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Environmental Sensing and Regulation of Motility in *Toxoplasma*

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Abstract

Toxoplasma and other apicomplexan parasites undergo a unique form of cellular locomotion referred to as 'gliding motility'. Gliding motility is crucial for parasite survival, as it powers tissue dissemination, host cell invasion and egress. Distinct environmental cues lead to activation of gliding motility and have become a prominent focus of recent investigation. Progress has been made toward understanding what environmental cues are sensed and how these signals are transduced in order to regulate the machinery and cellular events powering gliding motility. In this review we will discuss new findings and integrate these into our current understanding to propose a model of how environmental sensing is achieved to regulate gliding motility in *Toxoplasma*. Collectively, these findings also have implications for the understanding of gliding motility across Apicomplexa more broadly.

Plain English Summary

Toxoplasma parasites cause toxoplasmosis which can lead to blindness, neurological problems and birth defects. Sensing of environmental cues is of central importance in regulating infectivity in *Toxoplasma*. Recently there has been major advances in understanding the molecular events underpinning this process in *Toxoplasma*; new environmental signals have been revealed, the first understanding of how parasites sense these and how they are transduced to regulate infectivity. Here we review these findings,

integrating these into what we already understand and propose new questions that appear most important to understand.

Introduction

Toxoplasma is an obligate intracellular parasite from the phylum Apicomplexa. *Toxoplasma* causes toxoplasmosis, which can lead to congenital neurological birth defects, disease in immunocompromised patients and progressive blindness (Tenter *et al.*, 2000) . Due to its facile genetics and wide-ranging experimental techniques, *Toxoplasma* has been instrumental in understanding conserved processes governing pathogenesis across other apicomplexan species such as *Plasmodium* spp. (malaria), *Cryptosporidium* (severe diarrhoea), as well as a range of agriculturally important species.

A key pathogenic process governing disease and life cycle progression across all Apicomplexa is a form of cellular locomotion called gliding motility (Frenal *et al.*, 2017). Gliding motility is essential for tissue dissemination, invasion and host cell egress and is driven by a unique actomyosin motor complex termed the 'glideosome'. The glideosome powers gliding motility by linking to parasite transmembrane adhesins, which are released from microneme organelles and bind to host cell receptors. The current model suggests that activation of the glideosome results in adhesins being dragged rearward through the plane of the membrane, thus driving forward motion.

The regulation of adhesin release from micronemes and activity of the glideosome are inextricably linked to environmental cues to ensure appropriate activation and suppression of gliding motility. For some time, cytosolic Ca^{2+} signalling has been implicated in the activation of gliding motility (Lourido & Moreno, 2015), as has the sensing of environmental [K^+] as a likely marker of host cell breakdown (Moudy *et al.*, 2001). Over recent years, many gaps in this rudimentary understanding have been filled; new environmental cues that activate motility have been discovered, a role for cyclic nucleotide signalling has been unveiled and a putative mechanism for how *Toxoplasma* parasites sense environmental cues has been postulated. Furthermore, the first insight into how *Toxoplasma* parasites negatively regulate gliding motility after host cell invasion has been discovered.

Overall, these new findings have led to many new exciting questions about the exact nature of environmental sensing in the regulation of *Toxoplasma* motility. Here we will review this latest work and put it into context of previous data. We also pose new questions that now appear the most pressing to answer in our quest to understand how *Toxoplasma* and indeed, apicomplexan parasites more generally, tune into environmental cues to appropriately regulate gliding motility in space and time.

What are the environmental cues that *Toxoplasma* receives to regulate motility?

Extrinsic signals

Tachyzoites can sense environmental cues that signal extracellularity. Factors leading to those cues could be derived from death of the host cell, induced by innate immune mechanisms, or perhaps by mechanical forces imposed on the host cell by parasites growing within. For some time, it has been known that tachyzoites potently activate motility upon a drop in $[K^+]$, as would be encountered when the host cell membrane is breached (Moudy *et al.*, 2001)(Fig 1). Likewise, there is recent evidence to suggest that tachyzoites are also able to sense elevated levels of serum albumin (Brown *et al.*, 2016)(Fig 1). Indeed, these environmental conditions are often artificially applied to parasites to initiate downstream signalling, to either activate or supress motility. What is less clear, however, is whether tachyzoites can also sense upstream immune attack to exit host cells before they are destroyed (i.e., before the host plasma membrane is breached). Recent evidence suggests that tachyzoites can also sense a drop in pH (rise in $[H^+]$), as would be expected to happen during lysosomal attack (Fig 1). Interestingly, increasing $[H^+]$ is dominant over high $[K^+]$ in inducing motility, suggesting that this decreasing pH overrides the suppressive effects of high host cell $[K^+]$. What is less clear is whether this is relevant to lysosomal attack or whether acidification of the vacuole is parasite driven, acting as an 'internal clock' (see below) (Fig 1). It has been suggested that parasites are resistant to lysosomal attack, even after stimulation with IFN-γ in murine cells (Coppens *et al.*, 2006, Selleck *et al.*, 2015). More recently however, it was shown in human cells that *Toxoplasma* can be killed through this pathway and parasite death could be induced through acidification(Clough *et al.*, 2016). This suggests that, in human cells at least, lysosomal attack

and vacuolar acidification does occur and perhaps is an environmental cue that parasites use to exit host cells to avoid death.

Cell death inducers such as IFN-γ, TNF-α, Fas ligand and perforin have also been shown to mediate death in *Toxoplasma*-infected host cells, which is associated with premature egress of parasites (Niedelman *et al.*, 2013, Persson *et al.*, 2007, Yao *et al.*, 2017). Yet it is unclear whether egress is caused by cell dismantling and activation of motility due to exposure to low extracellular [K^+] environment, or whether parasites sense an upstream event before membrane permeabilization. The immune modulator nitric oxide (NO) can also trigger egress from infected cells without compromising parasite virulence (Ji *et al.*, 2013, Yan *et al.*, 2015). Interestingly, NO is a known activator of mammalian soluble guanylate cyclase (Horst & Marletta, 2018), raising the possibility that NO could signal through the *Toxoplasma* TgGC (see more below). However, this protein does not appear to contain a nitric oxide-interacting domain.

Internal clocks

An increase in [H^+] could also be parasite induced and may also be considered an intrinsic signal for spontaneous egress, acting as an internal clock dictating the timing of egress. This is because acidification of the parasitophorous vacuole (PV) has been observed late in replication, near the time of egress, as monitored by changes in the fluorescence of a pH sensitive GFP variant (Roiko *et al.*, 2014). Whether it is host cell or parasite induced, this decrease in pH just prior to egress seems to have two outcomes; It both stimulates microneme secretion whilst also increasing membrane binding and cytolytic activity of Perforin-Like Protein 1 (PLP1) (Roiko *et al.*, 2014) (Fig 1). This latter effect potentially points to a reason as to why [H^+] is used as an environmental cue. If vacuolar acidification is parasite induced, how does this take place? Are proton pumps on the vacuolar or parasite membrane used to drive this? How is vacuolar acidification sensed?

Another potential internal signal that dictates the timing of 'natural' egress has also recently emerged. It has been reported that *Toxoplasma* tachyzoites can respond to self-produced phosphatidic acid (PA) in the vacuolar space (Fig 1). The model here is that intracellular parasites temporally accumulate phosphatidic acid (PA) in the PV and when this reaches a certain level, natural egress is triggered (Bisio *et al.*, 2019). This was discovered by the finding

that deletion of a PV-localised Diacyl Glycerol Kinase 2 (DKG2) resulted in delayed egress from fibroblasts, and that tachyzoites secrete their micronemes in response to exogenously added extracellular PA (Bisio *et al.*, 2019).

