Current Topics in Medicinal Chemistry, 2017, *17*, 2158-2165

REVIEW ARTICLE

$InsP_3$ Signaling in Apicomplexan Parasites

Celia R.S. Garcia[1,*], Eduardo Alves[2], Pedro H.S. Pereira[1,2], Paula J. Bartlett[3], Andrew P. Thomas[3], Katsuhiko Mikoshiba[4], Helmut Plattner[5] and L. David Sibley[6]

[1]*Departamento de Fisiologia, Instituto de Biociências, Universidade de São Paulo. Sao Paulo 05508-090, Brazil;* [2]*Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo. Sao Paulo 05508-000, Brazil;* [3]*New Jersey Medical School, Rutgers, The State University of New Jersey, New Jersey, USA;* [4]*Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, Wako, Saitama, Japan;* [5]*Department of Biology, University of Konstanz, 78457 Konstanz, Germany;* [6]*Department of Molecular Microbiology, Washington University Sch. Med., Saint Louis, USA*

ARTICLE HISTORY

Received: September 01, 2016
Revised: October 20, 2016
Accepted: October 30, 2016

DOI:
10.2174/1568026617666170130121042

Abstract: ***Background:*** Phosphoinositides (PIs) and their derivatives are essential cellular components that form the building blocks for cell membranes and regulate numerous cell functions. Specifically, the ability to generate myo-inositol 1,4,5-trisphosphate ($InsP_3$) via phospholipase C (PLC) dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to InsP3 and diacylglycerol (DAG) initiates intracellular calcium signaling events representing a fundamental signaling mechanism dependent on PIs. $InsP_3$ produced by PI turnover as a second messenger causes intracellular calcium release, especially from endoplasmic reticulum, by binding to the $InsP_3$ receptor ($InsP_3R$). Various PIs and the enzymes, such as phosphatidylinositol synthase and phosphatidylinositol 4-kinase, necessary for their turnover have been characterized in Apicomplexa, a large phylum of mostly commensal organisms that also includes several clinically relevant parasites. However, $InsP_3Rs$ have not been identified in genomes of apicomplexans, despite evidence that these parasites produce $InsP_3$ that mediates intracellular Ca^{2+} signaling.

Conclusion: Evidence to supporting IP_3-dependent signaling cascades in apicomplexans suggests that they may harbor a primitive or non-canonical $InsP_3R$. Understanding these pathways may be informative about early branching eukaryotes, where such signaling pathways also diverge from animal systems, thus identifying potential novel and essential targets for therapeutic intervention.

Keywords: Calcium signaling, $InsP_3$ signaling, Apicomplexan parasites, Phosphoinositides, *Plasmodium*.

1. INTRODUCTION

Phosphoinositides (PIs) and their derivatives are important for controlling a variety of vital cell functions including intracellular signaling cascades, regulated secretion, and cytoskeleton integrity [1]. The inositol containing phospholipids are abundant constituents of cell membranes of Archaea and all eukaryotes [2]. The importance of inositol signaling has been known for many years. However, the seminal discovery that ligation of cell surface receptors and PLC activity coupled to PI turnover and calcium release [3,4] paved the way for the discovery of the secondary messengers $InsP_3$ and diacylglycerol (DAG) and the downstream signaling cascades regulated by their generation and metabolism. $InsP_3$ formation depends on phospholipase C (PLC) that is activated by a variety of specific cell surface receptors. PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), thus releasing soluble $InsP_3$ [5,6]. Pharmacological evidence supports $InsP_3$ signaling in unicellular eukaryotes such as members of the phylum Apicomplexa, a diverse group that includes *Plasmodium*, *Toxoplasma* and *Cryptosporidium*. The mobilization of intracellular Ca^{2+} level by $InsP_3$ in single cell pathogens was described in *Trypanosoma,* a Kinetoplastid member of the phylum Euglenozoa. Although the cell surface receptor mediated signaling cascades that lead to $InsP_3$-dependent, and potentially DAG-dependent, signaling cascades have not been fully delineated, there is mounting evidence to suggest these pathways are operative in apicomplexans.

Interest in Ca^{2+} signaling in these parasites is motivated by a history of studies demonstrating the requirement of Ca^{2+} signaling for infection by apicomplexan parasites, as recently reviewed [7]. Elevation of intracellular calcium regulates secretion of adhesins from microneme organelles, as well as activating actin-myosin dependent motility. As described below, parasite pathways that recruit proteins sufficiently divergent from their mammalian hosts may be exploited for development of novel therapeutic interventions (Fig. **1**).

Apicomplexans share many molecules pertinent to Ca^{2+} signaling with their closest relatives, the ciliates (phylum Ciliophora), despite these two groups being evolutionarily

*Address correspondence to this author at the Rua do Matão, travessa 14, n. 321. Cidade Universitária, São Paulo, Brazil. – CEP 05508-090; Tel: +55 (11) 30917518; Fax: +55 (11) 30918095; E-mail: cgarcia@usp.br

