that a cyclic GMP (cGMP)-regulated kinase, protein kinase G (PKG), controls the synthesis of PIP_2, which is the precursor of IP_3 and therefore could be involved in modulation of the Ca^{2+} signaling pathway that controls ookinete gliding, gametocyte activation, and schizont rupture [22].

3.1. The endoplasmic reticulum

The endoplasmic reticulum (ER) is the largest membrane-bound compartment of eukaryotic cells and plays critical roles in calcium signaling. Ca^{2+} influx into the ER is driven by SERCA-type Ca^{2+} ATPases, which have been characterized in both *T. gondii* [23] and *P. falciparum* [24]. Inhibition of the *T. gondii* SERCA-type Ca^{2+}-ATPase by thapsigargin [25], results in increase of cytosolic Ca^{2+} in *T. gondii* [1], the result of Ca^{2+} leakage through an unknown pathway. Ca^{2+} leak is the passive calcium efflux from the ER that is thought to prevent ER calcium overload and thus allow cytosolic Ca^{2+} signaling [26]. In mammalian cells it has been proposed that leak channels in the membrane of the ER play a role in the steady state concentration of the ER luminal Ca^{2+} [27]. Four types of membrane proteins have been proposed to be involved in the ER calcium leak pathway, Bcl-2, pannexin 1, presenilins and TRPC1. The *Toxoplasma* genome contains ortholog genes for a presenilin (TGME49_204040) and two TRP channels (TGME49_247370 and TGME49_310560) [7]. These *Toxoplasma* molecules have not been studied.

The SERCA Ca^{2+}-ATPase from *P. falciparum* (PfATP6) has 39% sequence identity to the SERCA1a of skeletal muscle and has the typical Ca^{2+}-binding, nucleotide-binding, and phosphorylation sites that occur in the mammalian enzyme. It also has a 200 amino acid insertion rich in asparagine residues in its N-terminus [28]. The recombinant enzyme is highly sensitive to cyclopiazonic acid, insensitive to artemisinin, and weakly inhibited by thapsigargin [28]. However, this enzyme showed inhibition by thapsigargin when expressed in *Xenopus* oocytes [24], and thapsigargin is able to mobilize intracellular Ca^{2+} from *P. chabaudi*-permeabilized cells and from *P. berghei* and *P. falciparum* intact parasites [2, 21], which suggests that the recombinant enzyme loses is sensitivity to artemisinin and thapsigargin.

Although there is physiological evidence of a mechanism for Ca^{2+} release from intracellular stores by inositol 1,4,5-trisphosphate (IP_3) and cylcic ADP ribose (cADPR) (see above) there is no molecular evidence of the presence of either an IP_3 or ryanodine receptor in any Apicomplexan parasite. These results suggest different mechanisms of Ca^{2+} release from the ER.

3.2. Acidic organelles

Acidic Ca^{2+} stores are widely recognized as having important roles in Ca^{2+} signaling [29]. Two main groups of acidic Ca^{2+} stores have been identified in Apicomplexan parasites. One is the acidocalcisome, an organelle that contains large amounts of Ca^{2+} and polyphosphate [30], and the other is a lysosome-like compartment, which has been named food or digestive

vacuole in malaria parasites, and plant-like vacuole (PLV) or vacuolar compartment (VAC) in *T. gondii* [31].