This then begs the question; how do parasites sense PA? One possibility is that the newly discovered unusual guanylate cyclase (TgGC) senses this (discussed more below) or a more intriguing idea is that PA may be a conduit for sensing [H^+]. Interestingly, PA has the capacity to act as a [H^+] sensor, as it has a pKa at around physiological pH, which means that even relatively small changes in acidification will lead to changes in protonation state and hence changes in the strength of interaction with PA effector proteins (Shin & Loewen, 2011, Zegarlińska *et al.*, 2018). While it still does not answer how this would allow PA accumulation to act to induce motility, it does suggest that [H^+] and levels of this lipid could interact somehow to induce egress, perhaps by affecting association of signalling effectors at the membrane, but this clearly needs more investigation.

What also remains unknown is where PA is localised upon its synthesis by DGK2 during parasite growth. This is important to understand as it will provide insight into how this internal clock system operates. Whilst DGK2 appears to have a somewhat dispersed PV localisation, PA must be associated within a lipid environment. Two possibilities are that PA is associated with the tubulovesicular network (TVN) found in the vacuolar space of tachyzoites, or it is deposited in the outer leaflet of the parasite plasma membrane. PA is generally present only at low levels in the plasma membrane compared to other phospholipids, therefore its generation could lead to noticeable differences in membrane properties. Secondly, it has a cone shape structure and thus its polar headgroup is not tightly packed with neighbouring lipid headgroups in the membrane bilayer. This enables interaction of hydrophobic amino acids of PA effector proteins with the hydrophobic acyl layer of surrounding phospholipids. Thirdly, depending on environmental pH, PA can have a more negatively charged headgroup (-2) compared to other membrane phospholipids (-1), allowing for strong interaction with positively charged regions of effector proteins (properties of PA reviewed here (Shin & Loewen, 2011) (Zegarlińska *et al.*, 2018)). Thus, PA is highly suitable for modulating changes in the parasite's environment.

Another outstanding question is how important this PA-dependent mechanism of egress is during in vivo infection? Typically, fewer parasites reside inside host cells in vivo, as compared to inside the enormous vacuoles that are much more prevalent in large fibroblasts often used

to cultivate *Toxoplasma* in vitro. Macrophages, for example, are a known reservoir for *Toxoplasma* in mouse models, yet vacuoles typically only get to 4-cell stage before egress (Tomita *et al.*, 2009). Certainly, this question will be difficult to answer in vivo, but perhaps investigation of primary cells in vitro, including immune cells, may yield evidence of a similar mechanism of natural egress. Of course, this mechanism of PA-dependent egress may not apply at all to activated immune cells, which may trigger early egress through other mechanisms, as discussed above. To answer these questions and fully elucidate how accumulation of PA leads to microneme secretion and egress, sensitive methods to monitor PA signalling, particularly in real-time, would be of great benefit. In addition, more effort is needed to identify PA effector proteins. Some protein domains are known to interact with PA, for example, pleckstrin homology domains (PHD), but there are no obvious PA binding motifs common to all PA binding proteins.

Over a decade ago evidence emerged for a role of abscisic acid (ABA) in *Toxoplasma* egress. ABA is an important phytohormone with multiple functions in higher plants. Its biosynthesis branches off the isoprenoid biosynthetic pathway, which is also found in the relict plastid (apicoplast) of Apicomplexa. Nagamune et al demonstrated that ABA could induce microneme secretion in *Toxoplasma* (Nagamune et al., 2008*)*. They could detect this phytohormone by mass spectrometry in parasite lysate and furthermore, showed that the herbicide fluridone, a phytoene desaturase inhibitor, which leads to loss of ABA production, prevents *Toxoplasma* growth and toxoplasmosis in a mouse model. This work suggested that ABA is synthesized by the parasite and used as an internal clock for parasites to know when to egress from host cells. Since then, however, there has been little follow-up work in this area and no genes have been identified in any apicomplexan species that are required for the synthesis of ABA. Phytoene desaturase, the target of fluridone, for example does not appear to be found in any apicomplexan species, according to OrthoMCL and no convincing matches are found by BLAST against the multiple near complete genomes of *Toxoplasma*. Furthermore, in one of the only follow-up studies in the related protist *Perkinsus* the authors found no evidence for ABA (Sakamoto *et al.*, 2017). Whilst it is hard to prove a negative, it seems like the field needs unequivocal genetic evidence to move forward in this area. Perhaps the increasing ability to perform elaborate forward genetic screens in *Toxoplasma* can further illuminate the role, if any, of ABA in *Toxoplasma* environmental sensing.

Sensing environmental cues

Apicomplexan parasites are devoid of common receptor platforms such as G-Protein Coupled Receptors (GPCRs) and Receptor Tyrosine Kinases (RTKs) that are used in mammalian cells for sensing environmental signals. It has therefore remained a complete mystery how this group of parasites sense the environment. A recent flurry of work by several groups provides a potential answer to this problem. Here a very large and unusual guanylate cyclase (TgGC) was functionally characterized and found to be the most upstream factor required for sensing environmental cues (Fig 2). TgGC is an enormous protein of ~477 kD with around 22 predicted TM domains (TMDs) and is localized to the apical end of tachyzoites in a crescent shape (Bisio *et al.*, 2019, Brown & Sibley, 2018, Günay-Esiyok *et al.*, 2019, Yang *et al.*, 2019). The most interesting aspect of TgGC is that it contains multiple accessory domains, which could all potentially act to sense or transduce extracellular signals, ultimately resulting in_cGMP production (Bisio *et al.*, 2019, Brown & Sibley, 2018, Günay-Esiyok *et al.*, 2019, Yang *et al.*, 2019)(Fig 2).

Both conditional knockdown and knockout of TgGC demonstrate that this protein is very important for lytic stage growth. Parasites lacking TgGC are severely impaired in egress, invasion and motility and are unable to secrete micronemes under standard conditions, or after blocking cGMP degradation through inhibition of phosphodiesterases (PDEs). Interestingly, microneme secretion can be triggered when Ca^{2+} ionophore A23187 is used, suggesting that TgGC and cGMP signaling are upstream of Ca^{2+} signaling (Fig 2). Furthermore, it appears that in the absence of TgGC, addition of A23187 leads to considerably more microneme secretion, suggesting loss of a negative feedback loop that also requires this protein. Most interestingly, tachyzoites that lack TgGC are unable to respond to changes in extracellular $[K^+]$ or $[H^+]$, suggesting that this protein either directly senses these environmental cues, or at very least participates in this in some way (Fig 2).

The TgGC domain architecture and presence of accessory domains that could be involved in environmental sensing are shared across the alveolates (Baker *et al.*, 2017, Linder *et al.*, 2000). Interestingly, TgGC and mammalian adenylate cyclases (ACs) appear to belong to the same family of cyclases, based on primary amino acid sequence (Shenoy *et al.*, 2002), where only a single amino acid substitution is predicted to confer specificity to cGMP over cAMP (Glu2987). The N-Terminal sequence of TgGC harbors a range of accessory domains including a putative lipid-translocating ATPase, an E1-E2-like ATPase, a cation-transporting ATPase-like and a haloacid dehalogenase (HAD)-like hydrolase domain (Günay-Esiyok *et al.*, 2019,

Yang *et al.*, 2019). These domains are speculated to somehow sense changes in environmental ionic concentration.

