

Analysis of Ca²⁺ mediated signalling regulating *Toxoplasma* infectivity reveals complex relationships between key molecules

Rebecca L. Stewart^{1, 2}, Lachlan Whitehead^{1, 2}, Brunda Nijagal³, Brad E. Sleebs^{1, 2}, Guillaume Lessene^{1, 2, 4}, Malcolm J McConville⁵, Kelly L. Rogers^{1, 2}, Christopher J. Tonkin^{1, 2*}

1. The Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia
2. Department of Medical Biology, The University of Melbourne, Melbourne, Victoria, Australia
3. Metabolomics Australia, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria, Australia.
4. Department of Pharmacology and Therapeutics, The University of Melbourne, Melbourne, Victoria, Australia
5. Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, 3010, Australia

*Corresponding Author

Chris Tonkin

Division of Infection and Immunity

The Walter and Eliza Hall Institute of Medical Research

1G Royal Parade, Parkville 3052, Victoria, Australia

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/cmi.12685](https://doi.org/10.1111/cmi.12685)

ph: +61 3 9345 2555 and +61 3 9345 2926

Fax: +61 3 9347 0852

email: tonkin@wehi.edu.au

Competing Interests: The authors declare that no competing interests exist.

Word count (excluding materials and methods and references): 5,939 words

Running title: Ca²⁺ and cGMP signalling in *Toxoplasma*

Author Manuscript

Abstract

Host cell invasion, exit and parasite dissemination is critical to the pathogenesis of apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium* spp. These processes are regulated by intracellular Ca^{2+} signalling although the temporal dynamics of Ca^{2+} fluxes and downstream second messenger pathways are poorly understood. Here we use a genetically encoded biosensor, GFP-Calmodulin-M13-6 (GCaMP6), to capture Ca^{2+} flux in live *Toxoplasma* and investigate the role of Ca^{2+} signalling in egress and motility. Our analysis determines how environmental cues and signal activation influence intracellular Ca^{2+} flux, allowing placement of effector molecules within this pathway. Importantly, we have identified key interrelationships between cGMP and Ca^{2+} signalling that are required for activation of egress and motility. Furthermore, we extend this analysis to show that the Ca^{2+} Dependent Protein Kinases - *TgCDPK1* and *TgCDPK3* - play a role in signal quenching before egress. This work highlights the interrelationships of second messenger pathways of *Toxoplasma* in space and time, which is likely required for pathogenesis of all apicomplexan species.

Author Manuscript

Introduction

Malaria and toxoplasmosis are caused by infection with the apicomplexan parasites *Plasmodium* and *Toxoplasma*, respectively. Malaria kills more than 700,000 people annually, while *Toxoplasma* chronically infects 30% of world's population and can cause birth defects, blindness and disease in immunocompromised individuals (World Health Organization, 2014). These ancient parasites have evolved mechanisms to sense their environment responding to cues to activate host cell invasion, intracellular replication, egress and motility for parasite dissemination, a process collectively termed the lytic cycle. External cues activate parasite signalling cascades, most notably Ca^{2+} signalling, to drive progress through the lytic cycle including transitions from intracellular replication to extracellular motility (For review see (Lourido and Moreno, 2014)). Specifically, Ca^{2+} signalling controls the regulated release of specialised organelles called micronemes, which harbour adhesins and other factors required for invasion and egress that are central to apicomplexan pathogenicity (Carruthers and Sibley, 1999; Lovett *et al.*, 2002; Wetzel *et al.*, 2004). Parasite motility is also thought to be regulated by Ca^{2+} signalling via activation of the 'glideosome' - an actomyosin motor which provides the force required for a distinctive form of cellular locomotion termed 'gliding motility'. However, little is understood about the spatio-temporal dynamics and hierarchy of signalling pathways and how key molecules relate to one another during motility and egress.

The cyclic nucleotide second messenger, cyclic GMP (cGMP), activates Protein Kinase G (PKG), and also plays a vital role in the activation of apicomplexan egress, motility and differentiation (Wiersma *et al.*, 2004; Eaton *et al.*, 2006; Taylor *et al.*, 2008; Collins *et al.*, 2013). cGMP signalling is tightly regulated by guanylyl cyclases and phosphodiesterases that are

responsible for *de novo* synthesis and degradation of cGMP, respectively. As a result, targeted inhibition of parasite phosphodiesterases with zaprinast or BIPPO prevents cGMP breakdown leading to an accumulation of cellular cGMP, which activates PKG and in turn signals for microneme secretion and egress (Howard *et al.*, 2015). Conversely, inhibition of PKG interrupts cGMP signalling leading to microneme secretion defects in both *Toxoplasma* and *Plasmodium* spp. (Wiersma *et al.*, 2004; Donald *et al.*, 2006; Collins *et al.*, 2013). Recent work suggests that this pathway plays a role in regulating of phosphoinositide metabolism and Ca²⁺ signalling during stage conversion and motility in *Plasmodium* (Brochet *et al.*, 2014) and Ca²⁺ signalling driving microneme secretion during the asexual cycle of *Toxoplasma* (Brown *et al.*, 2016, Sidik *et al.*, 2016).

