CHAPTER 5

Calcium Regulation and Signaling in Apicomplexan Parasites

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Abstract

Apicomplexan parasites rely on calcium-mediated signaling for a variety of vital functions including protein secretion, motility, cell invasion, and differentiation. These functions are controlled by a variety of specialized systems for uptake and release of calcium, which acts as a second messenger, and on the functions of calcium-dependent proteins. Defining these systems in parasites has been complicated by their evolutionary distance from model organisms and practical concerns in working with small, and somewhat fastidious cells. Comparative genomic analyses of *Toxoplasma gondii, Plasmodium* spp. and *Cryptosporidium* spp. reveal several interesting adaptations for calcium-related processes in parasites. Apicomplexans contain several P-type Ca^{2+} ATPases including an ER-type reuptake mechanism (SERCA), which is the proposed target of artemisinin. All three organisms also contain several genes related to Golgi PMR-like calcium transporters, and a Ca^{2+}/H^{+} exchanger, while plasma membrane-type (PMCA) Ca^{2+} ATPases and voltage-dependent calcium channels are exclusively found in *T. gondii*. Pharmacological evidence supports the presence of IP_3 and ryanodine channels for calcium-mediated release. Collectively these systems regulate calcium homeostasis and release calcium to act as a signal. Downstream responses are controlled by a family of EF-hand containing calcium binding proteins including calmodulin, and an array of centrin and caltractin-like genes. Most surprising, apicomplexans contain a diversity of calcium-dependent protein kinases (CDPK), which are commonly found in plants. *Toxoplasma* contains more than 20 CDPK or CDPK-like proteases, while *Plasmodium* and *Cryptosporidium* have fewer than half this number. Several of these CDPKs have been shown to play vital roles in protein secretion, invasion, and differentiation, indicating that disruption of calcium-regulated pathways may provide a novel means for selective inhibition of parasites.

Defining Calcium Regulation in an Early Branching Eukaryote

Apicomplexan parasites are most similar to ciliates and dinoflagellates and only distantly related to plants, fungi, and animals typically used as model organisms.[1] Apicomplexans contain a remnant plastid derived from a secondary endosymbiont, called the apicoplast.[2] A number of plant-like metabolic systems are found in apicomplexans either due to retention of

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plastid-related functions or due to the acquisition of nuclear genes from the endosymbiont. For example, plant like enzymes have been identified in glycolysis[3] and some structural elements such as microtubules are more closely related to plants than animals.[4] As a consequence, comparison to model organisms is not always informative about the biology of apicomplexans either because their early origin predates the specialization of systems found in yeast, plants, and animals, or because it is not always certain where the closest affinities lie, even in systems that are conserved. Fortunately, there are a large number of whole genomes completed for the Apicomplexa, making it possible to delineate many conserved pathways through sequence-based phylogenetic comparisons.[5] Additionally, experimental tools for direct manipulation have advanced significantly, making it possible to explore specific systems directly in parasites.

Calcium is an important second messenger for signaling cascades that regulate protein secretion, motility, gene expression, and cellular development in eukaryotic cells. While calcium is an abundant cation in the environment, it is maintained at a very low resting level in the cytosol of eukaryotic cells. Rapid release of stored calcium is thus a potent signal for controlling a variety of downstream effectors. Calcium-binding motifs in several key cytosolic proteins, (e.g., the EF-hand found in calmodulin and other related proteins), allows this signal to be propagated through different pathways. Because calcium is such a potent signal, it must be rapidly dampened or it becomes highly toxic for cells. Consequently, cells have developed elaborate ways to control release in response to environmental stimuli, and then to rapidly sequester calcium to shut off the signal.