One of the next major questions that needs to be addressed is the mechanism by which changes in ionic environment are sensed and what role TgGC plays in this? What seems important now is to apply membrane physiology methods to characterize the ATPase-like domains of TgGC. These methods could be used to measure whether the transporter-like domains actually transport K^+ or H^+ or rather just sense these ions. The fact that they are ATPase-like transporter domains suggests that they use energy and thus transport up the concentration gradient. In this light, it also appears pertinent to know the intracellular [K^+] and [H^+] so that this can be compared to the environmental concentrations that trigger motility. This will give us clues as to whether hyperpolarization or depolarization of the parasite membrane plays are role in activation (and suppression) of motility. If these techniques are combined with the facile genetics on offer with *Toxoplasma,* we will no doubt build up an understanding of the mechanism by which [K^+] and [H^+] activate motility. Indeed, this would provide an interesting paradigm in biology as most links between membrane transporters and environmental sensing operate in the reverse, ie. sensing of environmental cues leads to changes in membrane transporter activity, for example as found in neurons to mediate action potentials, or in plant guard cells to control respiration.

Natural egress triggered by intrinsic PA was shown to be dependent on TgGC (Bisio *et al.*, 2019)(Fig 2). This work is particularly interesting in light of the role of intracellular PA in microneme secretion. Both sequence analysis and involvement of TgGC in PA signaling has led to speculation that the P4-ATPase domain may act as a lipid flippase, resulting in increased concentrations of PA in the inner leaflet of the plasma membrane, which has been implicated in activation of microneme secretion and the glideosome (see below) (Bisio *et al.*, 2019, Günay-Esiyok *et al.*, 2019, Takada *et al.*, 2018, Yang *et al.*, 2019, Segawa *et al.*, 2018). However, direct evidence of PA flipping by TgGC is lacking. Experiments designed to detect flipping of fluorescent PA show only minimal activity compared to phosphatidylserine and no change in PA accumulation could be observed after TgGC depletion (Bisio *et al.*, 2019). As a point of interest, a general search of the biological literature reveals a distinct lack of experimental evidence of PA flipping, suggesting that it may be hard to detect for reasons unknown, but which may include flipping occurring at an extremely slow rate under the conditions employed. There is, however, circumstantial evidence for flipping by TgGC -

depletion of cofactor CDC50.1 (typically required for correct membrane trafficking and activity of flippases (Gomès *et al.*, 2000)) phenocopies DKG2 depletion. Also, TgGC has a catalytically important conserved aspartate residue (Asp782) which is known to be auto-phosphorylated in P-type ATPases during flipping events. Mutation of this residue impairs parasite survival, indicating that lipid flipping through TgGC is essential (Bisio *et al.*, 2019, Kühlbrandt, 2004). Using complementation of a conditional knockdown of TgGC, Brown et al. observed diminished microneme secretion after insertion of a point mutation within the catalytic ATPase domain, which was unable to restore the massive growth defect induced by the loss of the endogenous copy (Brown & Sibley, 2018). The exact localization of TgGC is somewhat unresolved and is important to determine if we wish to fully understand how this protein functions in environmental sensing. One study showed that in intracellular stages containing multiple parasites in one vacuole, TgGC localizes to the apical end in both N- and C-terminally tagged lines (Brown & Sibley, 2018), while another study observed an apical localization only when tagged to the C-terminus (Yang *et al.*, 2019). Extracellular parasites always showed apical staining, regardless of tag localization (Bisio *et al.*, 2019, Brown & Sibley, 2018, Yang *et al.*, 2019). Whilst reasons for differences in the observed localisation are unknown, this may be influenced by swapping of promoters when making transgenic lines, which has previously been seen to affect protein localisation in *Toxoplasma* (Jacot *et al.*, 2016, Daher *et al.*, 2010). Furthermore, how a multi-pass TMD protein could have such a disperse cytoplasmic localisation is certainly puzzling. Which membrane TgGC resides in is also an important but unresolved question. To be directly involved in environmental sensing of ions, TgGC would be expected to be on the PM exposed to the extracellular milieu. Whilst this is most likely the case, it has not been formally proven.

Whether or not TgGC is proteolytically cleaved is another important point of discussion. Western blotting of TgGC displays several molecular weight species that vary depending on which end is being detected. The full-length form (>460 kD) is detected regardless of which end the protein tag is found (Brown & Sibley, 2018, Yang *et al.*, 2019), indicating that at least some proportion of TgGC has the N- and C-termini connected. Mapping of putative proteolytic fragments performed by Yang et al. suggests that the detected species are cleaved at sites that would disable protein function in both the ATPase and GC domains (Yang *et al.*, 2019). Colocalization of C- and N-terminal tags via IFA by Brown et al, suggests that the processed forms are likely artifacts resulting from sample preparation, despite every attempt by

researchers to avoid this. This notion is further backed up by functional data suggesting that both C- and N-terminal activities are dependent on an intact protein (Brown & Sibley, 2018).

Direct or indirect sensing of environmental cues by TgGC likely results in the generation of cGMP (Fig 2). In mammalian cells levels of cGMP are dictated by the balance of its synthesis through GCs and degradation by cGMP phosphodiesterases (PDEs). Whilst there is yet to be any functional analysis of the 18 PDEs predicted to be encoded in the *Toxoplasma* genome, zaprinast, a compound that blocks mammalian PDE5 and PDE6 (Gibson, 2001, Nakamizo *et al.*, 2003), has some activity against *Toxoplasma* at high concentrations and induces egress. A more potent apicomplexan-specific small molecule PDE inhibitor 5-benzyl-3-isopropyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (BIPPO) (Howard *et al.*, 2015), also induces egress and motility by stimulating microneme secretion (Fig 2). The use of these and other small molecules combined with genetic mutants, strongly suggests that cGMP signaling occurs upstream of Ca^{2+} release and subsequent activation of motility (see more below) (Fig 2).

Cyclic nucleotide phosphodiesterases (PDEs).

What is clearly missing in our understanding of regulation of gliding motility is the function of each of the PDE orthologues, especially those that are expressed in tachyzoites. A good place to start would be to consider those genes with low CRISPR fitness scores on ToxoDB (Sidik *et al.*, 2016b). These are thought to be essential given what is currently understood about cGMP (and cAMP) in tachyzoites. It is noteworthy that ten of the PDE genes have negative CRISPR scores, with four genes showing scores of less than -1.8, indicative of their importance to *Toxoplasma* survival. Determining localization of different PDEs will also be important as they can function as regulators of different cyclic nucleotide pools within the cell.

It will also be important to understand the specificity and selectivity of the *Toxoplasma* PDEs towards cGMP and cAMP. It is possible that some of these PDEs can degrade both cyclic nucleotides, as is the case in mammalian cells, and are involved in crosstalk between cGMP and cAMP signalling pathways. Understanding the cyclic nucleotide specificity of individual PDEs may shed light on this crosstalk.

Cyclic Nucleotide dynamics.

The field also currently lacks understanding of the spatial and temporal dynamics of cGMP. Whilst several groups have wrestled with the use of ELISA-based kits and metabolomic

approaches to follow cyclic nucleotide levels, this continues to be an unreliable method and can only measure cGMP at a population level. Whilst many groups have used Ca^{2+} biosensors in *Toxoplasma* to understand signaling at single cell resolution (Stewart *et al.*, 2017, Sidik *et al.*, 2016a, Borges-Pereira *et al.*, 2015, Brown *et al.*, 2016), no one yet has had success at using the several available cGMP biosensors. This would no doubt give a much better understanding of the temporal dynamics of cGMP and this could be further enhanced by targeting sensors to different cellular compartments. There are also biosensors for cAMP and these too could be instrumental in understanding signaling in *Toxoplasma*.

Protein kinase G (PKG) signalling.