1873-4294/17 $58.00+.00

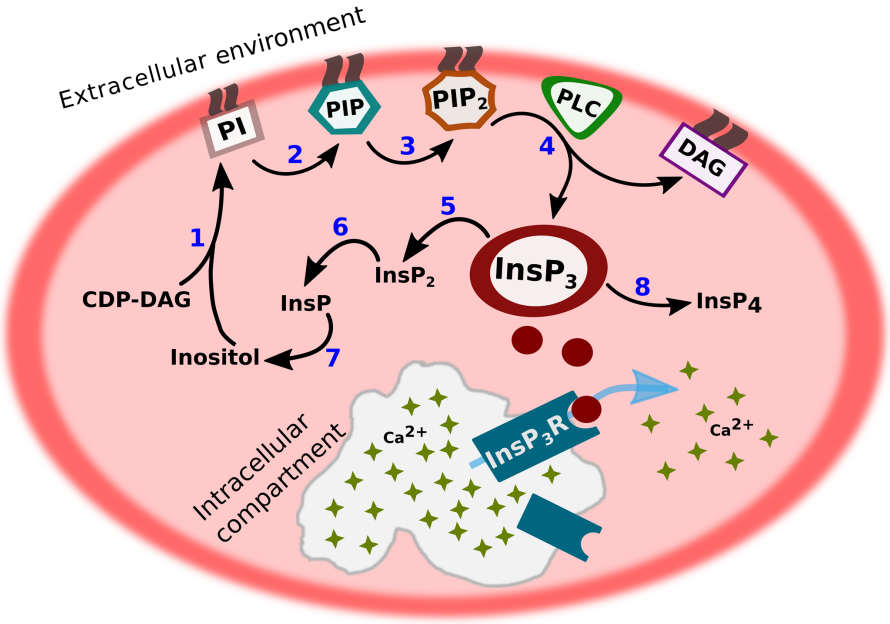

Fig. (1). Schematic representation of the myo-inositol 1,4,5-triphosphate ($InsP_3$) turnover and its function in intracellular Ca^{2+} release. Step 1: Inositol incorporation into CDP-DAG (CDP = cytidine diphosphate DAG = diacylglycerol) catalyzed by phosphatidylinositol synthase generating phosphatidylinositol (PI). Step 2: Phosphorylation of PI by phosphatidylinositol 4-kinase forming phosphatidylinositol 4-phosphate (PIP). Step 3: Phosphorylation of PIP by phosphatidylinositol 4-phosphate 5-kinase forming phosphatidyl 4,5-bisphosphate (PIP_2). Step 4: PIP_2 is cleaved by phospholipase C (PLC) generating a soluble molecule, inositol 1,4,5-trisphosphate ($InsP_3$), that is capable to bind to a $InsP_3$ receptor ($InsP_3R$) present in the membranes of intracellular compartments. $InsP_3$ binding temporarily opens the $InsP_3R$ that acts as a Ca^{2+}-release channel, so that Ca^{2+} can flow into the cytoplasm. The insoluble fraction formed by cleavage of PIP_2 is diacylglycerol (DAG). Step 5: $InsP_3$ is dephosphorylated by inositolpolyphosphate 5-phosphatase forming inositol 1,4-bisphosphate ($InsP_2$). Step 6: $InsP_2$ is desphosphorylated by inositol polyphosphate 1-phosphatase generating inositol 1-monophosphate (InsP). Step 7: Dephosphorylation of InsP to inositol by inositol monophosphatase. Step 8: phosphorylation of $InsP_3$ by inositol 1,4,5-trisphosphate 3-kinase generating inositol 1,3,4,5-tetrakisphosphate ($InsP_4$).

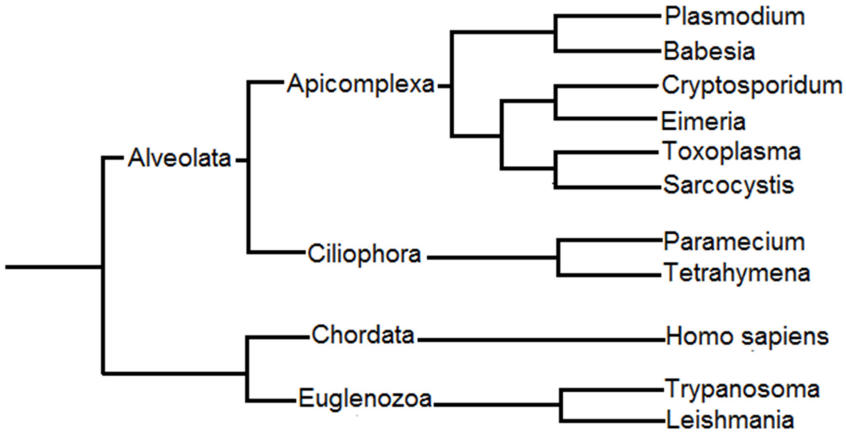

Fig. (2). Phylogenetic tree showing evolutionary relationships among organisms discussed in this review.

quite diverse [8]. Both groups are combined in the Alveolata, due to their endowment with subplasmalemmal flattened membranes, *i.e.* alveolar sacs in ciliates and inner membrane complex in apicomplexans. In ciliates, Ca^{2+} signaling mechanisms are known in some detail, including the molecular identity of intracellular Ca^{2+} channels involved in Ca^{2+} release from intracellular Ca^{2+} stores [9,10]. In contrast, identifying Ca^{2+}-channels in apicomplexans such as *Plasmodium* and *Toxoplasma* has proved a major challenge. However, the recent development of transgenic parasites expressing genetically encoded sensors for detection of Ca^{2+} in *P. falciparum* and *T. gondii* may reveal new aspects of Ca^{2+} signaling [11,12] (Fig. **2**).

2. EVIDENCE OF INSP$_3$ TURNOVER AND INSP$_3$-INDUCED CA^{2+} RELEASE IN APICOMPLEXA

Inositol and inositol phospholipids have been described in most Archaea and eukaryotes indicating this system developed in a common ancestor of the Archaea kingdom approximately 2 billion years ago [2,13]. Inositol phospholipids (PI) are ubiquitous components of cell membranes, there is not a unique PI found only in apicomplexan. Table **1**

Table 1. Occurrence of proteins important for the $InsP_3$ signaling pathway in some model organisms. The corresponding genes were searched for in the databases of *Plasmodium*, *Toxoplasma*, *Homo sapiens*, *Trypanosoma* and *Paramecium* (PlasmoDB, ToxoDB, PDB/NCBI, TriTrypDB and ParameciumDB, respectively). Phospholipase C of *Paramecium tetraurelia* is described in Klöppel *et al.*, 2009 [27]. (1) Putative protein; (2) -like proteins; (3) encoded by pseudogene; (4) Leondaritis *et al.* (2013) [28], described 62 highly homologous phosphoinositide kinases genes; *genes that could not be found in databases.

	Phosphatidyl Inositol 4-kinase	Phosphatidyl Inositol 4-phosphate 5-kinase	Phospholipase C	Inositol Polyphosphate 5-phosphatase	Inositol 1,4,5-Trisphosphate 3-kinase	Inositol Triphosphate Receptor
***Plasmodium falciparum* 3D7**	PF3D7_0509800; PF3D7_0419900[(1)]	PF3D7_0110600; PF3D7_1129600[(1)]	PF3D7_1013500	PF3D7_1354200[(1)]	*	*
***Toxoplasma gondii* ME49**	TGME49_328200	TGME49_245730	TGME49_248830	*	*	*
Homo sapiens	AAA56839.1; BAA21661.1	CAD99242.1	AAA60112.1; BAA07688.1	CAA74743.1; CAA67071.1	AAH26331.1; CAC40650.1	NP_002214.2
***Leishmania major* Friedlin**	LmjF.34.3590[(1)]; LmjF.29.1450[(1)]	LmjF.36.0370[(2)]; LmjF.34.3090[(2)]; LmjF.35.0560[(1,2)]	LmjF.30.2950[(2)]; LmjF.22.1680[(1)]	*	*	LmjF.16.0280[(1)]
***Trypanossoma brucei* gambiense**	Tbg972.4.970[(1)]	Tbg972.10.4830[(1)]	Tbg972.11.6720[(1)]	*	*	Tbg972.8.2330[(1)]
***Trypanossoma cruzi* CL Brener**	TcCLB.505987.70[(1)]	TcCLB.510289.30[(1)]; TcCLB.511001.70[(1)]	TcCLB.508039.90[(3)]	*	*	BAM68694.1
Paramecium tetraurelia	XP_001346932.1	XP_001347030.1; XP_001347013.1	XP_001432600.1; XP_001426835.1	*	[(4)]	PTMB.445c