Acidocalcisomes were first found in trypanosomatids [32, 33] and *T. gondii* [1] and later found to be similar to the previously described “volutin granules” in bacteria and polyphosphate bodies in algae [30]. More recent work in *Leishmania donovani* [34] and *Trypanosoma brucei* [35] suggest that acidocalcisomes are lysosome-related organelles (see Docampo and Huang in this issue). Acidocalcisomes were also found in *P. falciparum* [36] and more recently in *Eimeria* parasites [37]. Acidocalcisomes of *T. gondii* possess a plasma membrane-type Ca^{2+}-ATPase (PMCA) for Ca^{2+} uptake that has been named TgA1 [11]. Purified acidocalcisome fractions from *T. gondii* tachyzoites show vanadate-sensitive Ca^{2+} uptake supporting the presence of this enzyme [38]. The acidocalcisome is acidified by two enzymes with proton pumping activity. One is the vacuolar-H^+-pyrophosphatase, an enzyme that hydrolyses pyrophosphate (PPi) and uses the energy released to pump protons toward the lumen of the organelle. This enzyme was described and characterized in *T. gondii* (TgVP1) and also in *P. falciparum* (PfVP1) [30]. The second enzyme is the vacuolar-H^+-ATPase, which pumps protons using the energy provided by the hydrolysis of ATP. Acidification of the acidocalcisomes by these pumps is important to maintain organellar Ca^{2+} and polyphosphate. Alkalinizing agents, such as NH_4Cl, release Ca^{2+} to the cytoplasm probably through a Ca^{2+}/H^+ exchanger and hydrolyze polyphosphate. VP1 also localizes to other compartments like the plant-like vacuole of *T. gondii* [31, 39], the food vacuole in *Plasmodium* spp. [40], the plasma membrane in both parasites, and endocytic compartments [31]. This is different to what occurs in mammalian cells, where acidic compartments are acidified by the V-H^+-ATPase. It has been suggested that this difference is because the V-H^+-pyrophosphatase uses PP_i as an energy source, which is very abundant in these parasites. Some of the life cycle stages of these parasites might run short in ATP supply because of their suboptimal mitochondrial function [41].

The PLV described in *T. gondii*, was also found to store Ca^{2+}. Addition of glycyl-L-phenylalanine-naphthylamide (GPN) to intact parasites loaded with the Ca^{2+} indicator Fura-2/AM results in its hydrolysis by a cathepsin C protease, increase in its osmolarity, and a swelling effect that leads to Ca^{2+} leaking out to the cytoplasm. GPN-dependent Ca^{2+} release is independent of other Ca^{2+} stores such as the ER [31].

There is clear evidence for the presence of acidic Ca^{2+} stores in malaria parasites. For example, bafilomycin A_1, an inhibitor of the vacuolar H^+-ATPase (V-ATPase), aminomethylenediphosphonate (AMDP), an inhibitor of the vacuolar H^+-pyrophosphatase (VP1), the ionophores nigericin, and monensin, and the alkalinizing agent NH_4Cl can all increase cytosolic Ca^{2+} in *P. berghei* isolated throphozoites [42]. Bafilomycin A_1 and NH_4Cl can also release Ca^{2+} from the food vacuole of *P. falciparum*, which is mainly involved in hemoglobin digestion. Both the V-H^+-ATPase and VP1 localize to acidocalcisomes and the food vacuole [40]. It has been shown that thapsigargin and cyclopiazonic acid can release Ca^{2+} from the food vacuole of *P. falciparum*, suggesting the presence of a SERCA-type Ca^{2+}-ATPase in this compartment [43]. However, little is known about the mechanisms used by acidocalcisomes of these parasites for uptake and efflux of Ca^{2+} or of the mechanism of acidocalcisome Ca^{2+} release in *T. gondii*.

3.3. Mitochondria

As occurs with yeast mitochondria [44], the mitochondria of *T. gondii* [45] and malaria parasites [46] are able to maintain a membrane potential and oxidative phosphorylation but lack a mitochondrial Ca^{2+} uniporter (MCU) in their inner membrane [47]. A Ca^{2+}/H^{+} antiporter (CAX) was localized to *P. falciparum* mitochondria and sensitivity to ruthenium red and ruthenium 360 suggested the authors the presence of a Ca^{2+} uniporter in these mitochondria [48]. However, there is no molecular evidence for the presence of an MCU [47] and the inhibitors might have had off site effects. It is not known whether any mitochondrial enzyme is regulated by mitochondrial Ca^{2+} as occurs in mammalian cells [49]. The *P. berghei* CAX appears essential for ookinete differentiation and is not localized to the mitochondria [50].