The only known cGMP effector in *Toxoplasma,* or indeed any other apicomplexan parasite, is TgPKG (Fig 2). *Toxoplasma* appears to have two isoforms of TgPKG, one cytosolic and the other associated with the plasma membrane via N-terminal myristoylation and palmitoylation, which likely arise from the use of 2 different start codons. However, functional analysis suggests that only the membrane bound (i.e. full length) version is functionally important in tachyzoites (Brown *et al.*, 2017, Donald & Liberator, 2002). Understanding the function of TgPKG has been greatly aided by the PKG inhibitor pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl]pyridine (Compound 1), the affinity of which can be ablated using a single point mutation in the 'Gatekeeper' position of TgPKG (Donald & Liberator, 2002). These studies have demonstrated that depleting/inhibiting TgPKG ablates egress, motility and subsequent invasion, likely as a result of defective microneme secretion (Donald & Liberator, 2002). More recently a conditional knockdown of TgPKG has been generated and shows a similar phenotype (Brown *et al.*, 2017). Since compound 1 also inhibits CDPK1(Donald *et al.*, 2006), which acts downstream of PKG, care must be taken in assigning phenotypic changes observed upon addition of compound 1 to PKG, as some phenotypes attributed to PKG may be dependent in part to inhibition of CDPK1.

What is becoming increasingly clear is that cGMP signalling through TgPKG is required for the initiation of Ca^{2+} signalling (Brown *et al.*, 2017, Yang *et al.*, 2019, Stewart *et al.*, 2017) (Fig 2). This comes from the observation that treatment of parasites with the PDE inhibitor BIPPO, which is thought to increase cGMP levels thereby activating TgPKG, (Yang *et al.*, 2019) leads to amplification of Ca^{2+} flux prior to egress (Fig 2). This does not occur when TgPKG is inhibited by treatment of parasites with Compound 1 (Brown *et al.*, 2017, Stewart *et al.*, 2017, Yang *et al.*, 2019). The molecular events linking cGMP and Ca^{2+} signalling, are

however, completely unknown in *Toxoplasma.* One possible mechanism of how PKG initiates Ca^{2+} release into the cytoplasm is via phosphoinositide signalling. Whilst this is yet to be explored in *Toxoplasma* PKG is required for the phosphorylation of putative phosphoinositide signalling proteins in *Plasmodium* (Brochet *et al.*, 2014) (Fig 2). Drawing on knowledge from mammalian cells this could activate the production of inositol1,4,5 triphosphate (IP_3), which then augments Ca^{2+} release in the ER by the IP_3-receptor. Much is to be gained in this space by identifying the substrates of TgPKG. Here newly developed 'substrate capture' approaches may be useful (Lourido *et al.*, 2013, Rothenberg *et al.*, 2016) or looking for PKG interacting proteins.

There is also some potential redundancy in cGMP and Ca^{2+} signalling. Both TgPKG and Ca^{2+}-Dependent Protein Kinase 3 (CDPK3) are localized to the parasite plasma membrane by dual acylation and both have a role in Ca^{2+} dependent (ionophore induced) egress (Brown *et al.*, 2017, McCoy *et al.*, 2017)(Fig 2). It has been reported that loss of CDPK3 can be overcome by treatment with the mammalian PDE inhibitor zaprinast, suggesting similar mechanisms of action (Lourido *et al.*, 2012). However, the use of BIPPO only delays egress in ΔCDPK3 tachyzoites (Stewart *et al.*, 2017), thereby suggesting that CDPK3 just acts to amplify the signalling event required for egress. Whilst these results may appear contradictory, these differences may be due to differential PDE targeting by BIPPO and zaprinast (where inhibition of some PDEs allow PKG to completely complement the role of CDPK3, while inhibition of other PDEs allow only partial complementation by PKG), or these differences could be due to the time points used in each study, or even be a consequence of signalling being artificially induced by use of PDE inhibitors (Lourido *et al.*, 2012, Stewart *et al.*, 2017).

PI-PLC and PA sensing

In mammalian and yeast systems, phosphoinositide (PI) signalling is central to signal transduction upon changes in environment. Typically, GPCRs activate Phosphoinositide-Phospholipase C (PI-PLC) which cleaves PIP_2 into diacylglycerol (DAG) and inositol triphosphate (IP_3) second messengers. This then results in IP_3 opening up Ca^{2+} channels in the ER, causing a rapid rise in cytosolic [Ca^{2+}] (Berridge, 2009). DAG can be further converted into PA, but also activates protein kinase C (Newton, 2018), which has numerous downstream targets. However, PKC appears to be absent in *Toxoplasma*, as is the other DAG-producing enzyme phospholipase D. To date there have been few studies focussed on understanding PI

signalling in apicomplexan parasites, so clearly this is an area that requires greater attention, but based on a study with *Plasmodium* parasites, it has been suggested that cGMP signalling stimulates downstream intracellular Ca^{2+} flux following activation of PI signalling (Brochet *et al.*, 2014). It is thought that phosphoinositol-4-kinase (PI4K) and subsequent PI4P5K-driven reactions culminate in the conversion of PI into $PI(4,5)P_2$, which is the substrate for Phosphoinositide Phospholipase C (PI-PLC) to produce DAG and IP_3 (Fig 2). Whilst the biochemical activity and exact function of most of these enzymes remains a mystery in *Toxoplasma,* recent work suggests that PI-PLC plays an important role in intracellular growth, but its role is yet to be established during microneme secretion and motility (Bullen *et al.*, 2016). PI-PLC-produced DAG leads to accumulation of phosphatidic acid (PA) at the plasma membrane through the action of membrane-associated diacylglycerol kinase 1 (DGK1) (Fig 2). While conditional knockout of DGK1 blocks microneme secretion, inhibition of the PA phosphatase (PAP) leads to an accumulation of PA, inducing microneme secretion (Bullen *et al.*, 2016). PA is suggested to bind to an acylated PH domain-containing protein found on the surface of micronemes (APH), which is proposed to induce microneme-plasma membrane fusion in a manner that also involves the Ca^{2+} binding protein DOC2.1, to allow for exocytosis (Farrell *et al.*, 2012, Bullen *et al.*, 2016) (Fig 2). These findings imply that a build-up of PA is required for microneme secretion, which must therefore occur in a regulated manner. The only evidence so far connecting PA lipid levels to cGMP signalling is a decrease in PA levels observed by lipidomic profiling following genetic depletion of TgGC (Yang *et al.*, 2019).

Interestingly, the recently identified Glideosome Associated Connector (GAC), which likely connects actin filaments and the cytoplasmic tails of adhesins, also has a PA binding domain that provides plasma membrane affinity and represents another potential mechanism of regulation of glideosome function (Jacot *et al.*, 2016). This elegantly makes PA a central nexus in the regulation of gliding motility, controlling both microneme secretion and glideosome activity. Several pressing questions have now arisen; What is the enzyme(s) that- synthesises DAG, the substrate of DGK1, and how is it regulated? Does PA accumulate in localised areas to promote microneme secretion and glideosome activity? Clearly being able to monitor the production and degradation of inositol lipid species is important here, as is the biochemical characterisation and analysis of genetic mutants. Furthermore, to get a spatial and temporal understanding of inositol signalling, it would be illuminating to use inositol lipid binding domain sensors. Another outstanding and important question is whether there is a link between

synthesis of PA by DGK2 in the vacuolar space (as described above) and accumulation of PA in the inner leaflet of the parasite plasma membrane? This would require a PA flippase, which is yet to be identified, but may be present in the N-terminal domain of TgGC (as discussed above). This could indeed be the mechanism through which PA is used as an internal clock and is utilized to activate motility.