shows some important PI specific enzymes that have been reported in Apicomplexa and other unicellular parasites. The pathway by which PI is generated appears conserved amongst all eukaryotes [13]. The genes encoding the enzyme phosphatidylinositol synthase that catalyzes the formation of phosphatidylinositol (PI) are well characterized in *P. falciparum* and *P. knowlesi* [14], *T. gondii* [15] and *T. brucei* [16]. Phosphatidylinositol transfer protein encoding genes are also present, suggesting the existence of systems responsible for transporting PI from the ER to the plasmamembrane [1]. In *Plasmodium*, Gardner *et al.*, 2002 [17] reported a gene encoding the enzyme phosphatidylinositol 4-kinase (PI4K) that catalyses the formation of phosphatidylinositol 4-phosphate (PIP) from phosphatidylinositol (PI). Thus, apicomplexans have the machinery to generate the precursor of $InsP_3$. Table **1** summarizes known and putative proteins from the $InsP_3$ pathway in model organisms and parasites. In mammalian systems the ability of $InsP_3$ to act as a second messenger requires the enzymes to rapidly release $InsP_3$ from PIP_2, as well as the ability to reduce $InsP_3$ levels to basal. Importantly, genetic evidence for phospholipase C enzymes can be found in all apicomplexans, except Sacrocysis (presumable due to a lack of genetic data), and Kinetoplastids (See Table **1**). The presence of the archetypal PLCδ isoform in all genomes indicates the ability to generate $InsP_3$ and DAG in unicellular parasites arising from a common ancestor. The enzymes involved in $InsP_3$ metabolism are less well conserved. *Plasmodium falciparum* have an annotated inositol polyphosphate 5-phosphatase, an indication that they too can metabolize $InsP_3$, to $InsP_2$, whilst a specific inositol 1,4,5 trisphosphate 3-kinase has not been annotated. However, inositol polyphosphate kinase genes can be found in both *P. falciparum* and *T. gondii* genomes and may metabolize $InsP_3$ to $InsP_4$.

3. PHARMACOLOGICAL EVIDENCE FOR $INSP_3$ SIGNALING IN APICOMPLEXA

Importantly, biochemical evidence to support PI turnover in apicomplexan parasites has also been reported in the literature. Despite the fact that mammalian RBCs have all the enzymes enabling the production of PIP_2 from PI [18], a non-infected RBC has limited biosynthesis of PIP_2 and other PIs [19]; in contrast, when a RBC is infected with *Plasmodium knowlesi; P falciparum*, [20] and *Babesia bovis* [21] PI turnover increases due parasite metabolism. Incubating red blood cells (RBC) infected with *P. falciparum* with radiolabeled myo-inositol [22] results in the biosynthesis of PI, PIP and PIP_2. Moreover, treatment with the Ca^{2+} ionophore, ionomycin, caused an increase in inositol phosphate production with $Ins1,4,5P_3$ levels being the highest. The increase of intracellular Ca^{2+} promoted by ionomycin presumably activates the Ca^{2+} sensitive PLC [23] to cleave PIP_2, thus generating $InsP_3$. Martin *et al.* [24] demonstrated the formation of $InsP_3$ and diacylglycerol (DAG) during exflagellation of *P. falciparum* gametocytes, an important event in the sexual cycle that takes place in the *Anopheles* mosquitos. Fang *et al.,*[25] identified a PI-PLCδ in *T. gondii* that cleaves PIP_2. Whilst recent bioinformatic approaches have failed to identify intracellular Ca^{2+} release channels such as IP_3Rs and Ryanodine receptors (RyRs) within the genomes of apicomplexans [7, 26-28], a large body of pharmacological evidence suggests that a Ca^{2+} release channel sensitive to $InsP_3$ does indeed

exist in these species. Prompted by evidence that malaria parasites maintain intracellular Ca^{2+} stores, Passos *et al.* [29] published the first work demonstrating $InsP_3$ mobilized Ca^{2+} from an intracellular store in *P. chabaudi* using a permeabilized cell system. The response was blocked by heparin (an inhibitor of $InsP_3Rs$) providing pharmacological evidence for an $InsP_3$ dependent Ca^{2+} channel in *P. chabaudi*. Similarly, treatment of microsome preparations from *T. gondii* tachyzoites with $InsP_3$ initiated Ca^{2+} release which could be fully blocked by pharmacological inhibitors of $InsP_3Rs$ (Xestospongin C and heparin) [30].

Inhibitors of the $InsP_3$ signaling pathway, including agents that block $InsP_3R$, such as heparin, xestospogin C and 2-aminoethoxydiphenyl borinate (2-APB), as well as inhibitors of PLC, such as U73122, have also been used to study Ca^{2+} signaling in Apicomplexa [31]. In *T. gondii*, the release of adhesins that mediate parasite attachment to host cells is an event regulated by Ca^{2+}-mediated microneme secretion [32,33]. Lovett *et al.*, (2002) also reported that *T. gondii* mobilizes Ca^{2+} when exposed to ryanodine or caffeine, both RyR ligands [34]. Addition of xestospongin C inhibited the caffeine- or ethanol-induced increase of intracellular Ca^{2+}, thus preventing microneme secretion in *T. gondii* [35], and suggesting a role for $InsP_3R$. In *Paramecium* this pathway controls stimulated trichocyst exocytosis, as found by energy-dispersive X-ray microanalysis and fluorochrome analysis [36], via activation of RyR-type Ca^{2+} release channels in alveolar sacs [10,37]. RyRs share common features and an evolutionary history with $InsP_3Rs$ [9,38,39], yet RyRs are activated by the intracellular messenger cADPR (cyclic ADP-ribose) [40]. In fact, cADPR has been shown to activate Ca^{2+} signaling pathways in *T. gondii* and *P. falciparum* [30,41].