Defining the molecular pathways for the regulation of intracellular calcium in parasitic protozoa is of importance for three primary reasons. First calcium controls several vital cellular processes including secretion of adhesive proteins and motility. Second, the homeostatic mechanisms for controlling calcium may provide drugable targets as shown in the case of artemisinin, which acts on the calcium reuptake pump in the ER (i.e., SERCA).[6] Finally, as an early branching eukaryotic group, apicomplexans occupy a useful position phylogenetically that provides insights into the early origin of complex signaling pathways. This review summarizes what is known about calcium homeostasis, including clues derived form the recently completed genomes, and outlines pathways for calcium signaling in *Toxoplasma*, *Plasmodium*, and *Cryptosporidium*.

Calcium Homeostasis

Regulation of Intracellular Ca^{2+} Concentration

In eukaryotic cells, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained at 10,000-fold lower levels than the extracellular environment.[7,8] Maintaining this steep calcium gradient requires the concerted operation of several mechanisms present in the plasma membrane and intracellular organelles. Calcium enters through the plasma membrane, which in eukaryotic cells contains a number of calcium channels, some of which are under the control of receptors (receptor-operated Ca^{2+} channels), the electrical potential across the plasma membrane (voltage-gated Ca^{2+} channels), and the content of intracellular Ca^{2+} stores (store-operated Ca^{2+} channels), in addition to nonselective channels.[9]

In the case of the *T. gondii* tachyzoites, the $[Ca^{2+}]_i$ is about 100 nM, as detected in fura-2-loaded cells in the presence of 1 mM extracellular Ca^{2+}.[10] Similar $[Ca^{2+}]_i$ levels have been reported for extracellular *P. chabaudi* and *P. falciparum* parasites using fura-2 loading and ratiometric imaging.[11] Studies of intracellular *P. falciparum* parasites using Fura-red for ratiometric imaging indicate much higher levels of $[Ca^{2+}]_i$ (370-480 nM), which is likely an average of the cytosol and extensive ER compartment within these cells.[12] It is likely the cytosolic concentration of calcium in parasites is within the concentration range observed in other eukaryotic cells (i.e., 90-100 nM).[13] Red blood cells also maintain a low level of $[Ca^{2+}]_i$; however, following infection with malaria, the cytosolic concentration of calcium appears to rise, due to enhanced permeability and reduced export via the membrane Ca^{2+} ATPases (reviewed in ref. 11).

Storage Organelles

Intracellular organelles contain transporters, channels, and pumps that contribute to homeostasis by releasing calcium or taking it up according to the cellular requirements. The most important compartments are the mitochondria, acidocalcisome, and endoplasmic reticulum. While mitochondria are important to control large fluctuations of cytosolic calcium and the acidocalcisomes are important for storage, the endoplasmic reticulum serves as the primary source of mobilizeable calcium for signaling.

Mitochondria

Mitochondria possess a high capacity to sequester calcium. The inner mitochondrial membrane possesses a uniport carrier for Ca^{2+}, which allows the electrogenic entry of the cation driven by the electrochemical gradient generated by respiration or ATP hydrolysis. Calcium efflux takes place by a different pathway, which appears to catalyze the electrically neutral exchange of internal calcium by external sodium or protons. Previous studies have provided biochemical evidence for mitochondrial Ca^{2+} uptake in malaria parasites[14,15] and preliminary evidence suggests the presence of a uniport mechanism in *T. gondii* (Vercesi and Moreno, unpublished observations).

Acidocalcisomes

A large store of Ca^{2+} in *T. gondii* and *Plasmodium spp.* is found in the acidocalcisomes,[10,16,17] which are acidic calcium-storage organelles found in a diverse range of microorganisms from bacteria to man.[18] Acidocalcisomes are characterized by their acidic nature, high density (both in weight and by electron microscopy), and high content of pyrophosphate, polyphosphate, calcium, magnesium, and other elements.[18] Acidocalcisomes in *T. gondii* have been shown to possess a plasma membrane type ATPase (PMCA), involved in Ca^{2+} influx, with similarity to vacuolar Ca^{2+}-ATPases of other unicellular eukaryotes.[16,19] Acidocalcisomes also contain two proton pumps, a vacuolar H^+-ATPase (V-H^+-ATPase) and a vacuolar H^+-pyrophosphatase (V-H^+-PPase), involved in their acidification.[16,20,21] Although the Ca^{2+} content of acidocalcisomes is very high (probably in the molar range), most of it is bound to polyphosphate and can only be released upon alkalinization[22] or after polyphosphate hydrolysis.[16] No second messengers have been demonstrated to be involved in Ca^{2+} release from acidocalcisomes of *T. gondii*, and it seems likely this store is not directly involved in calcium-mediated signaling.