Ca^{2+} signalling

In mammalian systems, the other product of PI-PLC activity, IP_3, stimulates Ca^{2+} secretion (Fig 2). There is some evidence to suggest that both PI-PLC and IP_3 are also involved in Ca^{2+} mobilization and activation of motility in *Toxoplasma*, but there is still much to be learned. Cytosolic [Ca^{2+}] is intimately involved in motility, egress and invasion, and recently more details on how this operates to regulate motility have been revealed. Several groups have utilized genetically encoded Ca^{2+} biosensors with interesting findings (Borges-Pereira *et al.*, 2015, Brown *et al.*, 2016, Sidik *et al.*, 2016a, Stewart *et al.*, 2017). Ca^{2+} oscillations occur just before egress and invasion (Lovett & Sibley, 2003, Stewart *et al.*, 2017, Wetzel *et al.*, 2004, Singh *et al.*, 2010) and are essential for microneme release and motility (Lovett & Sibley, 2003, Stewart *et al.*, 2017, Wetzel *et al.*, 2004), but what is not yet determined is the source of intracellular Ca^{2+} and how it is mobilized. There are several excellent reviews in this area and in the interests of space we will not repeat this here (Billker *et al.*, 2009, Lourido & Moreno, 2015). Suffice to say that, apart from intracellular stores, extracellular Ca^{2+} has also been shown to play a role, likely by cell entry through L-type channels (Pace *et al.*, 2014). There are no obvious genes for many known Ca^{2+} channels or IP_3 receptors that normally release Ca^{2+} from the ER (as reviewed by (Hortua Triana *et al.*, 2018)). It may be that IP_3 receptors and other Ca^{2+} channels are present in *Toxoplasma*, but their primary sequence is sufficiently diverged so that they cannot be found though standard genomic searches. Indeed, there is a recent report of a PKG binding protein in *Plasmodium* spp (named ICM1), which has little primary sequence homology but with some structural homology to the Ca^{2+} channels (Aurélia C. Balestra, 2020). This same study shows that this protein is required for the initiation of Ca^{2+} signalling, adding further weight to this being the long sought-after ER Ca^{2+} channel. Interestingly, homology searches show a clear orthologue of this protein in *Toxoplasma*, which according to the LOPIT cellular localisation data maps to the ER (Barylyuk *et al.*, 2020). This clearly should be followed up in conjunction with organelle specific Ca^{2+} biosensors that differ in their Ca^{2+} affinity, which are now available and may be useful in answering questions around how Ca^{2+} is stored in *Toxoplasma*. One interesting point to mention; if the ICM1 orthologue in

Toxoplasma is localised in the ER then it is unlikely a PKG interacting protein as reported in *Plasmodium*. This is because the only functionally important pool of PKG has been reported to be at the plasmamembrane thus physically separated from the ICM1 orthologue. Regardless, it will be interesting for the function of ICM1 to be followed up in *Toxoplasma* and whether it is regulated by PKG, directly or indirectly.

The Ca^{2+}-Dependent Protein Kinases (CDPKs).

An important consequence of the mobilisation of intracellular Ca^{2+} is the activation of Ca^{2+}-dependent protein kinases (CDPKs) and a Ca^{2+}-dependent phosphatase – Calcineurin A (CnA). There is not much literature on CnA but current data suggests it is involved in host cell attachment, potentially through by regulating phosphorylation of cytoplasmic tails of adhesins (Paul *et al.*, 2015). CDPKs on the other hand are much better understood, being found across Apicomplexa and related protists as well as plants, but not metazoans (including humans), making them interesting drug targets (Hui *et al.*, 2015). CDPKs are a fusion between CaM-like domain and a kinase domain and are regulated by a conformational change upon binding of Ca^{2+}, leading to rotation of the CaM domain, thereby freeing the active pocket for phosphorylation of substrates. There are 12 CDPKs in *Toxoplasma,* and the functions of some of them have emerged over the last decade. The role of many of the atypical CDPKs (containing an uneven number of EF hands for binding Ca^{2+}) is not clear when using genetic deletion mutants in the asexual cycle (Long *et al.*, 2016), possibly because some have redundant functions or roles in other lifecycle stages.

Interestingly, several members have cellular functions other than gliding motility; CDPK7 and CDPK2 have roles in cell division and regulation of amylopectin metabolism, respectively (Morlon-Guyot *et al.*, 2014, Uboldi *et al.*, 2015) suggesting that Ca^{2+} regulates more during growth than just motility. CDPK1 is required for microneme secretion and thus egress, tissue dissemination and invasion (Lourido *et al.*, 2010). Intriguingly, CDPK3 has a role confined to egress (Garrison *et al.*, 2012, Lourido *et al.*, 2012, McCoy *et al.*, 2012) (Fig 2). What remains unknown is how these CDPKs promote egress and motility. Typically, phosphorylation will change a target protein's conformation, localisation or ability to interact with other proteins, and this is how CDPKs are thought to activate motility. Several studies using quantitative phosphoproteomics and substrate capture methodologies have generated lists of candidates for Ca^{2+}-dependent CDPK phosphorylation (Lourido *et al.*, 2013, McCoy *et al.*, 2017, Treeck *et al.*, 2014). In one such study, uusing a combination of substrate capture and ^{32}P labelling,

Lourido et al provided evidence that CDPK1, which is required for microneme secretion, phosphorylates Dynamin related protein B (DrpB) (Lourido *et al.*, 2013). DrpB has previously been shown to be involved in microneme and rhoptry organelle biogenesis (Breinich *et al.*, 2009), yet CDPK1 deficient parasites are capable of forming these organelles (Lourido *et al.*, 2010). Instead, given that dynamins are typically involved in endocytosis, the authors speculate that DrpB might also be involved in membrane dynamics or the size of fusion pores during microneme secretion (Lourido *et al.*, 2013). It will indeed be interesting to see if phospho-null and mimetic mutants can find a role for CDPK1-dependent DrpB phosphorylation in microneme secretion genesis. What the field really lacks is an understanding on the functional importance of specific phosphorylation events during motility. One study functionally assessed a putative CDPK3 substrate Suppressor of Ca^{2+}-dependent egress (SCE1) (McCoy *et al.*, 2017) (glideosome phosphorylation is addressed below). This protein acts as a suppressor of egress, but its role in this process is only clear in the absence of CDPK3. It is known, however, that CDPK3-dependent phosphorylation does regulate its function by relieving its suppressive activity. The precise role of SCE1 in Ca^{2+} signalling and egress pathways requires further investigation. Only a few candidates for CDPK-dependent phosphorylation identified in global screens have been pursued further. In general, more effort needs to be made to characterize these proteins phenotypically, particularly with phospho-mimetic and phospho-null mutations of phenotypically variable phosphorylation sites, bearing in mind that some effects of phosphorylation may be subtle or unanticipated. In addition, substrate capture techniques need to be improved to give less non-specific hits that are also spatially confined by using intact parasites instead of cell lysates (Rothenberg *et al.*, 2016).

Regulation of the glideosome

Tissue dissemination, invasion and egress all require the activation of the glideosome, an actomyosin-dependent motor that provides the force for motility (Fig 2). Spikes of Ca^{2+} flux coincide with bursts of motility and it has been a long-held hypothesis that the motor is regulated by signalling through this axis (Lovett & Sibley, 2003, Stewart *et al.*, 2017). Ca^{2+} seems to be involved in the activation of the actomyosin motor via stabilization of the interface between MLC1 and ELCs (Lovett & Sibley, 2003, Nebl *et al.*, 2011, Stewart *et al.*, 2017, Williams *et al.*, 2015, Pazicky *et al.*, 2019). However, it remains unclear if this is a mechanism of regulation (Bookwalter *et al.*, 2017, Williams *et al.*, 2015). Several components of the actomyosin motor complex are phosphorylated in a Ca^{2+}-dependent manner (Nebl *et al.*, 2011) and in vitro evidence suggests that TgCDPK3 (Gaji *et al.*, 2015) and its orthologue in *P.*

falciparum, PfCDPK1 (Green *et al.*, 2008), (Gaji *et al.*, 2015) (Gaji *et al.*, 2015) are responsible for some of these phosphorylation events. Interestingly, mutants of these sites show only mild effects on levels of egress and motility, suggesting that the function of these phosphorylation sites is not the only mechanism regulating glideosome activity. Phosphomutants of MyoA on sites phosphorylated by TgCDPK3 (*in vitro*), and addition of the compound 130038, (which stimulates Ca^{2+} signalling) lead to delays in egress rather than complete ablation. On the other hand, phosphomutants of MLC1 or GAP45 did not impact on assembly or function of the actomyosin motor (Jacot *et al.*, 2014). This latter study, however, did not assess MyoA phosphosites. It may be that the cumulative effects of all phosphorylation events in the glideosome complex need to be considered simultaneously, but given the large number identified, this would be a complicated experiment.