In malaria melatonin dependent signaling via calcium is well established [29,42,43] enabling the investigation of a natural ligand to induce intracellular signaling. Hotta *et al.*, [42] reported that intracellular Ca^{2+} mobilization induced by melatonin in *P. falciparum* and *P. chabaudi* is abolished by the PLC inhibitor U73122, but not by its inactive analogue, indicating that melatonin may activate signaling via PLC/$InsP_3$. Enomoto *et al.*, [44] blocked spontaneous Ca^{2+} mobilization in the ring and trophozoite forms of intraerythrocytic stage of *P. falciparum* using the $InsP_3R$ inhibitor, 2-APB [45]. The presence of 2-APB during intraerythrocytic development of *P. falciparum* compromised the asexual replication of this parasite suggesting that blocking the $InsP_3R$ can be a potential target for antimalarial treatment. Raabe *et al.*, [46] investigated the role of PLP_2/PLC/$InsP_3$ during *P. berghei* gametocyte exflagellation induced by xanthurenic acid (XA), a small metabolic intermediate found in *Anopheles* mosquito gut. Under XA stimulus, addition of the PLC inhibitor U73122 inhibits Ca^{2+} mobilization in this model. Together this work suggested a vital role of the $InsP_3$/PLC signaling pathway at different points during the *Plasmodium* life cycle, and the important nature of this pathway suggests it may contain potential antimalarial targets expressed during all life cycle phases.

Evidence for a ryanodine sensitive store in *T. gondii* [35] motivated Raabe *et al.*, [46] to investigate the role of Ca^{2+} release via the RyR in *P. berghei* during gametocyte maturation. In the presence of RyR inhibitors, dantrolene and ruthenium red (RR), XA mediated Ca^{2+} increases were attenuated. Remarkably, RR treatment decreased $InsP_3$ levels and dantrolene inhibited gametocyte maturation during XA stimulation. These data also suggest the existence of ryanodine sensitive channels in *P. berghei*. The development of cell permeant caged Ins1,4,5P_3,[47], enabled the investigation of $InsP_3$ dependent calcium signaling without compromising membrane integrity. With this tool, Alves *et al.* [43] reported that *P. falciparum* trophozoites within intact RBCs release Ca^{2+} from thapsigargin-sensitive stores due to liberation of caged Ins1,4,5P_3. This observation was the first demonstration of a Ca^{2+}-increase induced by exogenous $InsP_3$ under physiological conditions in Apicomplexa. Furthermore, the authors reported an increase of $InsP_3$ in infected RBC treated with melatonin. These data further support the concept of PIP_2 hydrolysis to $InsP_3$ as a signaling pathway activated by melatonin in *P. falciparum,* as reported previously [42,48].

Mossaad *et al.* [49] first reported a complete reversal of chloroquine resistance in malaria parasites after treatment with 2-APB *in vitro* and *in vivo*, and this effect was justified by disturbance of Ca^{2+} homeostasis in the parasite cell. This result showed that 2-APB and other related compounds that block the $InsP_3$ pathway might be promising candidates in the search for new-resistance reversing agents that aid in treatment of the disease.

Beraldo *et al.* [50] showed that increase in cytosolic Ca^{2+} concentration in melatonin induced *P. falciparum* is abolished with the use of 2-APB, U73122 (PLC inhibitor) and luzindole (a melatonin antagonist). In the same work, capacitative calcium entry was also reported in malaria parasites.

Since then, other pharmacological agents have further been applied to investigate this pathway. It should be noted that most drugs used to interfere with signaling in protozoa are used solely on the basis of effects observed in mammalian cells [31], and all these drugs have potential off target effects. Despite this caveat, the combined evidence of a number of studies suggest the presence of an $InsP_3R$ and/or RYR calcium release channels in *Plasmodia* and *Toxoplasma*.

4. $INSP_3RS$ AND RYRS IN PROTOZOANS

The history of $InsP_3R$ discovery and the impact of this receptor on mammalian cell signaling has been previously reviewed [51]. Knowledge of receptor sequences and functional proprieties in mammals, together with increasing information and access to genome databases for different organisms, made bioinformatics tools such as BLAST searches a major strategy to identify orthologs to putative $InsP_3R$ and RyRs in metazoans.

Until recently no information about such channels has been available from protozoa. After successful identification of $InsP_3Rs$ and RyR-like proteins [9,37,52] in the ciliated protozoan, *Paramecium,* partial sequences suggested the occurrence of $InsP_3Rs$ in several protozoan phyla, including trypanosomatids [37,39]. Similarity with established $InsP_3Rs$ is often scattered throughout the protein, with highest similarity in the carboxy-terminal region containing the pore do-

main. Prole and Taylor [53] also used conserved regions of $InsP_3R$ and RyR, specifically the amino-terminal RIH (RyR and $InsP_3R$ homology) domain and the N-terminal $InsP_3$-binding domain, to successfully identify candidates for $InsP_3R$ in *T. cruzi* and *T. brucei*. Such work supported the identification of an $InsP_3R$-type protein in *T. cruzi* [54,55] and *T. brucei* [56]. Hashimoto *et al.* [54] demonstrated that *TcInsP$_3$R* is essential for *T. cruzi* epimastigote survival, contributes to parasite invasiveness in mammalian cells, mediates Ca^{2+} release in trypomastigotes upon attachment to host cells, modulates parasite development and contributes to parasite virulence. These findings are consistent with previous evidence for a $InsP_3$/DAG pathway in *T. cruzi* (*49*) and *T brucei* [47].

The identification of an $InsP_3R$ candidate in apicomplexan parasites is a much more challenging task. When the same sequences of mammalian $InsP_3Rs$ were used for BLAST searches, no $InsP_3R$ orthologs were identified for *Plasmodium*, *Toxoplasma, Cryptosporidium* or *Babesia* species [53]. Previously, Naganume *et al.* [58], using a collection of Ca^{2+}-associated protein orthologs to identify proteins participating in Ca^{2+} signaling, were also unable to find either any orthologs of $InsP_3Rs$, or of protein kinase C (PKC), a classic target for DAG activation [59]. Ladenburger *et al.,* (2009) [37] were also unable to detect any $InsP_3R$ or RyR orthologs in genomes of apicomplexans, in contrast to ciliates where they are readily found.

Recently, it was reported that, when *TcInsP$_3$R* expression is decreased by two thirds compared to wildtype, the invasion of trypomastigotes of *T. cruzi* is blocked, indicating *TcInsP$_3$R* as a potential therapeutic target [60]. After treatment of trypomastigotes with specific antisense oligonucleotides of *TcInsP$_3$R*, a reduction in infectivity, thus suggesting that the suppression of transcription of this gene led to reduced levels of *TcInsP$_3$R* protein. In Chagas disease, Ca^{2+} signaling mediated by $InsP_3R$ plays a key role in multiple parasite differentiation steps [61]. A therapeutic approach using antisense transcripts is particularly feasible in the acute phase of infection in which trypomastigotes predominate in the bloodstream. It is possible that a treatment with antisense transcripts is effective in preventing the development of the disease by blocking the proliferation of trypomastigotes by inhibition of production of $TcInsP_3R$ [62].