In addition to the biochemical[17] and morphological[23] evidence for the presence of acidocalcisomes in malaria parasites it has been proposed that the acidic food vacuole of *Plasmodium falciparum* is also a dynamic internal Ca^{2+} store, which possess a thapsigargin (and cyclopiazonic acid)-sensitive Ca^{2+} pump.[24] However, these studies were based on the changes in fluorescence of vacuole-localized Fluo-4 after addition of inhibitors or alkalinizing agents. More recent studies using more quantitative methods questioned the validity of the results using Fluo-4 and failed to confirm that the food vacuole acts as a major internal Ca^{2+} store containing a thapsigargin-sensitive Ca^{2+} pump.[12] Importantly, these studies did reveal the presence of electron-dense organelles rich in phosphorus (i.e., the acidocalcisomes) in *P. falciparum*.[12]

Endoplasmic Reticulum

The endoplasmic reticulum also contains a large store of Ca^{2+} with local concentration reaching millimolar levels, and this pool is generally considered to be the primary source of mobilizable calcium for signaling. The ER possesses two pathways for calcium efflux that are stimulated by IP_3 and ryanodine or cyclic ADPribose (described further below). The influx is catalyzed by the very well known sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA), which actively translocates two Ca^{2+} ions for the hydrolysis of one ATP molecule.

Calcium Transporters and Channels

The active export of calcium across the plasma membrane of eukaryotic cells is accomplished by the action of Na^+/Ca^{2+} or Ca^{2+}/H^+ exchangers or P-type Ca^{2+}-ATPases, which are classified as plasma membrane transporters (PMCA), secretory compartment transporters (i.e., PMR1-like Golgi Ca^{2+} ATPase), and ER or SER transporters.[25]

A PMCA-type Ca^{2+}-ATPase (TgA1) has been characterized in *T. gondii*, and this protein is located in the plasma membrane and acidocalcisomes.[16] TgA1 is able to complement yeast deficient in the vacuolar Ca^{2+}-ATPase gene *PMC1*, providing genetic evidence for its function.[26] Mutants deficient in *tga1* were shown to have decreased infectivity in vitro and in vivo due to their deficient invasion of host cells.[26] Biochemical analysis revealed that the polyphosphate content of *tga1* mutants was drastically reduced, and that the basal Ca^{2+} levels were increased and unstable. These defects lead to impaired microneme secretion in response to agonists, thus demonstrating the importance of homeostasis in this calcium-activated pathway. In addition, a second PMCA-like gene is found in the *T. gondii* genome.[27] The deduced amino acid sequence (1200 residues) of this protein shows 45% identity with TgA1.[16] Surprisingly, no homologues of PMCAs are recognizable in the genomes of *Plasmodium* or *Cryptosporidium*, indicating they do not rely on this pump for calcium homeostasis.[27]

Apicomplexans contain two genes with similarity to the yeast PMR1 transporter, which is a Golgi-type Ca^{2+} ATPase.[27] One of these has previously been named ATPase4 in *P. falciparum* where it has been localized to the plasma membrane in asexual stages of malaria,[28] suggesting it may function more like a PMCAs. Characterization of in vitro activity in *Xenopus* revealed that it is inhibited by vanadate and cyclopiazonic acid but not thapsigargin or ouabain.[29] Apicomplexans contain well-conserved homologues of SERCA-type Ca^{2+} ATPases.[27] Apicomplexans also contain a gene previously named PfATPase2, which is most similar to phospholipids transporting ATPases and may not play a direct role in calcium homeostasis.[27] Finally, all three apicomplexans encode a transporter with similarity to Ca^{2+}/H^+ exchangers;[27] homologues are found in the vacuole in plants, and also present in bacteria and fungi.[25] The location of this exchanger in apicomplexans is uncertain, but it may be involved in calcium homeostasis in internal organelles.