Apicomplexa have evolved divergent signalling elements to regulate novel functions specific to this group of pathogens. Plant-like Ca²⁺ dependent protein kinases (CDPKs) are expanded across Apicomplexa and play pivotal roles in Ca²⁺ signal transduction throughout the lytic cycle (For review see (Billker *et al.*, 2009)). CDPKs contain Ca²⁺ binding EF hand domains fused with a calmodulin-like kinase domain and inhibitory region, an architecture seen only in protists and plants and not metazoans. Upon interactions with Ca²⁺ ions, conformational change relieves autoinhibition and reveal the active site for substrate phosphorylation (Wernimont *et al.*, 2010). CDPKs have been implicated in a range of processes, including stage conversion (Billker *et al.*, 2004; Sebastian *et al.*, 2012; Brochet *et al.*, 2014), invasion (Lourido *et al.*, 2010; Bansal *et al.*, 2013) and egress (Dvorin *et al.*, 2010; McCoy *et al.*, 2012; Garrison *et al.*, 2012; Lourido *et al.*, 2012). In the case of *TgCDPK1*, this kinase plays a role in regulation of adhesins from the microneme organelles (Lourido *et al.*, 2010). Despite the multifaceted role of Ca²⁺ signalling in

Apicomplexan parasites little is known about how Ca^{2+} signalling operates, in space and time, to activate these kinases.

In mammalian cells, Ca^{2+} signal transduction commonly involves the regulated release of Ca^{2+} from intracellular stores (i.e ER) into the cytoplasm (Berridge, 2009). Regulated pump and channel activation drive distinct spatiotemporal Ca^{2+} oscillatory patterns modulating amplitude, duration and localisation for diverse cellular outcomes (Berridge *et al.*, 2003; Uhlen and Fritz, 2010). This phenomenon has been documented in *Toxoplasma* where cytoplasmic Ca^{2+} flux coincides with a burst of parasite motility immediately preceding invasion (Lovett, 2003). However, studies such as these have been technically limited due the broad cellular targeting of Ca^{2+} sensitive dyes. The development of genetically encoded Ca^{2+} sensors has revolutionised the understanding of signalling processes in living cells (Chen *et al.*, 2013). In the case of Apicomplexa, the focused expression of the genetically encoded Ca^{2+} sensors has allowed for the differentiation between signals derived from the host and those initiated by the occupying parasites (Borges-Pereira *et al.*, 2015). Interestingly, the source of intracellular Ca^{2+} has been brought into question through use of GCaMP6s identifying a PKG regulated neutral Ca^{2+} store distinct from the ER (Sidik *et al.*, 2016).

In this study we examine the spatiotemporal dynamics of intracellular signal transduction and investigate the interplay of multiple signalling nodes in *Toxoplasma* egress and motility. We employ the genetically encoded Ca^{2+} indicator GCaMP6s (Chen *et al.*, 2013), and the new cGMP agonist, **DBPC** (Howard *et al.*, 2015), to interrogate the hierarchy of signal transduction pathways, identifying activation of cGMP upstream of Ca^{2+} in both egress and motility. Extending this approach to CDPKs we reveal a role for *Tg*CDPK1 and *Tg*CDPK3 in Ca^{2+} levels immediately prior to egress. Furthermore, we demonstrate the relationship of Ca^{2+} periodicity and amplitude as a

driver of speed and distance travelled by motile parasites, thus together revealing the importance of Ca^{2+} signalling as a key mediator of *Toxoplasma* infectivity.

Author Manuscript

Results

GCaMP6s detects parasite-derived Ca^{2+} during *Toxoplasma* motility and egress

We sought to monitor intracellular Ca^{2+} flux within live tachyzoites using the genetically encoded, Ca^{2+} biosensor GCaMP6s (Chen *et al.*, 2013). For ratiometric analysis in *Toxoplasma* we co-expressed mCherry with GCaMP6s, whilst integration into the *uprt* locus ensured consistent expression across cell lines (Figure 1A). Visualisation of these lines demonstrated that both fluorescent signals could be detected in the cytoplasm of live extracellular and intracellular tachyzoites (Figure 1B).