The presence of dense mitochondria in *T. cruzi* with nonfunctional $InsP_3R$ provides new insights for the contribution of $InsP_3Rs$ to organelle integrity and the fact that 2-APB does not completely inhibit the development of trypomastigotes [63] suggest structural difference to mammalian $InsP_3Rs$ [64]. On the other hand, at high concentrations, 2-APB might influence functions of proteins other than $InsP_3Rs$.

5. LESSONS FROM OTHER SYSTEMS

Clues about the composition of Ca^{2+} signaling pathways in apicomplexans can be drawn from their closest ancestral group, free living ciliates. It is estimated that ciliates originated between ~800 and 850 million years [65,66]. Apicomplexa are assumed to have arisen ~550, and the genus *Plasmodium* ~400 million years ago [65]. These events occurred much before Apicomplexa became parasites of vertebrates and then of mammalian species, which occurred only ~13 million years ago [67]. *Paramecium tetraurelia* contains several genes encoding $InsP_3Rs$ and RyR-like channels that have been identified at a genomic and proteomic level [9,37,52]. Considering the finding of $InsP_3Rs$ and of RyR-like channels in *Paramecium* [10], the argument that the old age of some phyla may explain the failure to detect Ca^{2+} release channels in apicomplexans appears unlikely.

The lack of conservation at the gene level, suggests that $InsP_3R$ in apicomplexans may diverge significantly from mammalian channels and those found in ciliates; one may ask whether they adopted a different, distinct protein or a complex of proteins for $InsP_3R$ function. If this is the case, successful identification of $InsP_3R$ in apicomplexans may require a different strategy focused on biochemical and physiological criteria. Specifically one should consider that any Ca^{2+} release channel requires a pore domain with six transmembrane stretches in $InsP_3Rs$ and RyRs, from ciliates to humans, and a rather conserved, though slightly variable selectivity filter [10].

Currently the lack of identified Ca^{2+} release channels in Apicomplexa leaves a significant gap in our understanding of PI and calcium signaling mechanisms. There are clear physiological effects of stimulating production of $InsP_3$, and of inhibiting putative channels, and yet the molecular machinery in Apicomplexa is clearly diverse from other cells. Ultrastructurally the inner membrane complex of Apicomplexa looks very much like the alveolar sacs of ciliates; however, whereas the sacs of ciliates are well established Ca^{2+} stores [36], they appear largely independent of such function in Apicomplexa [8]. The third enigma is the presence of well defined $InsP_3R$- and RyR-type Ca^{2+} release channels in ciliates [9,37,52], in contrast to their absence in their closest relatives, the Apicomplexa [68,69].

Similar to apicomplexans, plant genomes lack recognizable $InsP_3Rs$ in despite of evidence for an $InsP_3$ signaling pathway [70]. Despite responding to $InsP_3$ to regulate Ca^{2+} increases, land plants typically lack an $InsP_3R$ or RyR, leading to the suggestion that these channels were animal-specific, and perhaps evolved during the vertebrate lineage [70]. However, recent evidence from green algae *Chlamydomonas* indicates that chlorophyte plants contain $InsP_3R$, suggesting they were present in the ancestral eukaryotes prior to the plant-animal split [71]. Why $InsP_3R$ channels appear to have been lost in land plants remains a mystery, but it is intriguing to consider that like apicomplexans, they may have evolved different mechanism to release internal Ca^{2+} stores in response to $InsP_3$.

CONCLUSION

Differences in Ca^{2+} signaling between parasites and their hosts may present unique targets for developing interventions. For example, McNamara *et al.*, 2013 [72] reported PI4K as a target of imidazopyrazine, a class of new antimalarial drug that inhibits all stages of malaria parasite infection of the vertebrate host and also prevents transmission by mosquitos to a murine malaria model. Additionally, as mentioned above, agents that disrupt $InsP_3R$ channels such as 2-ABP block malaria growth *in vitro* [44]. Given the apparent

divergent mechanism by which $InsP_3$ is sensed in parasites, identification of the molecular basis for this pathway might also identify novel targets for intervention.

The pharmacological literature and functional studies with exogenous $InsP_3$ discussed herein seem to support the occurrence of $InsP_3R$-type Ca^{2+}-channels in ciliates, trypanosomatids and apicomplexans. $InsP_3R$ is the major missing piece of the PI signaling puzzle in Apicomplexa. Although $InsP_3R$ have been found in ciliates and tryponosomatids, the lack of ^3H-$InsP_3$ binding assays in parallel to a vast unknown protein function in apicomplexans genome has hampered the identification of potential candidates for $InsP_3R$. Although identification of these channels will certainly not be an easy task, it will be a rewarding engagement for groups willing to accept the challenge.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Supported by São Paulo Research Foundation (Process number 2011/51295-5) to CRSG, by a grant from the National Institutes of Health, USA (AI094098) to LDS, AI099277 to APT and by grants from the German Research Council to H.P.

REFERENCES

[1] Balla, T. Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol. Rev.,* **2013**, *93*, 1019-1137.

[2] Michell, R.H. Inositol derivatives: evolution and functions. *Nat. Rev. Mol. Cell Biol.,* **2008**, *9*(2), 151-161.

[3] Michell, R.H. Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta - Rev. Biomembr.,* **1975**, *415*(1), 81-147.

[4] Fain, J.N.; Berridge, M.J. Relationship between phosphatidylinositol synthesis and recovery of 5-hydroxytryptamine-responsive ca^{2+} flux in blowfly salivary glands. *Biochem. J.,* **1979**, *180*(3), 655-661.

[5] Streb, H.; Irvine, R.F.; Berridge, M.J.; Schulz, I. Release of ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature,* **1983**, *306*(5938), 67-69.

[6] Berridge, M.J.; Irvine, R.F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature,* **1984**, *312*(5992), 315-321.

[7] Lourido, S.; Moreno, S.N.J. The calcium signaling toolkit of the apicomplexan parasites toxoplasma gondii and plasmodium spp. *Cell Calcium,* **2014**, *57*(3), 186-193.

[8] Plattner, H.; Sehring, I.M.; Mohamed, I.K.; Miranda, K.; De Souza, W.; Billington, R.; Genazzani, A.; Ladenburger, E.M. Calcium signaling in closely related protozoan groups(alveolata): non-parasitic ciliates(*paramecium, tetrahymena*) vs. parasitic apicomplexa(*plasmodium, toxoplasma*). *Cell Calcium,* **2012**, *51*, 351-382.