Voltage-gated Ca^{2+} channels (VGCC) have been detected in free-living protozoa, including ciliates.[30] VGCCs regulate influx of calcium from the extracellular medium to the cell cytosol, usually in response to changes in membrane potential. No such channels have been characterized in apicomplexans, although several orthologues are present in the genome of *T. gondii*[27] One of these is similar to the previously described two-pore channel 1 (TPC1), the *Arabidopsis thaliana* Ca^{2+}-dependent Ca^{2+}-release channel.[27,31] The predicted protein has similarities to the channels present in the plant vacuoles and it is possible that this channel might be present in the acidocalcisomes or in other intracellular membranes. These calcium channels are apparently absent in *Plasmodium* spp. and *Cryptosporidium* spp.

Ca^{2+} release from the endoplasmic reticulum of eukaryotic cells is mediated by ryanodine receptors (RyR) and inositol 1,4,5-trisphosphate (IP_3) channels.[32,33] RyR are activated by a rise in $[Ca^{2+}]_i$ (Ca^{2+}-induced Ca^{2+} release, CICR).[34] In addition, there are RyR-like channels activated by cyclic ADP-ribose (cADPR), sphingosine, and a distinct Ca^{2+}-release pathway activated by nicotinic acid adenine dinucleotide phosphate (NAADP).[35] NAADP also mobilizes calcium from lysosomal like stores in sea urchin eggs,[36] and mammalian cells, although this pathway has not been explored in parasites. Pharmacological evidences indicate the presence of a IP_3/ryanodine-sensitive stores in *T. gondii*.[37] However, searching the genomes of apicomplexans failed to reveal genes for IP_3 or ryanodine channels as defined in metazoans. This apparent absence could be due to lack of homology with the channels of animal cells, as occurs in plants that also respond to these second messengers.[25]

SERCA: Structure and Function

SERCA is a large membrane protein of approximately 110-130 kDa with 10 transmembrane regions. SERCA is widely conserved from protozoa to mammals, although direct homologues are absent in yeast and fungi, which instead have a plasma membrane type Ca^{2+} ATPase and a secretory Ca^{2+} ATPase (summarized in ref. 38). Vertebrates contain three SERCA genes each of which exhibits multiple splice variants. SERCA1 is found in muscle, SERCA2 is expressed in muscle and a variety of cell types and is evolutionarily oldest, while SERCA3 is widely expressed but has a lower affinity for calcium.[38] Several human disease states are associated with dysfunction in SERCAs including Brody's disease (defect in ATP2A1) and Darier's disease (defect in ATP2A2). Plants also contain SERCA homologues with conserved transmembrane, phosphorylation and ATP binding domains.[25]

SERCA is one of the most well-characterized P-type ATPases, which are defined by the existence of phosphorylated intermediate.[38,39] Structurally, SERCA consists of 10 transmembrane regions (M1-M10) and three cytoplasmic domains (A domain, actuator; N domain, nucleotide binding; P domain, phosphorylation).[40] The N domain binds to ATP, and after the ATP hydrolysis, residue Asp351 in the P-domain becomes phosphorylated. Extensive X-Ray crystallographic studies have defined amino acids residues that form the ATP-binding and Ca^{2+}-binding pockets.[41,42] SERCA transports two Ca^{2+} molecules from the cytoplasm to the lumen of the ER during hydrolysis of one ATP molecule. The reaction mechanism involves transformation between two conformational states, known as E1 and E2, which bind calcium on the cytoplasmic side and release it into the lumen.[38,39]