4. Calcium binding proteins in apicomplexan parasites

An elevation in intracellular Ca^{2+} results in the activation of numerous cellular processes including the mechanisms that returns cytosolic Ca^{2+} concentrations to their basal, physiological state. These responses can be directly mediated by the membrane transporters discussed above, which sequester Ca^{2+} into intracellular organelles or actively pump it out of the cell. However, most responses involve signal transduction through calcium-binding proteins (CaBPs). The irregular coordination chemistry of Ca^{2+} and its affinity for carboxylate oxygen, allows it to complex with proteins [51]. Some proteins have evolved domains that specifically interact with Ca^{2+} in ways that change their biochemical properties, and these are formally regarded as the CaBPs. The most ubiquitous of these calcium-binding domains is the helix-loop-helix motif known as an EF-hand. EF-hands frequently occur in pairs (also called EF-hand domains) that allow the cooperative binding of two Ca^{2+} ions per domain. CaBPs with single or odd number of EF hands sometimes function via dimerization mechanisms. Analysis of apicomplexan genomes has revealed an expansion of EF-hand containing CaBPs. The most current annotations reveal 74 EF-hand domain-containing proteins are encoded by the *T. gondii* genome (ToxoDB), and 103 by the *P. falciparum* genome (PlasmoDB). However the latter is significantly inflated due to the presence of a highly divergent EF-hand motif in some rifins, one of the largest gene families in the *P. falciparum* genome. Excluding the rifins, *P. falciparum* encodes only 43 EF-hand containing CaBPs.

Most of the EF-hand containing CaBPs can be classified into three main families: the calmodulin (CaM) family, the calcineurin B-like (CBL) family, and the calcium-dependent protein kinase (CDPK) family. Both CaM and CBL family members lack effector domains and are thought to act by regulating other proteins. Broadly construed, the CaM family includes classical CaMs (with > 75% sequence identity to human CaM), calmodulin-like (CML) proteins (sequence identity with human CaM < 75%), and centrins or caltractins. Structurally, calmodulins are acidic proteins comprised of two globular domains (each with a pair of EF hands) linked by a flexible helical region. Most apicomplexan genomes encode single prototypical CaM and a variable number of CMLs/CaM-related proteins. Of these proteins, only the *T. gondii* CaM has been cloned and shown to bind Ca^{2+} *in vitro* [52]. The CaM inhibitors calmidazolium and trifluoperazine were shown to inhibit *T. gondii* host cell

entry [53]. CaM inhibitors are also toxic to *P. falciparum*, affecting erythrocyte invasion by merozoites [54, 55]. The myosin light chain (MLC) and myosin regulatory light chain-like proteins, are each represented by two and one conserved members among apicomplexans, respectively [56]. Although various members of this group have been shown to play important structural roles within the motor complex [56-58], they are unlikely to bind calcium given that most of their EF-hands are degenerate. Similarly, apicomplexan centrins have been shown to associate with various cytoskeletal components including the centriole and conoid [59, 60], but none have been demonstrated to be calcium regulated. The CBL family members have been identified only in some protist genomes. Some gene sequences potentially encode functional CBLs in *T. gondii* and *P. falciparum*. A reticulocalbin located in the ER of asexual stages of *P. falciparum* contains 6 EF-hand domains [61].

CDPKs comprise the third family of EF-hand containing CaBPs. Canonically, CDPKs are composed of a kinase domain followed by a CaM-like domain, also known as the CDPK activation domain (CAD) [62, 63]. The activity of these kinases is directly regulated by Ca^{2+} binding to the CAD. The important role of CDPKs in apicomplexans is reflected by their overrepresentation in the parasite genome. Given their known roles in various calcium-regulated processes, they will be further discussed below.

A second, less prominent Ca^{2+}-binding motif is the C2 domain, which was originally identified as the domain responsible for the Ca^{2+}-dependent phospholipid binding of protein kinase C (PKC). Although no PKC can be found in apicomplexan genomes, various C2-domain containing proteins have been identified. Among them, DOC2 has been shown to be required for the calcium-regulated secretion of micronemes in both *T. gondii* and *P. falciparum* [64]. Apicomplexan phospholipase C (PLC), which is thought to mediate intracellular store release through the production of inositol (1,4,5) triphosphate (IP_3), is also known to contain a C2 domain, in addition to an EF-hand, although it is unknown how Ca^{2+} impacts its regulation [16]. Finally, Ca^{2+} regulates various other proteins directly or indirectly, including other kinases like Ca^{2+}/CaMKs, proteases, phosphatases, and nucleoside triphosphatases (NTPases). These proteins, which have not been completely characterized in apicomplexans, could have vital unknown functions in the signaling pathways regulated by Ca^{2+}.