[9] Ladenburger, E.-M.; Plattner, H. Calcium-release channels in paramecium. genomic expansion, differential positioning and partial transcriptional elimination. *PLoS One,* **2011**, *6*(11), e27111.

[10] Plattner, H. Molecular aspects of calcium signalling at the crossroads of unikont and bikont eukaryote evolution - the ciliated protozoan paramecium in focus. *Cell Calcium,* **2014**, *57*(3), 174-185.

[11] Borges-Pereira, L.; Campos, B.R.K.L.; Garcia, C.R.S. The GCaMP3 - A GFP-based calcium sensor for imaging calcium dynamics in the human malaria parasite *plasmodium falciparum*. *MethodsX,* **2014**, *1*, 151-154.

[12] Borges-Pereira, L.; Budu, A.; McKnight, C.A.; Moore, C.A.; Vella, S.A.; Hortua Triana, M.A.; Liu, J.; Garcia, C.R. S.; Pace, D.A.; Moreno, S.N.J. Calcium signaling throughout the *toxoplasma gondii* lytic cycle. a study using genetically encoded calcium indicators. *J. Biol. Chem.,* **2015**, *3*, jbc.M115.652511.

[13] Michell, R.H. Inositol and its derivatives: their evolution and functions. *Adv. Enzyme Regul.,* **2011**, *51*(1), 84-90.

[14] Wengelnik, K.; Vial, H.J. Characterisation of the phosphatidylinositol synthase gene of *plasmodium* species. *Res. Microbiol.,* **2007**, *158*, 51-59.

[15] Séron, K.; Dzierszinski, F.; Tomavo, S. Molecular cloning, functional complementation in *saccharomyces cerevisiae* and enzymatic properties of phosphatidylinositol synthase from the protozoan parasite *toxoplasma gondii*. *Eur. J. Biochem.,* **2000**, *267*(22), 6571-6579.

[16] Martin, K.L.; Smith, T.K. Phosphatidylinositol synthesis is essential in bloodstream form *trypanosoma brucei*. *Biochem. J.,* **2006**, *396*(2), 287-295.

[17] Gardner, M.J.; Hall, N.; Fung, E.; White, O.; Berriman, M.; Hyman, R.W.; Carlton, J.M.; Pain, A.; Nelson, K.E.; Bowman, S.; Paulsen, I.T.; James, K.; Eisen, J.A.; Rutherford, K.; Salzberg, S.L.; Craig, A.; Kyes, S.; Chan, M.-S.; Nene, V.; Shallom, S.J.; Suh, B.; Peterson, J.; Angiuoli, S.; Pertea, M.; Allen, J.; Selengut, J.; Haft, D.; Mather, M.W.; Vaidya, A.B.; Martin, D.M. A.; Fairlamb, A.H.; Fraunholz, M.J.; Roos, D.S.; Ralph, S.A.; McFadden, G.I.; Cummings, L.M.; Subramanian, G.M.; Mungall, C.; Venter, J.C.; Carucci, D.J.; Hoffman, S.L.; Newbold, C.; Davis, R.W.; Fraser, C.M.; Barrell, B. Genome sequence of the human malaria parasite *plasmodium falciparum*. *Nature,* **2002**, *419*(6906), 498-511.

[18] Allan, D. Inositol lipids and membrane function in erythrocytes. *Cell Calcium,* **1982**, *3*(4-5), 451-465.

[19] van Deenen, L.L. M.; de Gier, J.; Demel, R.A.; de Kruyff, B.; Blok, M.C.; van der Neut-Kok, E.C. M.; Haest, C.W. M.; Ververgaert, P.H. J.T.; Verkleij, A.J. Lipid-lipid and lipid-protein interaction in model systems and membranes. *Ann. N. Y. Acad. Sci.,* **1975**, *264*(1), 124-141.

[20] Vial, H.J.; Ancelin, M.L.; Philippot, J.R.; Thuet, M.J. Biosynthesis and dynamics of lipids in *plasmodium*-infected mature mammalian erythrocytes. *Blood Cells,* **1990**, *16*, 531-555.

[21] Florin-Christensen, J.; Suarez, C.E.; Florin-Christensen, M.; Hines, S.A.; McElwain, T.F.; Palmer, G.H. Phosphatidylcholine formation is the predominant lipid biosynthetic event in the hemoparasite babesia bovis. *Mol. Biochem. Parasitol.,* **2000**, *106*(1), 147-156.

[22] Elabbadi, N.; Ancelin, M.L.; Vial, H.J. Characterization of phosphatidylinositol synthase and evidence of a polyphosphoinositide cycle in *plasmodium*-infected erythrocytes. *Mol Biochem Parasitol,* **1994**, *63*(2), 179-192.

[23] Abdel-Latif, A.A. Calcium-mobilizing receptors, polyphosphoinositides, generation of second messengers and contraction in the mammalian iris smooth muscle: historical perspectives and current status. *Life Sci.,* **1989**, *45*(9), 757-786.

[24] Martin, S.K.; Jett, M.; Schneider, I. Correlation of phosphoinositide hydrolysis with exflagellation in the malaria microgametocyte. *J. Parasitol.,* **1994**, *80*(3), 371-378.

[25] Fang, J.; Marchesini, N.; Moreno, S.N.J. A *Toxoplasma gondii* phosphoinositide phospholipase c(tgpi-plc) with high affinity for phosphatidylinositol. *Biochem. J.,* **2006**, *394*(Pt 2), 417-425.

[26] Moreno, S.N. J.; Ayong, L.; Pace, D.A. Calcium storage and function in apicomplexan parasites. *Essays Biochem.,* **2011**, *51*, 97-110.

[27] Klöppel, C.; Müller, A.; Marker, S.; Simon, M. Two isoforms of eukaryotic phospholipase c in *paramecium* affecting transport and release of gpi-anchored proteins *in vivo*. *Eur. J. Cell Biol.,* **2009**, *88*(10), 577-592.

[28] Leondaritis, G.; Siokos, J.; Skaripa, I.; Galanopoulou, D. Genome-Wide analysis of the phosphoinositide kinome from two ciliates reveals novel evolutionary links for phosphoinositide kinases in eukaryotic cells. *PLoS One,* **2013**, *8*(11).

[29] Passos, A.P.; Garcia, C.R. Inositol 1,4,5-trisphosphate induced ca^{2+} release from chloroquine-sensitive and -insensitive intracellular stores in the intraerythrocytic stage of the malaria parasite *p.*

chabaudi. *Biochem. Biophys. Res. Commun.,* **1998**, *245*(1), 155-160.