It is also worth pointing out that it is unlikely that phosphorylation of the glideosome is the only mechanism regulating motility. Recent work has shown that GAC – the connector between actin filaments and adhesins, is held in an apical localisation when parasites are non-motile (Jacot *et al.*, 2016). Activation of motility leads to GAC being released from this location and translocated to the basal end of parasites. This implies that a signalling event leads to the release of GAC from this complex for subsequent association with adhesins released from micronemes on one side and newly synthesised actin filaments on the other. Interestingly, Formin1, a protein that likely nucleates the production of F-actin used by the motor to drive motility, is also found in this same apical localisation (Jacot *et al.*, 2016). This suggests that this apical punctum could be a central regulatory hub that controls motility. Indeed, an apical methyl transferase (AKMT) also co-localises with GAC and Formin1 (Heaslip *et al.*, 2011). AKMT is required for GACs apical localisation and activation of motility (Jacot *et al.*, 2016, Tosetti *et al.*, 2019, Heaslip *et al.*, 2011), suggesting that methylation is also a potential regulatory mechanism controlling motility. However, how AKMT and GAC regulation might be linked to Ca^{2+}, cGMP and phosphorylation events, let alone to environmental signals, remains completely unexplored.

Protein Kinase A (PKA) and negative regulation of motility

PKA is classically understood as a heterotetrametric holoenzyme comprised of two regulatory subunits and two catalytic subunits, which associates with several cellular localisations using

'A-kinase associated proteins' (AKAPs) and is often tied in with cellular receptors such as GPCRs and adenyl cyclases (ACs), that produce cyclic AMP (cAMP) to relay the environmental signals these proteins receive. Following production of cAMP by ACs, each regulatory subunit can bind two cAMP molecules, which induces a conformational change in the complex, leading to dissociation of the two catalytic subunits and allowing for phosphorylation of downstream substrates.

The *Toxoplasma* genome encodes four putative adenylyl cyclases, which show redundancy in function based on genetic ablation studies (Mueller *et al.*, 2013, Jia *et al.*, 2017) (Brown & Sibley, 2018). (Jia *et al.*, 2017)Of the PKA-encoding genes in *Toxoplasma,* one PKA regulatory subunit (PKAr1; TGME49_242070) and three catalytic subunits (PKAc1-3; TGME49_226030, TGME49_228420, and TGME49_286470) have been identified, however at least two other putative regulatory subunits exist in the genome (TGME49_311300 and TGME49_ 219070) (Jia *et al.*, 2017, Kurokawa *et al.*, 2011, Sugi *et al.*, 2016, Uboldi *et al.*, 2018) The *Toxoplasma* PKA holoenzyme likely comprises both subunits in a one-to-one ratio as PKAr lacks the N-terminal dimerization domain found in most eukaryotes, which provides an anchor point to AKAPs in bigger protein complexes (Kurokawa *et al.*, 2011, Haste *et al.*, 2012). In *Toxoplasma*, PKAc1 and PKAc3 are both expressed in the tachyzoite and bradyzoite stages, while PKAc2 expression is highest during the sexual cycle in felids (Sugi *et al.*, 2016). PKAc1 and PKAc2 both localise to the IMC via binding to the regulatory subunit, which is sequestered to the IMC membrane by myristoylation and palmitoylation (Jia *et al.*, 2017, Uboldi *et al.*, 2015) (Uboldi *et al.*, 2018) (Fig 2). Conversely, PKAc3 is cytosolic, indicating the potential importance of spatially regulating cAMP signalling in the parasite (Sugi *et al.*, 2016). PKAc1 is the most extensively studied of the three catalytic subunits. Overexpression of PKAc1 or genetic depletion of PKAr showed defects in intracellular growth (Jia *et al.*, 2017). However, the most interesting finding relating to PKAc1 function is the 'premature egress' and 'restless invasion' phenotypes observed following PKAc1 depletion or dominant negative expression of PKAr. Here PKAc1-deficient parasites that invade continue to be motile, demonstrating successive rounds of premature egress followed by invasion, destroying host cells in the process (Jia *et al.*, 2017, Uboldi *et al.*, 2015)Uboldi *et al.*, 2018). Dual knockdown of the upstream adenylyl cyclases TgACα1 and TgACβ also results in this phenotype (Jia *et al.*, 2017). This premature egress phenotype can be partially rescued by blocking vacuolar acidification as well as by genetic deletion of DGK2 or PLP1, the latter which relies on PV acidification for activation in order to mediate egress (Bisio *et al.*, 2019, Jia *et al.*, 2017, Uboldi

et al., 2015)Uboldi *et al.*, 2018). Artificial vacuole acidification following permeabilization with digitonin can still trigger egress in DGK2 knockout parasites, suggesting that natural egress is likely mediated by both DGK activity -and PV acidification (Bisio *et al.*, 2019). PKAc1 likely acts as a 'handbrake,' preventing DGK2-dependent and/or acidification-dependent premature egress under normal conditions (Fig 2).

It has been known for some time that cytosolic Ca^{2+} levels dampen very quickly after invasion (Lovett & Sibley, 2003) and these recent studies also highlighted an important role for PKAc1 in regulating cytosolic Ca^{2+} levels, as parasites showed an inability to rapidly dampen Ca^{2+} levels following invasion – a necessary event for motile invasive parasites to switch to the replicative stage inside the cell (Uboldi *et al.*, 2018), despite being able to undergo invasion normally. This work is an important milestone in understanding regulation of motility as it is the first description of a likely mechanism which allows gliding motility to be switched off after invasion is complete. It also suggests that cAMP is somehow implicated in sensing environmental cues to dampen motility upon completion of invasion. What now seems pertinent to understand is how PKAc1 and cAMP tie in with environmental sensing. What we do know is that loss of PKAc1 causes an inability to regulate levels of cytosolic [Ca^{2+}] upon increasing concentrations of extracellular [Ca^{2+}] (Uboldi *et al.*, 2018). Furthermore, PKAc1-deficient parasites have abnormally high levels of cytosolic [Ca^{2+}] in buffers containing high and low concentrations of K^+. The most likely explanation is that changes in environmental cues (ie [K^+]) upon invasion of a host cell activates cAMP production, which activates PKAc1, which then represses Ca^{2+} signalling, causing parasites to inhibit motility and begin the processes of replication. Is there a separate membrane transducer that kickstarts this process or is TgGC somehow involved in this process too, such that the suppression of cGMP levels causea rise in cAMP in a reciprocal relationship (discussed more below)(Fig 1)?