SERCA as a Target for Artemisinin

The SERCA orthologue of *P. falciparum* (PfATPase6) encodes a 1228 amino acid protein that shares 41% identity and 65% similarity with human SERCA.[43] All of the key amino acids residues that are predicted to form the Ca^{2+}-binding pocket and ATP-binding site are conserved in PfATPase6[43] and the TgSERCA homologue.[27] Thapsigargin is a sesquiterpene lactone, derived from the plant *Thapsia garganica* that was first reported as a tumor promoter.[44] Thapsigargin inhibits mammalian SERCA at subnanomolar concentrations by locking the molecule in the E2 form, which is unable to bind calcium.[45,46] Biochemical evidence that calcium homeostasis depends on the action of the SERCA pump has been provided by previous studies showing that *T. gondii*[10,17,47] and *P. falciparum*[48] are sensitive to thapsigargin. Consistent with this, 10 of 14 amino acids that are thought to interact with thapsigargin are conserved in PfATPase6[43] and TgSERCA.[27]

Other plant alkaloids also offer potential as selective antiparasitic compounds due to inhibition of SERCA. Artemisinin is an antimalarial agent isolated from sweet wormwood (*Artemisia annua*)[49,50] and artemisinin and derivatives are used to treat multidrug-resistant malaria. Artemisinin has a unique peroxide bridge that is essential for anti-malarial activity. The peroxide is activated to a radical by the presence of Fe^{2+}, and this activation step is essential for its potent anti-malarial activity.[49,50] One potential mechanism for its antimalarial activity is the demonstration that artemisinin inhibits malarial SERCA (PfATPase6) expressed in *Xenopus laevis* oocytes.[6] More recently, Uhlemann et al[51] reported that a single amino acid residue could determine the sensitivity of PfATPase6 to artemisinin in *X. laevis* oocytes. An artemisinin-binding cleft was predicted by computer modeling based on the thapsigargin-binding pocket, which was previously demonstrated by structural studies of the mammalian SERCA.[51] Transmembrane domains M3, M5, and M7 form the putative artemisinin binding-site. Within this region, Leu263 in malarial SERCA was found to be essential for determining the sensitivity to artemisinin. When this residue was mutated to Glu, which is the corresponding residue to mammalian SERCA, the K_i value to artemisinin increased more than 300-fold. Furthermore, the K_i values of artemisinin for SERCA harboring L263A and L263S mutations (corresponding to *P. vivax* and *P. berghei* orthologues, respectively) were in proportion to the sensitivity of these *Plasmodium* species to drug treatment.[51] The sequence of SERCA in *T. gondii* contains

Glu at the residue corresponding to Leu263, consistent with the ~30-fold lower sensitivity of *T. gondii*.[52,53] to artemisinin vs. *P. falciparum*.[54] While these results suggest that SERCA is a possible target for artemisinin, further experiments are required to confirm that the inhibition of SERCA is responsible for the major antimalarial effects of atremisinin.

Calcium-Regulated Signaling Pathways in Apicomplexans

Motility, cell invasion, and egress from infected cells have all been linked to intracellular calcium in *T. gondii*. The relatively robust nature of *T. gondii* tachyzoites has made it possible to probe these events in real-time using time-lapse video recording with dyes such as fluo-4, and to provide quantitative measurements of calcium levels using fura-2. Moreover, efficient systems are available to quantitatively monitor gliding motility, cell invasion, and egress of the parasite. The genome of *T. gondii* has recently been completed and excellent systems for genetic manipulation are available to test the role of specific genes by knock-out[55] or knock-down studies.[56] In this regard, *T. gondii* provides a model system for studying the role of intracellular calcium in apicomplexans.