[30] Chini, E.N.; Nagamune, K.; Wetzel, D.M.; Sibley, L.D. Evidence that the cadpr signalling pathway controls calcium-mediated microneme secretion in *toxoplasma gondii*. *Biochem. J.,* **2005**, *389*(Pt 2), 269-277.

[31] Cruz, L.N.; Guerra, M.T.; Kruglov, E.; Mennone, A.; Garcia, C.R. S.; Chen, J.; Nathanson, M.H. Regulation of multidrug resistance-associated protein 2 by calcium signaling in mouse liver. *Hepatology,* **2010**, *52*(1), 327-337.

[32] Carruthers, V.B.; Giddings, O.K.; Sibley, L.D. Secretion of Micronemal Proteins Is Associated with *Toxoplasma* Invasion of Host Cells. *Cell. Microbiol.,* **1999**, *1*(3), 225-235.

[33] Vieira, M.C. F.; Moreno, S.N. J. Mobilization of intracellular calcium upon attachment of *toxoplasma gondii* tachyzoites to human fibroblasts is required for invasion. *Mol. Biochem. Parasitol.,* **2000**, *106*(1), 157-162.

[34] Zucchi, R.; Ronca-Testoni, S. The sarcoplasmic reticulum ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states . *Pharmacol. Rev. ,* **1997**, *49*(1), 1-52.

[35] Lovett, J.L.; Marchesini, N.; Moreno, S.N. J.; Sibley, L.D. *Toxoplasma gondii* microneme secretion involves intracellular ca^{2+} release from inositol 1,4,5-triphosphate(ip(3))/ryanodine-sensitive stores. *J. Biol. Chem.,* **2002**, *277*(29), 25870-25876.

[36] Plattner, H. Calcium regulation in the protozoan model, *paramecium tetraurelia*. *J. Eukaryot. Microbiol.,* **2014**, *61*, 95-114.

[37] Ladenburger, E.-M.; Sehring, I.M.; Korn, I.; Plattner, H. Novel Types of Ca^{2+} Release channels participate in the secretory cycle of *paramecium* cells. *Mol. Cell. Biol.,* **2009**, *29*(13), 3605-3622.

[38] Mackrill, J.J. Ryanodine receptor calcium release channels: an evolutionary perspective. *Adv Exp Med Biol,* **2012**, *740*, 159-182.

[39] Plattner, H.; Verkhratsky, A. Ca^{2+} signalling early in evolution--all but primitive. *J. Cell Sci.,* **2013**, *126*(Pt 10), 2141-2150.

[40] Lee, H.C. Cyclic adp-ribose and nicotinic acid adenine dinucleotide phosphate(naadp) as messengers for calcium mobilization. *J. Biol. Chem.,* **2012**, *287*(38), 31633-31640.

[41] Jones, M.L.; Cottingham, C.; Rayner, J.C. Effects of calcium signaling on *plasmodium falciparum* erythrocyte invasion and post-translational modification of gliding-associated protein 45(pfgap45). *Mol. Biochem. Parasitol.,* **2009**, *168*(1), 55-62.

[42] Hotta, C.T.; Gazarini, M.L.; Beraldo, F.H.; Varotti, F.P.; Lopes, C.; Markus, R.P.; Pozzan, T.; Garcia, C.R. S. Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. *Nat. Cell Biol.,* **2000**, *2*(7), 466-468.

[43] Alves, E.; Bartlett, P.J.; Garcia, C.R. S.; Thomas, A.P. Melatonin and ip3-induced ca^{2+} release from intracellular stores in the malaria parasite *plasmodium falciparum* within infected red blood cells. *J. Biol. Chem.,* **2011**, *286*(7), 5905-5912.

[44] Enomoto, M.; Kawazu, S.; Kawai, S.; Furuyama, W.; Ikegami, T.; Watanabe, J.; Mikoshiba, K. Blockage of spontaneous ca^{2+} oscillation causes cell death in intraerythrocitic *plasmodium falciparum*. *PLoS One,* **2012**, *7*(7), e39499.

[45] Maruyama, T.; Kanaji, T.; Nakade, S.; Kanno, T.; Mikoshiba, K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of ins(1, 4, 5) p_3-induced ca^{2+} release. *J. Biochem.,* **1997**, *122*(3), 498-505.

[46] Raabe, A.C.; Wengelnik, K.; Billker, O.; Vial, H.J. Multiple roles for *plasmodium berghei* phosphoinositide-specific phospholipase c in regulating gametocyte activation and differentiation. *Cell. Microbiol.,* **2011**, *13*(7), 955-966.

[47] Dakin, K.; Li, W.-H. Cell membrane permeable esters of d-myo-inositol 1,4,5-trisphosphate. *Cell Calcium,* **2007**, *42*(3), 291-301.

[48] Beraldo, F.H.; Almeida, F.M.; Da Silva, A.M.; Garcia, C.R. S. Cyclic AMP and calcium interplay as second messengers in melatonin-dependent regulation of *plasmodium falciparum* cell cycle. *J. Cell Biol.,* **2005**, *170*(4), 551-557.

[49] Mossaad, E.; Furuyama, W.; Enomoto, M.; Kawai, S.; Mikoshiba, K.; Kawazu, S. Simultaneous Administration of 2-aminoethyl diphenylborinate and chloroquine reverses chloroquine resistance in malaria parasites. *Antimicrob. Agents Chemother.,* **2015**, *59*(5), 2890-2892.

[50] Beraldo, F.H.; Mikoshiba, K.; Garcia, C.R. S. Human malarial parasite, *plasmodium falciparum*, displays capacitative calcium entry: 2-aminoethyl diphenylborinate blocks the signal transduction pathway of melatonin action on the p. falciparum cell cycle. *J. Pineal Res.,* **2007**, *43*(4), 360-364.

[51] Mikoshiba, K. IP3 receptor/Ca^{2+} Channel: from discovery to new signaling concepts. *J. Neurochem.,* **2007**, *102*(5), 1426-1446.

[52] Ladenburger, E.-M.; Korn, I.; Kasielke, N.; Wassmer, T.; Plattner, H. An ins(1,4,5)p3 receptor in *paramecium* is associated with the osmoregulatory system. *J. Cell Sci.,* **2006**, *119*(Pt 17), 3705-3717.

[53] Prole, D.L.; Taylor, C.W. Identification of Intracellular and Plasma Membrane Calcium Channel Homologues in Pathogenic Parasites. *PLoS One,* **2011**, *6*(10), e26218.

[54] Hashimoto, M.; Enomoto, M.; Morales, J.; Kurebayashi, N.; Sakurai, T.; Hashimoto, T.; Nara, T.; Mikoshiba, K. Inositol 1,4,5-trisphosphate receptor regulates replication, differentiation, infectivity and virulence of the parasitic protist *Trypanosoma Cruzi*. *Mol. Microbiol.,* **2013**, *87*(6), 1133-1150.