Interestingly, the function of PKA in blood stage *P. falciparum* appears to be somewhat different. PfPKAc depletion appears to have no effect on intracellular Ca^{2+} levels (Patel *et al.*, 2019) and instead, a major function is phosphorylation of the short cytoplasmic tail of AMA1. PfPKAc is responsible for phosphorylating AMA1 at Ser610, an event which is crucial for successful invasion (Leykauf *et al.*, 2010, Patel *et al.*, 2019, Prinz *et al.*, 2016, Wilde *et al.*, 2019). This phosphorylation site is not conserved in *Toxoplasma* and it remains to be seen if phosphorylation of another residue in the TgAMA1 cytoplasmic domain is required for

invasion. These findings highlight a key difference between *Toxoplasma* and *Plasmodium* PKAc function.

It is interesting to note the similarities between the restless invasion phenotype of *Toxoplasma* PKAc1 mutants and cell-traversing *Plasmodium* sporozoites. CDPK6 and recognition of surface proteoglycans have been implicated in the transition by *Plasmodium* sporozoites from traversal to productive invasion of hepatocytes, but this is generally an understudied area (Coppi *et al.*, 2007). It is possible that cAMP and PKA are also somehow involved in regulating sporozoite traversal and the transition to productive invasion of hepatocytes. *Toxoplasma* sporozoites too can traverse cells (Tartarelli *et al.*, 2020), which puts forward the notion that PKA and cAMP regulate this process also. We hypothesise that sporozoites may require low cAMP levels as they traverse cells, enabling PLP1 to remain active following host cell invasion through the maintenance of an acidic vacuolar environment. Upon receiving appropriate signals, perhaps those that are specific for hepatocytes and not those that sporozoites transverse through, cAMP levels then rise and allow for the switch to productive invasion of a host cell (Fig 1). It is however unlikely to be this simple as sporozoites also seem to be on a molecular timer, whereby even when applied to hepatocytes they all undergo traversal for some time before undergoing productive invasion. It is also important to highlight that sporozoites also do not release certain rhoptry markers during traversal and this must also be regulated by some internal signalling event (Risco-Castillo *et al.*, 2015).

Recent work has also suggested that cAMP has a role in negatively regulating *Toxoplasma* bradyzoite differentiation, suggesting that this cyclic nucleotide may play a central role in stage transition. PKAc3 has been shown to be important for suppressing bradyzoite differentiation, as knockout leads to increased conversion rates into these latent forms (Sugi *et al.*, 2016). Conversely, treatment of parasites with the PDE inhibitor 3-isobutyl-1methylxanthene, represses bradyzoite differentiation (Eaton *et al.*, 2006, Sugi *et al.*, 2016). These data, together with the role of PKAc1 in negative regulation of egress and motility, leads to the hypothesis that high levels of cAMP mark a state of intracellular acute stage replication, suppression of Ca^{2+} signalling and bradyzoite differentiation (Fig 1). Upon environmental signals, including possible danger signals, [cAMP] drops and parasites either differentiate into bradyzoites or undergo egress. This interesting idea would put cAMP at a critical decision point for parasite developmental transition and thus warrants further investigation. There are other outstanding questions; Do ACs become activated to produce cAMP under certain environmental

conditions? Which proteins allow for relay of these signals? Are levels of cAMP higher in intracellular replicating tachyzoites as compared with extracellular motile forms or parasites differentiating into bradyzoites? Clearly, monitoring levels of cAMP would be required here. Perhaps an option would be the use of FRET sensors that have been established in mammalian cells. Furthermore, for us to understand how cAMP functions, we need to identify the proteins regulated by cAMP through PKA-dependent phosphorylation. Whilst PKAc1-dependent phosphorylation sites have been identified, the complexity of downstream signalling and cross-talk (see below) makes it very hard to deconvolve which substrates are directly phosphorylated by PKAc1 versus those that are phosphorylated downstream by other kinases. Our lab has attempted to make a PKAc1 gatekeeper mutant to accept ATP analogues and use chemistry to pull our direct substrates, but the enzymatic activity of this variant is very low, making this experimental setup challenging.

Crosstalk between Ca^{2+}, cGMP and cAMP

As discussed above, cGMP and cAMP appear to have opposing regulatory effects on intracellular Ca^{2+} levels in *Toxoplasma*. cGMP signalling leads to increased intracellular [Ca^{2+}], which is important for microneme secretion, motility, invasion and egress. Conversely, cAMP likely plays an important role in negatively regulating the Ca^{2+} signal following invasion through PKAc1. The major question that remains to be tackled is how do cAMP and cGMP signals interact, and potentially regulate each other? Previous literature hints at the interaction between these two signalling pathways. For example, premature egress by PKAc1-depleted parasites is blocked following PKG inhibition with Compound 1, and phosphoproteomic studies indicate that PKA may be required for phosphorylation of a putative cGMP-specific PDE, although direct phosphorylation of this PDE by PKA has not been demonstrated (Jia *et al.*, 2017). These findings suggest that premature egress induced by host cell acidification is PKG dependent, and PKAc1 may work to suppress this via regulation of cGMP levels, whether directly or indirectly, by acting on TgGC, or PDEs.

Previous studies have looked extensively at the relationship between cAMP and cGMP in regulation of cardiac contractility, revealing this cross-talk is primarily mediated by PDEs, whereby each cyclic nucleotide can either stimulate or inhibit the hydrolysis rate of the other, depending on its abundance (Zhao *et al.*, 2017). Differential expression and localisation of PDEs with single or dual specificity for cAMP and/or cGMP is likely important in mediating the co-regulation of these two cyclic nucleotides and enabling fine tuning of cAMP and cGMP

levels to coordinate invasion, motility, egress and bradyzoite differentiation. As discussed above, the functional characterisation of the 18 PDEs in *Toxoplasma* will be paramount to uncover interaction dynamics between cyclic nucleotides.

Concluding remarks

Recent years has seen significant advances in our understanding of regulatory signalling proteins and pathways involved in initiation of tachyzoite motility and egress. We have gained an appreciation of the complex interplay between Ca^{2+}, cGMP and cAMP signalling, the role acidification plays in initiating motility and egress, and the surprising discovery of an internal clock governing the timing of egress that is dependent on intravacuolar accumulation of PA. The fine details of these processes await future investigation. The characterization of TgGC means that a likely candidate for the transduction of extracellular signals into downstream cellular signalling events has been identified and further attention will be given to understanding how extracellular cues are transduced into downstream signals by this protein. There are still some sizeable gaps in our knowledge of the activation of motility to be deciphered, particularly the roles that PI signalling and the many PDEs have in this process. Furthermore, what is the mechanism by which changes in ionic composition are sensed? It will also be fascinating to see whether there is a conserved role for cAMP/PKA signalling across the Apicomplexa and whether it truly does have a role in switching Plasmodium sporozoites from cell traversal to productive invasion. We look forward to the coming years to see these new insights emerge.