5. Ca^{2+} signaling and its biological downstream effects in apicomplexan parasites

The phenotypic consequences of perturbing Ca^{2+} signaling range from developmental defects in *Plasmodium spp.* to premature egress from host cells in the case of *T. gondii*. Gliding motility, the substrate-dependent, adhesin-mediated movement unique to apicomplexans, depends on Ca^{2+} signaling in all apicomplexans where it has been studied. This seems, at least in part due to the reliance of gliding motility on Ca^{2+}-regulated secretion of adhesins from specialized organelles called micronemes. Secretion of micronemes also precedes entry and exit from host cells, and its perturbation alone could account for many of the phenotypes we attribute to Ca^{2+} signaling. However, the plethora of calcium-binding proteins and the widespread calcium-dependent changes on the phosphoproteome, suggest a much broader response to elevated cytoplasmic Ca^{2+}. How Ca^{2+} signaling affects other

cellular processes like development, transcription or parasite physiology will need to be addressed by future work in this field.

5.1 Microneme secretion, motility, host cell invasion and egress

Micronemes are specialized, apical, secretory organelles found in most apicomplexan zoites. They are the smallest of the apical organelles and are readily distinguished by their elongated morphology and electron-dense structure. It has been observed, that zoites that migrate extensively, such as *Plasmodium spp.* ookinetes, have numerous micronemes, while those that rapidly invade like the merozoites of *Eimeria spp.* or *Plasmodium spp.*, have relatively few [65]. Proteomic analysis of microneme-enriched fractions from *P. berghei* ookinetes [66], and *T. gondii* secreted proteins [67] show a wide range of secreted proteins are released from micronemes. Most prominently, micronemes contain many of the adhesins that participate in the interactions with the host-cell surface, and participate in the early stages of host-cell invasion. Microneme secretion can be induced by calcium ionophores, in contrast to the constitutive secretion of dense granules and the secretion of rhoptries upon host-cell contact [68, 69]. This effect is blocked by chelating intracellular Ca^{2+} with BAPTA-AM, suggesting that microneme secretion is triggered by an increase in intracellular Ca^{2+}. BAPTA-AM also prevents conoid extrusion [70], gliding motility [71] and host cell invasion [72] by *T. gondii* suggesting a role of Ca^{2+}, perhaps through the common requirement of microneme secretion for all of these processes. The stimulation of microneme secretion by Ca^{2+} has also been demonstrated in *P. berghei* [73]. In agreement with the role of Ca^{2+} in these processes, *TgA1 null* mutant tachyzoites, which exhibit altered intracellular-Ca^{2+} levels, are deficient in microneme secretion, are deficient in invasion, and show reduced virulence *in vivo* [12]. Calcium ionophores, like A23187 and ionomycin, also stimulate egress of *T. gondii* from host cells [74]. Resistance to ionophore-induced egress (or delayed response) has been used to isolate mutant parasites bearing defects in Ca^{2+} signaling in *T. gondii*. One such mutant, defective in a Na^+/H^+ exchanger (TgNHE1), has been shown to have elevated intracellular Ca^{2+} levels and reduced pathogenicity [75]. The same screen also identified TgCDPK3 as a mediator of ionophore-induced egress [76]. A different study characterized temperature sensitive mutants defective in the same process and identified a DOC2 protein required for microneme secretion, presumably mediating Ca^{2+}-dependent vesicular trafficking [64]. Conditional down-regulation of the homologous protein in *P. falciparum* similarly affected parasite viability and reduced secretion of the micronemal protein PfEBA-17 [64].

Ca^{2+} fluctuations have been observed in actively moving parasites. Periodic oscillations in intracellular Ca^{2+} levels linked to gliding have been observed by live video microscopy of *T. gondii* parasites loaded with the Ca^{2+}-indicator Fluo 4 [71]. Similar oscillations were also observed during gliding motility in *P. berghei* sporozoites expressing a fluorescence energy transfer (FRET) biosensor [77]. Many of the same perturbations of Ca^{2+} signaling affect gliding motility and microneme secretion [78, 79], which has been used to deduce our current understanding of this form of parasite movement. Significant experimental evidence now supports the model that links the secretion of micronemal adhesins and their directional translocation via an acto-myosin motor complex anchored in the inner membrane complex (IMC). This makes it difficult to assess whether Ca^{2+} also regulates aspects of gliding

motility beyond secretion of the adhesins. The same argument can be extended to our understanding of parasite invasion and egress from host cells, although certain cellular components may affect the different phenotypes to different extents.