Microneme Secretion Pathway

During interaction of the host cell, microneme proteins are secreted at the apical tip of the parasite where they engage host cell receptors and mediate polarized attachment to the host cell.[57] Discharge of microneme proteins is triggered by treatment with calcium ionophores and conversely blocked by chelation of intracellular calcium with BAPTA-AM, thus demonstrating that a rise in intracellular calcium is both necessary and sufficient for secretion.[57] Since this initial demonstration of the requirement for calcium in secretion in *T. gondii*, similar studies have revealed that this is likely a conserved process in *C. parvum*[58] and *P. berghei*.[59] Treatment with short chain alcohols and acetaldehyde are also potent triggers of microneme release in *T. gondii*, a property that may result from activation of phospholipase C.[47] Activation of microneme secretion also requires protein kinases, as initially shown by studies that the general S/T kinase inhibitor staurosporine blocks microneme secretion, host cell attachment, and invasion in both *T. gondii*[57] and *P. knowlesi*.[60] Subsequently, two classes of protein kinases have been identified that act downstream of the calcium signal and which are essential for microneme secretion: plant-like calmodulin-like protein kinases (CDPKs)[61] and cyclic GMP-dependent protein kinase (cGMP-PK)[62] (described further below).

Induction of microneme secretion involves both IP_3-like and ryanodine-like response channels for elevating intracellular calcium (Fig. 1). Treatment with ethanol increased IP_3 and $[Ca^{2+}]_i$ and this pathway is sensitive to inhibitors of IP_3 channels.[37] Recent evidence indicates that IP_3 is produced in *T. gondii* by a phosphoinositide-specific phospholipase C, delta type.[63] Studies with *T. gondii* indicate that apicomplexans also responded to agonists of cADPR-gated channels such as ryanodine and caffeine[37] (Fig. 1). Consistent with this model, *T. gondii* contains cADPR cyclase and hydrolase activities, the two enzymes that control cADPR levels.[64] Pharmacological evidence suggests that both IP_3 and ryanodine response pathways contribute to calcium-mediated secretion (Fig. 1).

Calcium Oscillations and Motility

Calcium-dependent secretion of microneme proteins is also required for motility of *T. gondii*, presumably due to the ability of these adhesive proteins to bind to the substratum, cross the membrane, and link to the cytoskeleton.[65] Imaging of gliding parasites with the calcium-sensitive dye fluo-4 revealed a surprising result that intracellular calcium levels undergo periodic fluctuations in *T. gondii*, rising and falling in repeated cycles.[66] Calcium oscillations are associated with gliding and treatment with several agonists of calcium release revealed that these oscillations control motility.[67] Treatment with caffeine causes a prolonged elevation in intracellular calcium and blocks motility. In contrast, treatment with calmidazolium, which decreases the amplitude but increases the frequency of calcium spikes, increases microneme secretion and