[55] Ulrich, P.N.; Jimenez, V.; Park, M.; Martins, V.P.; Atwood, J.; Moles, K.; Collins, D.; Rohloff, P.; Tarleton, R.; Moreno, S.N. J.; Orlando, R.; Docampo, R. Identification of contractile vacuole proteins in *Trypanosoma Cruzi*. *PLoS One,* **2011**, *6*(3), e18013.

[56] Huang, G.; Bartlett, P.J.; Thomas, A.P.; Moreno, S.N. J.; Docampo, R. Acidocalcisomes of *Trypanosoma Brucei* have an inositol 1,4,5-trisphosphate receptor that is required for growth and infectivity. *Proc. Natl. Acad. Sci. U. S. A.,* **2013**, *110*(5), 1887-1892.

[57] Racagni, G.; García de Lema, M.; Domenech, C.E.; Machado de Domenech, E.E. Phospholipids in *Trypanosoma Cruzi*: Phosphoinositide Composition and Turnover. *Lipids,* **1992**, *27*(4), 275-278.

[58] Nagamune, K.; Sibley, L.D. Comparative genomic and phylogenetic analyses of calcium atpases and calcium-regulated proteins in the apicomplexa. *Mol. Biol. Evol.,* **2006**, *23*(8), 1613-1627.

[59] Berridge, M.J.; Irvine, R.F. Inositol trisphosphate as a second messenger in signal transduction. *Nature,* **1987**, *494*, 39-51.

[60] Hashimoto, M.; Nara, T.; Hirawake, H.; Morales, J.; Enomoto, M.; Mikoshiba, K. Antisense oligonucleotides targeting parasite inositol 1,4,5-trisphosphate receptor inhibits mammalian host cell invasion by Trypanosoma Cruzi. *Sci. Rep.,* **2014**, *4*, 4231.

[61] Hashimoto, M.; Morales, J.; Uemura, H.; Mikoshiba, K.; Nara, T. A Novel method for inducing amastigote-to-trypomastigote transformation *in vitro* in *trypanosoma cruzi* reveals the importance of inositol 1,4,5-trisphosphate receptor. *PLoS One,* **2015**, *10*(8), e0135726.

[62] Hashimoto, M.; Nara, T.; Mita, T.; Mikoshiba, K. Morpholino antisense oligo inhibits trans-splicing of pre-inositol 1,4,5-trisphosphate receptor mrna of *Trypanosoma Cruzi* and suppresses parasite growth and infectivity. *Parasitol. Int.,* **2015**, *65*(3), 175-179.

[63] Hashimoto, M.; Nara, T.; Enomoto, M.; Kurebayashi, N.; Yoshida, M.; Sakurai, T.; Mita, T.; Mikoshiba, K. A dominant negative form of inositol 1,4,5-trisphosphate receptor induces metacyclogenesis and increases mitochondrial density in *Trypanosoma Cruzi*. *Biochem. Biophys. Res. Commun.,* **2015**, *466*(3), 475-480.

[64] Yoshikawa, F.; Iwasaki, H.; Michikawa, T.; Furuichi, T.; Mikoshiba, K. Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor: structural and functional coupling of cleaved ligand binding and channel domains. *J. Biol. Chem.,* **1999**, *274*(1), 316-327.

[65] Douzery, E.J. P.; Snell, E. a; Bapteste, E.; Delsuc, F.; Philippe, H. The Timing of eukaryotic evolution: does a relaxed molecular clock reconcile proteins and fossils? *Proc. Natl. Acad. Sci. U. S. A.,* **2004**, *101*(43), 15386-15391.

[66] Parfrey, L.W.; Lahr, D.J. G.; Knoll, A.H.; Katz, L.A. Estimating the timing of early eukaryotic diversification with multigene molecular clocks. *Proc. Natl. Acad. Sci. USA,* **2011**, *108*(33), 13624-13629.

[67] Ricklefs, R.E.; Outlaw, D.C. A molecular clock for malaria parasites. *Science,* **2010**, *329*, 226-229.

[68] Aravind, L.; Iyer, L.M.; Wellems, T.E.; Miller, L.H. *Plasmodium* biology: genomic gleanings. *Cell,* **2003**, *115*(7), 771-785.

[69] Adl, S.M.; Simpson, A.G. B.; Lane, C.E.; Lukeš, J.; Bass, D.; Bowser, S.S.; Brown, M.W.; Burki, F.; Dunthorn, M.; Hampl, V.; Heiss, A.; Hoppenrath, M.; Lara, E.; Le Gall, L.; Lynn, D.H.; McManus, H.; Mitchell, E.A. D.; Mozley-Stanridge, S.E.; Parfrey, L.W.; Pawlowski, J.; Rueckert, S.; Shadwick, R.S.; Shadwick, L.; Schoch, C.L.; Smirnov, A.; Spiegel, F.W. The revised classification of eukaryotes. *J. Eukaryot. Microbiol.,* **2012**, *59*(5), 429-493.

[70] Krinke, O.; Novotná, Z.; Valentová, O.; Martinec, J. Inositol trisphosphate receptor in higher plants: is it real? *J. Exp. Bot.,* **2007**, *58*(3), 361-376.

[71] Wheeler, G.L.; Brownlee, C. Ca^{2+} signalling in plants and green algae - changing channels. *Trends Plant Sci.,* **2008**, *13*(9), 506-514.

[72] McNamara, C.W.; Lee, M.C. S.; Lim, C.S.; Lim, S.H.; Roland, J.; Nagle, A.; Simon, O.; Yeung, B.K. S.; Chatterjee, A.K.; McCormack, S.L.; Manary, M.J.; Zeeman, A.-M.; Dechering, K.J.; Kumar, T.R. S.; Henrich, P.P.; Gagaring, K.; Ibanez, M.; Kato, N.; Kuhen, K.L.; Fischli, C.; Rottmann, M.; Plouffe, D.M.; Bursulaya, B.; Meister, S.; Rameh, L.; Trappe, J.; Haasen, D.; Timmerman, M.; Sauerwein, R.W.; Suwanarusk, R.; Russell, B.; Renia, L.; Nosten, F.; Tully, D.C.; Kocken, C.H. M.; Glynne, R.J.; Bodenreider, C.; Fidock, D. A; Diagana, T.T.; Winzeler, E.A. Targeting *plasmodium* pi(4)k to eliminate malaria. *Nature,* **2013**, *504*(7479), 248-253.