Intracellular stores are known to be sufficient to initiate gliding motility and host-cell invasion [18]. However, a correlation between extracellular Ca^{2+} influx and invasion-linked traits was recently demonstrated. The study demonstrated that Ca^{2+} entry enhanced motility, conoid extrusion, microneme secretion and invasion [8]. Because *T. gondii* replicates through endodyogeny, its lytic cycle can be complex and parasites commonly invade and exit with little or no replication [80, 81]. This complex behavior, all of which depends on Ca^{2+}, likely requires replenishing intracellular Ca^{2+} stores with extracellular Ca^{2+}. It would be advantageous for the parasite to utilize the high concentration of available extracellular Ca^{2+} to trigger invasion-related processes and to resupply intracellular stores for use during subsequent rounds of invasion and egress. Experimental evidence supports a general mechanism for Ca^{2+} entry, which may not be sensitive to the filling state of the ER, as is the case in mammalian cells, but which could work in parallel with store release to elevate intracellular Ca^{2+}, and activate downstream events like invasion [8].

5.2 Ca^{2+}-dependent protein kinases

Ca^{2+}-dependent protein kinase (CDPK) are important transducers of Ca^{2+} signals in plants and protists. CDPKs are canonically composed of an N-terminal serine/threonine kinase domain, connected to four EF-hands in a calmodulin-like domain (CaM-LD). Apicomplexan genomes reveal a large family of CDPKs and related kinases, many of which are conserved throughout the phylum. The absence of CDPKs from mammalian genomes, and their recognized importance to many cellular pathways in apicomplexan parasites have made them attractive drug targets and the focus of intense study over the past decade.

Ca^{2+} activates CDPKs by binding directly to their EF-hands. Biochemical studies of plant CDPKs have defined the region between the kinase domain and the EF-hands as an autoinhibitory domain that maintains the enzyme in an inactive state [82, 83]. More recently, solution of the structures of *T. gondii* and *Cryptosporidium parvum* CDPK1 in the presence and absence of Ca^{2+}–the first full-length structures for any CDPK–have revealed the molecular details of their activation [63, 84]. In the absence of calcium, the autoinhibitory region forms an alpha helix that extends from the base of the kinase domain, occupies the substrate-binding region, and occludes the catalytic pocket of the enzyme. Ca^{2+} binding to the EF-hands causes a dramatic structural rearrangement that leads to a segmentation of the autoinhibitory helix around the CaM-LD and packing of the entire regulatory domain against the opposite face of the kinase [63]. Despite many similarities to the activation of mammalian CaMK by CaM, the distinct fold of the regulatory domain with the CaM-LD forms a distinct regulatory domain now termed the CDPK-activation domain (CAD) [63].

In plants, CDPKs regulate diverse pathways including stomatal closure, cell cycle progression, stress responses, and root-nodule formation [62]. A similar breadth of functions has been attributed to CDPKs in apicomplexans, with roles in differentiation, motility, invasion, and egress. Various CDPKs can be knocked out in the blood stages of *Plasmodium spp.*, yet display diverse defects in subsequent developmental stages. Knockouts of *P.*

berghei PbCDPK4 and PbCDPK3 are defective in male gametocyte exflagellation and ookinete motility, respectively [85, 86]. Both mutants are severely impaired in their ability to go through the sexual cycle. In *T. gondii* the homologue of PbCDPK4, TgCDPK1, had been shown to be a target of KT5926, a potent kinase inhibitor that blocks microneme secretion [87]. Conditional down-regulation of TgCDPK1 was shown to abrogate microneme secretion resulting in loss of parasite motility, host-cell invasion and egress, demonstrating its essentiality [88]. In contrast to the general effects of TgCDPK1 on Ca^{2+}-regulated processes, TgCDPK3 has been shown to have a more specific function in parasite egress, as demonstrated by a chemical-genetic approach as well as direct knockout of the gene [76, 89, 90]. The homologue of TgCDPK3 in *Plasmodium spp*., PfCDPK1 and PbCDPK1, cannot be knocked out despite repeated attempts, suggesting it plays an essential role in the asexual cycle [91]. More recently, the unconventional approach of inhibiting PfCDPK1 through expression, or exogenous addition, of its junction domain has been used to further assert its essentiality [92, 93]. A different kinase in *P. falciparum*, PfCDPK5, has also been demonstrated to regulate egress of merozoites during the erythrocytic cycle [94]. Together these studies demonstrate the diverse and important roles CDPKs play in transducing Ca^{2+} signals.