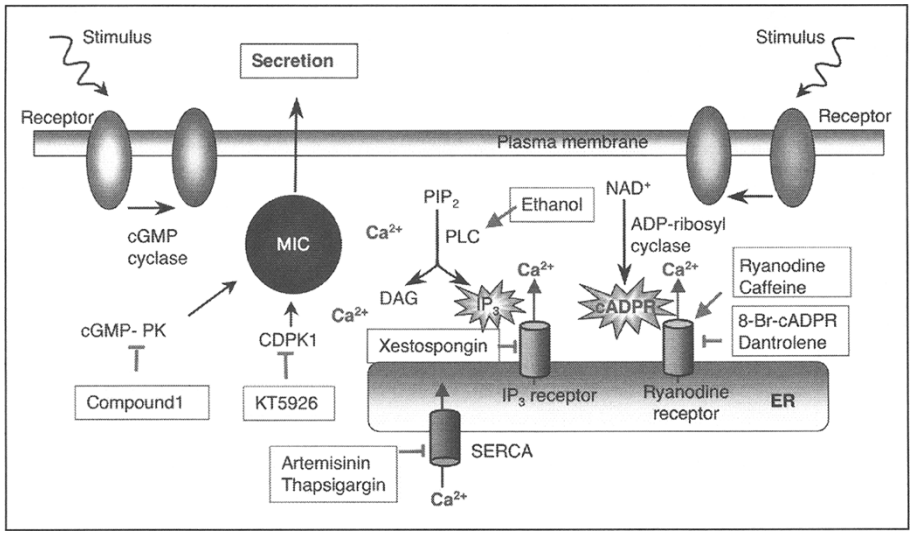

Figure 1. Calcium-mediated secretions in *T. gondii*. Elevated calcium controls microneme secretion in *T. gondii*.[47,57] Two pathways for calcium-mediated release have been described in *T. gondii*: generation of cyclic ADP ribose (cADPR) by ADP-ribose cyclase[64] and generation of IP_3 [37] Although specific release channels have not been identified, pharmacological evidence supports the presence of channels that respond to caffeine and ryanodine.[37] Ethanol is a potent trigger of secretion and is thought to act by stimulating phospholipase C (PLC) to generate IP_3. Ryanodine response channels are blocked specifically by 8-Bromo-cADP (8-Br-cADP) and dantrolene,[64] while IP_3 channels are blocked by xestospongin.[37] A calcium reuptake mechanism is provided by SERCA. Artemisinin and thapsigargin are thought to act by inhibiting this reuptake mechanism ([6] and unpublished data). Downstream of calcium release, two different kinases play an important role in secretion: calmodulin-like domain kinase 1 (CDPK1)[61] and cGMP- protein kinase (cGMP-PK).[62] The role of these kinases has been partially defined by the selective inhibition of cGMP-PK by compound 1[62] and CDPK1 by KT5926.[61] The natural agonists and cell surface receptors involved in sensing have not been defined.

prolongs motility.[67] While increased calcium levels within the parasite cytosol are important for motility and invasion of host cells by *T. gondii*, extracellular calcium appears to play little role.[66,68] Following completion of intracellular replication, the parasite exits the host cell through an active process of egress, which can be stimulated by calcium ionophores.[69] A decrease in extracellular K levels (which occurs in host cells due to loss of membrane integrity) has been linked to increases in intracellular calcium in the parasite and activation of egress.[70] However, this transition is not linked to changes in membrane potential, but rather to the activation of PLC and subsequent release of intracellular calcium.[70]

Ca^{2+}-Binding Proteins

Release of intracellular calcium or influx from outside the cell can activate a number of downstream signaling pathways that are mediated by calcium-binding proteins. Calmodulin (CaM) was initially characterized in *T. gondii* as a small (16 kDa) acidic calcium-binding protein with four calcium-binding sites (EF-hands) and with a high level of identity (92.5%) with human CaM.[71] By immunofluorescence analysis using monoclonal antibodies reactive against CaM from different species, *T. gondii* CaM was found in the apical end of released tachyzoites and also beneath the membrane in intracellular parasites.[71] Immunogold electron microscopy using monoclonal antibodies against mammalian CaM confirmed the localization of CaM in

the anterior region of tachyzoites.[72] Such heterologous antibodies may in fact recognize multiple CaM-like proteins in *T. gondii*. Whole genome analysis indicates there is a single conventional CaM, three centrins (CETN), and an additional 9 CETN-like genes in *T. gondii*.[27] Several of these more divergent CETN-like genes have been referred to as CAM1 and CAM2 and have been localized to the conoid.[73] Calmodulin has also been cloned in *P. falciparum*[74] and inhibitors of CaM block invasion of red blood cells, consistent with its apical location.[75]

Apicomplexans also contain a variety of centrin and caltractin-like genes, defined by the presence of EF-hands.[27] Centrins are a key component of centrioles at the core of the centrosome, which serves as a microtubular organizing center.[76] Centrioles also play an important role in cytokinesis, and centrin has been shown to localize to the dividing apicoplast in *Toxoplasma*.[77] It is unclear why apicomplexans have multiple centrins, but divergent centrins have been implicated in controlling voltage-gated calcium channels in *Paramecium*,[78] homologous DNA recombination and excision repair in *Arabidopisis*,[79] and axonemal functions in *Tetrahymena*.[80] *Toxoplasma* also contains a homologue of calnexin, a calcium-binding lectin in the ER that functions as a chaperone. None of the apicomplexans examined here have obvious homologues of other calcium sequestering proteins including calcineurin, endoplasmin, or troponin C.[27]