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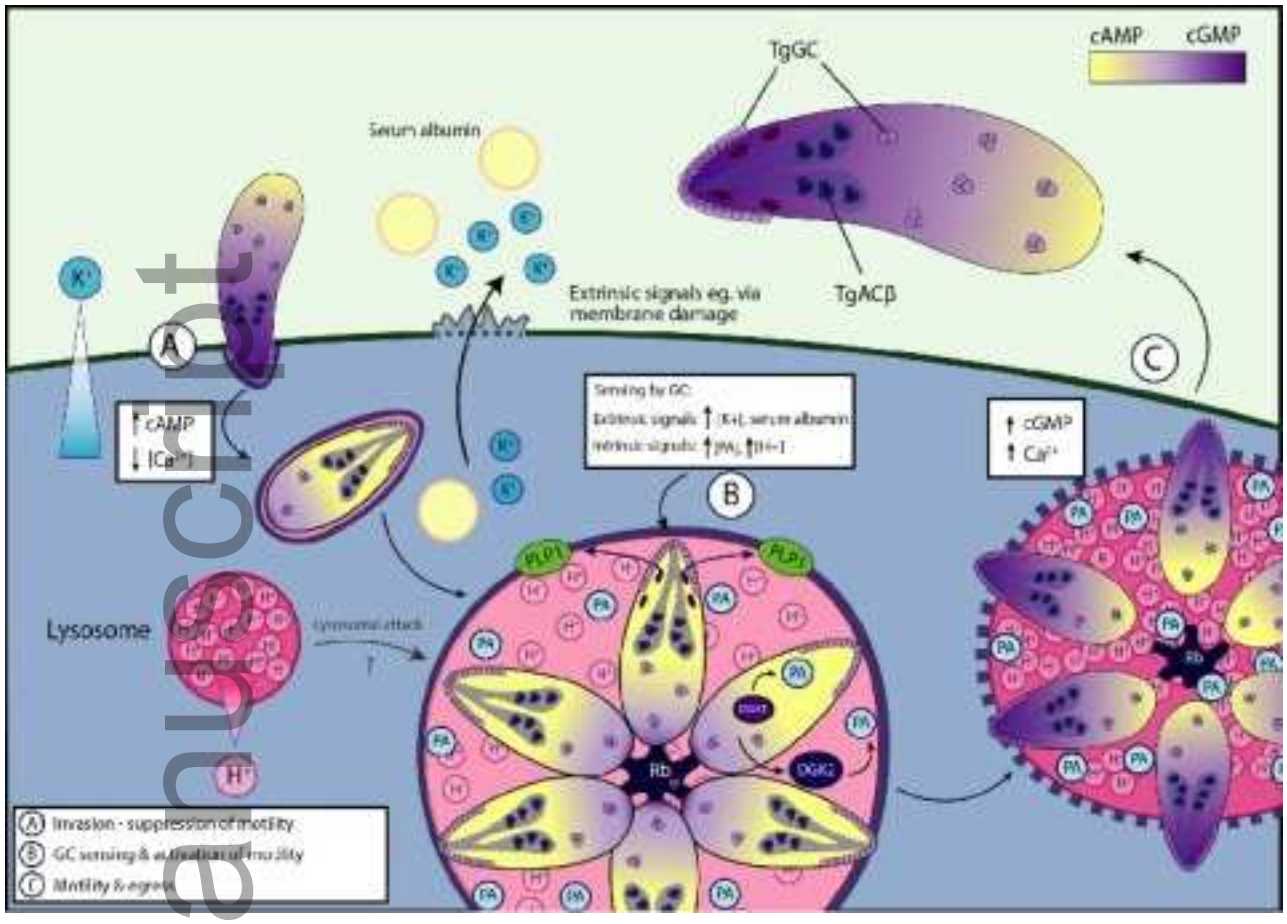
Figure 1: A Model of environmental sensing in Toxoplasma

A. Extracellular tachyzoites have high levels of cytosol Ca^{2+} and cGMP and are motile. Upon invasion tachyzoites experience a change in environmental conditions and switch off motility using cAMP/PKAc1 signaling. Intracellular replicative parasites then have high levels of cAMP and low levels of cGMP and cytosolic Ca^{2+} (B) During replication parasites can sense environmental cues that are signs of cellular damage (eg low $[K^+]$) or immune attack (eg lysosomes; high $[H^+]$). Parasites also have 'internal clocks' such as synthesis of phosphatidic acid (PA) or changes in $[H^+]$ in the vacuolar space that allow for correct timing of egress in the absence of other environmental cues. (C) Sensing of these signals requires an apically located guanylate cyclase (TgGC) that may directly or indirectly sense these environmental cues. This results in synthesis of cGMP and activation of downstream Ca^{2+} signaling. Parasites secrete Perforin-Related Protein 1 (PLP1) from micronemes and in an acidic environment leads to membrane permeabilization. Motility is activated and parasites exit the cell.

Figure 2: A model of molecular events governing environmental sensing and signal transduction that regulate *Toxoplasma* motility

Tachyzoites sense changes in ionic concentration or phosphatidic acid (PA) levels through ATPase domain in TgGC. This induces synthesis of cGMP and activation of the only known cGMP-effector Protein Kinase G (PKG). Phosphoinositol lipid signaling is activated and

results in synthesis of Diacyl Glycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP3 can then agonize Ca^{2+} release from the ER using an unknown transporter(s). High cytosolic [Ca^{2+}] activates Ca^{2+}-Dependent Protein Kinases (CDPKs) and Ca^{2+}-dependent phosphatase Calcinuerin A, which directly or indirectly phosphorylates/dephosphorylates downstream targets, including the glideosome. Synthesis of PA also occurs internally from DAG and augments the secretion of micronemes and association of the Glideosome Associated Connector (GAC) to drive glideosome function.



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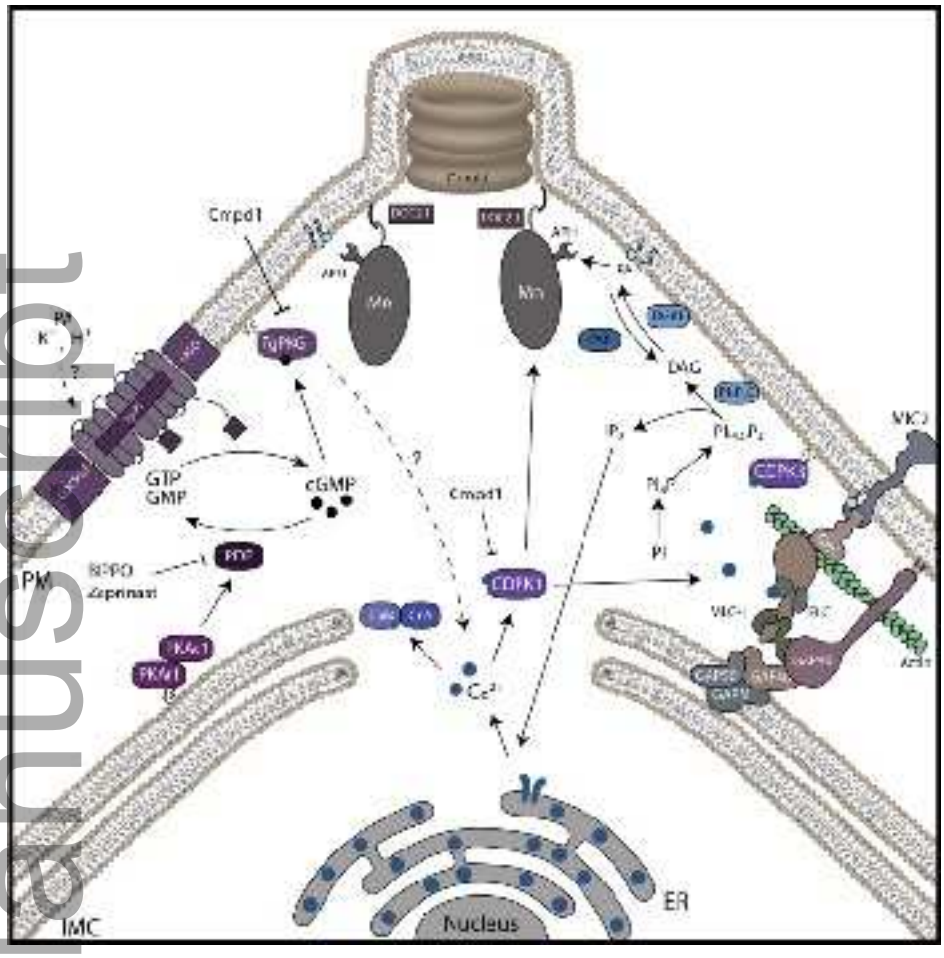

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