A variety of studies have targeted CDPKs for the development of antiparasitic compounds. An *in vitro* screen against recombinant PfCDPK1 identified purfalcamine as a potent inhibitor that blocks *P. falciparum* development at the late schizont stage, immediately prior to merozoite egress [91]. A rational approach has been used to target TgCDPK1. The ATP-binding pocket of TgCDPK1 is unusual in that it harbors a glycine at a conserved position termed the gatekeeper, which is typically occupied by bulky residue that restricts the size of the pocket. This atypical feature has enabled specific inhibition of TgCDPK1 using bulky ATP-analogues originally designed for kinases with expanded ATP-binding pockets [84, 88, 95], as well as similar compounds designed for their improved antiparasitic properties [96-98]. Although conserved, the small gatekeeper of TgCDPK1 can be mutated to methionine without significant effects on parasite viability. These strains are resistant to compounds targeting TgCDPK1 and have allowed transfer of drug sensitivity to other kinases in order to study their function [89].

Apicomplexans also harbor various atypical CDPKs that diverge from the canonical domain structure. Some of the atypical kinases display different numbers of EF-hands, N-terminal EF hands, or additional domains. Some of these kinases are conserved throughout the phylum. Such is the case for CDPK6, although the different homologues have different numbers of EF-hands, in all cases the kinase domain is flanked on both sides by multiple EF-hands [99]. *P. berghei* sprorozoites defective in PbCDPK6 do not respond to the signals that trigger circumsporozoite protein cleavage upon contact with hepatocytes [100]. In another group of atypical CDPKs, the CDPK7s found in both *Plasmodium spp.* and *T. gondii*, the kinase domain is preceded by a pleckstrin homology (PH) domain and two or more EF-hands. Conditional knockout of TgCDPK7 had no impact on microneme secretion, but led to aberrant cell division [101]. Similarly, knockout of PfCDPK7 stalled the early stages of parasite development during the asexual cycle [102]. The biochemical consequences of these divergent domain structures remain unknown. Although the

biological processes these kinases participate in have not been associated with Ca^{2+} signaling, it remains to be determined what role Ca^{2+} may play in the regulation of these important enzymes.

Proteomic studies show widespread changes in the phosphorylation state of diverse proteins following an increase in intracellular Ca^{2+} [58, 103], although linking these changes to the cellular phenotypes regulated by calcium remains a major challenge. A major study comparing the phosphoproteomes of wild-type and TgCDPK3-defficient parasites observed hundreds of changes in diverse cellular pathways including proteins associated with gliding motility [103]. Calcium-dependent phosphorylation of the motor complex is well documented in both *T. gondii* and *P. falciparum* [104, 105]. Components of the complex are phosphorylated *in vitro* by PfCDPK1 [104], although it is unclear how this reflects the substrate preference *in vivo*. Various mutational analyses have been undertaken to identify sites of phosphorylation that effect motor complex function [105, 106]. However, despite significant effort, most of the phosphorylation sites on GAP45 and MLC appear to be dispersible for glideosome function [105, 106]. A similar study looked phosphorylation sites on the major motor protein driving gliding motility, TgMyoA. By complementing a conditional knock-down of TgMyoA, the authors could demonstrate that the motor is subtly regulated by calcium-dependent phosphorylation [107]. Crucially, the defects in ionophore-induced egress displayed by non-phosphorylatable alleles could be rescued by expression of a MyoA bearing phospho-mimetic mutations [107]. It is conceivable that the important phenotypes that have been attributed to CDPKs are the consequence of many subtle regulatory functions. However, more work will be needed to untangle the net of calcium-dependent phosphorylation and understand the function of individual CDPKs in apicomplexan Ca^{2+} signaling.