CDPKs Control Secretion and Development

Apicomplexans resemble plants in that they contain a large number of calcium-dependent protein kinases (CDPK) while they do not contain calmodulin-dependent kinases, which are abundant in animal cells. CDPKs contain an N-terminal S/T kinase domain, a linker region and up to four partially conserved EF-hands that are similar to calmodulin. CDPKs are abundant in plants, for example *Arabidopsis* contains ~30 CDPK genes, which have been implicated in diverse functions including stress response, transport, and cytoskeletal functions.[81] Comparative genome analysis reveals a large number of CDPKs are found in the *T. gondii* genome, along with several members of a related family of CDPK-related kinases that lack conserved EF-hand domains.[27] Remarkably, *Toxoplasma* contains 22 members of this family of CDPK and CRK kinases, suggesting a wide range of cellular functions. *Plasmodium* (contains 12) and *Cryptosporidium* (contains 7) by comparison have fewer members of these families of kinases, but still show this bias to plant-like CDPKs.[27]

Among the diverse number of CDPKs in apicomplexans, only a few have been studied in any detail. CDPK1 has been described in *T. gondii* as a calcium-dependent kinase that phosphorylates several potential substrates required for microneme secretion and/or motility.[61] CDPK1 was shown to be the most probable target of KT5926, a S/T kinase inhibitor that blocks parasite motility and microneme secretion.[61,82] The substrates of CDPK1 have not yet been identified but may involve machinery that is required for vesicle transport or fusion to the membrane. Importantly, the requirement for kinase activity of CDPK cannot be bypassed by elevating intracellular calcium, indicating that protein phosphorylation is a downstream event that follows increases in intracellular calcium (Fig. 1). Additionally, cGMP-PK has been shown to play an essential role in microneme secretion, motility, and invasion.[62] cGMP-PK does not appear to be involved in generation of calcium release but likely acts downstream of this signal or at an independent step (Fig. 1).

In *Plasmodium*, CDPK1 and 2 are expressed in asexual stages while CDPK 3 and 4 are expressed in gametocytes and ookinetes. Recent data indicate that CDPK4 is essential for progression of the cell cycle during differentiation of microgametocytes of *P. berghei*,[83] while CDPK3 is necessary for ookinete migration and midgut invasion.[84,85] Intracellular calcium is an important signal for gametocyte development, and microgametocytes respond to xanthurenic acid by raising intracellular calcium.[83,86] Induction of gametocyte exflagellation by pH shift has been shown to induce IP_3, suggesting a route for release of Ca^{2+} from internal stores,[87] likely from the ER based on analogy to the studies mentioned above for *T. gondii*.

Future Challenges

Apicomplexan parasites exhibit several calcium-dependent activities that are vital to their survival including protein secretion, motility, and differentiation. Understanding the molecular basis for these processes will be greatly aided by the recent completion of parasite genomes, which allow predictions about possible functions of cation transporters, channels, and calcium-dependent effector proteins. Apicomplexans sit at an early branch point in the eukaryotic tree and yet they contain both IP_3-like and ryanodine-like calcium response channels that are characteristic of higher mammals. Hence studies of calcium storage and release mechanism in these parasites may inform us about the origins of complex signaling networks in eukaryotes. One of the most unusual features of calcium metabolism in apicomplexans is the presence of a diverse array of plant-like calcium-dependent protein kinases. Calcium signaling pathways offer multiple targets for selective inhibition either due to the presence of unusual members of these calcium response proteins in parasites or due to key molecular and structural differences in conserved machinery.

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