6. Outlook

In summary, Ca^{2+} has important roles in microneme secretion, gliding motility, host cell invasion and egress, and differentiation of Apicomplexan parasites. Ca^{2+} signaling is controlled by its uptake and release from different cellular compartments and there are important differences with the processes that control Ca^{2+} homeostasis in other eukaryotic cells, providing opportunities for targeting them for new therapies. Apicomplexan parasites contain SERCA-type Ca^{2+}-ATPases for Ca^{2+} uptake in the endoplasmic reticulum but PMCA-type Ca^{2+}-ATPases and putative voltage-dependent Ca^{2+} channels are only present in *T. gondii*. Ca^{2+} can be released from intracellular compartments by IP_3 or cADP ribose but no receptors for these second messengers have been identified. Many Ca^{2+}-binding proteins including calmodulins, calmodulin-like proteins, and Ca^{2+}-dependent protein kinases are present in these parasites. Both *Plasmodium* spp., and *T. gondii* possess acidocalcisomes. Other acidic Ca^{2+} stores include the food vacuole of malaria parasites and the plant-like vacuole of *T. gondii*. Studies of Ca^{2+} storage and signaling in these parasites could shed light about the origins of complex signaling networks in eukaryotes. The availability of experimental tools for genetic manipulation and molecular investigation of apicomplexan parasites will be extremely useful to decipher Ca^{2+} signaling pathways in these important eukaryotes.

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Highlights

- Ca^{2+} has important roles in virulence-related traits in apicomplexan parasites
- Apicomplexan parasites show important differences in Ca^{2+} homeostasis with other eukaryotic cells
- Calmodulins, calmodulin-like proteins, and Ca^{2+}-dependent protein kinases are present.
- *Plasmodium* spp., and *T. gondii* possess acidocalcisomes and other acidic stores
- Ca^{2+} signaling studies in these parasites could shed light of origins of complex signaling networks

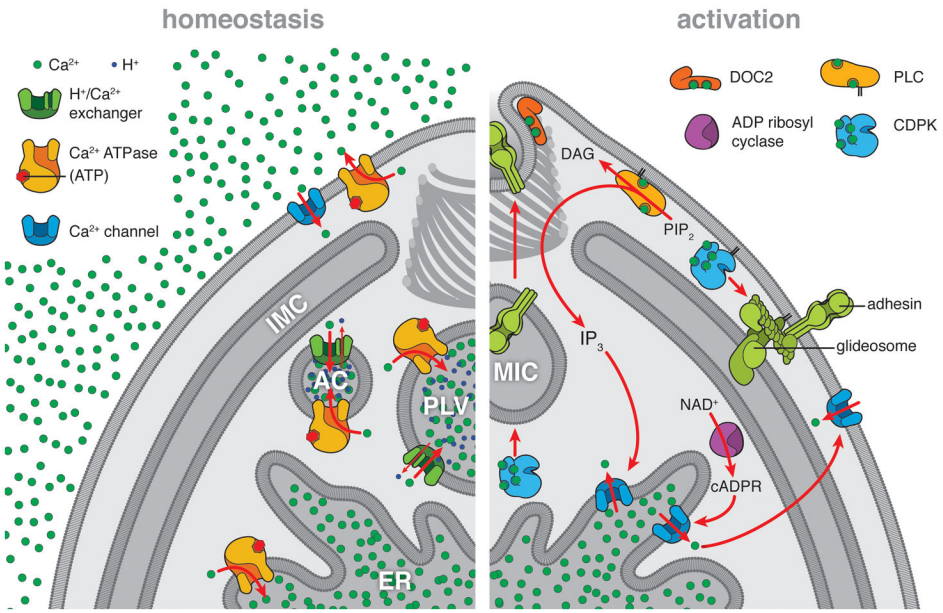

Figure 1.

Schematic representation of the distribution of Ca^{2+} in a *T. gondii* tachyzoite. Abbreviations: Ca^{2+} entry is probably through Ca^{2+} channels. Once inside the cells, Ca^{2+} can be translocated back to the extracellular environment, primarily by the action of a PMCA. In addition, Ca^{2+} will become sequestered by the ER by the action of the SERCA-Ca^{2+}-ATPase, sequestered by the acidocalcisome or the plant-like vacuole by the action of a Ca^{2+}-ATPase (TgA1). Ca^{2+} could also be released into the cytoplasm from internal stores as the ER through an uncharacterized channel by the action of IP_3 (generated from PIP_2 by a PLC) or cADP-ribose (generated from NAD^+ by an ADP ribosyl cyclase). It may also be released from the PLV and the acidocalcisome through a Ca^{2+}/H^+ exchanger. CDPKs stimulate gliding motility and microneme secretion, which is also stimulated by DOC2.