Ca^{2+} signalling can result from release of Ca^{2+} from internal stores, and/or influx from extracellular sources (Berridge, 2009; Uhlen and Fritz, 2010). To assess the contribution of internal Ca^{2+} stores for signalling in *Toxoplasma* motility GCaMP6s-expressing tachyzoites were harvested in modified Ringer's + EGTA (0.1 mM) solution away from host cells. Under resting conditions, non-motile parasites exhibited low cytoplasmic Ca^{2+} levels (Figure 1C, no stimulation - 60s time lapse projection, Ca^{2+} false coloured low = blue, high = red). In the absence of extracellular Ca^{2+} , the Ca^{2+} ionophore A23187 robustly activated Ca^{2+} signalling and motility of tachyzoites (Figure 1C, A23187 - lower panel). Further, ratiometric quantitation showed rapid induction of cytoplasmic Ca^{2+} with the population average increasing post stimulation (Figure 1D). Autofluorescence of A23187 was found not to influence our readings (Supplementary Figure 1A). Tachyzoite motility was strongly correlated with GCaMP6s fluorescence with ~60% of ionophore treated parasites exhibiting movement in the 60s following Ca^{2+} increase (Figure 1E) (motility discussed further in Figure 6 and 7). This analysis demonstrates the capacity of GCaMP6s to measure tachyzoites ability to store and respond to endogenous Ca^{2+} signalling in the absence of external Ca^{2+} sources.

Unlike Ca²⁺ sensitive dyes, the use of genetically encoded Ca²⁺ biosensors, such as GCaMP6s, allows detailed assessment of individual intracellular tachyzoites. We therefore monitored Ca²⁺ dynamics of intracellular tachyzoites at the single vacuole level within host cells in unstimulated and activated states. Basal, intracellular parasites maintained low Ca²⁺ levels (Figure 1F, basal panel). Upon stimulation with A23187, cytoplasmic Ca²⁺ rapidly increased followed by parasite egress from host cells (Figure 1F, amplification, peak, egress). This assessment of live Ca²⁺ flux defines two states; 1) an immotile, low Ca²⁺ state of intracellular tachyzoites and 2) an active, motile state where rapid induction of Ca²⁺ drives parasite egress for extracellular dissemination. Together this work validates GCaMP6s for Ca²⁺ detection and analysis in *Toxoplasma* egress and motility.

cGMP signalling through PKG precedes Ca²⁺ signalling in egress

Recent work has suggested the importance of cGMP pathway and its link with Ca²⁺ signalling (Brochet *et al.*, 2014; Brown *et al.*, 2016; Sidik *et al.*, 2016), however this work relied on the use of a human PDE inhibitor zaprinast. Here, activation of egress by zaprinast requires long incubation times and high concentrations, often resulting in heterogeneous responses (Howard *et al.*, 2015). We recently described a potent, apicomplexan-specific PDE inhibitor, 5-benzyl-3-isopropyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (BIPPO). BIPPO shows high selectivity to towards *Toxoplasma* and *P. falciparum* PDE and rapidly induces host cell egress and microneme secretion in a PKG dependent manner (Howard *et al.*, 2015). To determine first whether treatment with BIPPO induces an increase in cyclic nucleotide production we measured cAMP and cGMP levels using LC-MS (Figure 2A). Extracellular tachyzoites were treated with either 1µM of BIPPO or 2µM of the Ca²⁺ ionophore A23187 for 1 minute followed by rapid quenching. Levels of the cyclic

nucleotides, cGMP and cAMP, were expressed relative to their nucleotide triphosphate counterparts, GTP and ATP respectively. This analysis showed that BIPPO treatment elicited a rise in levels of both cGMP and cAMP as compared to DME growth media alone. The Ca^{2+} ionophore A23187 also caused a rise in these cyclic nucleotides (Figure 2Ai and ii).

We then used BIPPO to explore the relationship of cGMP and Ca^{2+} signalling. We monitored Ca^{2+} dynamics of tachyzoites induced to egress with BIPPO and showed a clear amplification of cytoplasmic Ca^{2+} concentrations prior to parasite egress (Figure 2Bi). By performing ratiometric analysis and normalising the GCaMP6s signal to mCherry fluorescence, we analysed specific aspects of the Ca^{2+} response and compared these dynamics across multiple conditions. In the case of BIPPO stimulation of WT cells, all tachyzoites responded with robust amplification of Ca^{2+} before egressing within 75 s of the first instance of Ca^{2+} increase (Figure 2Bii - response normalised to $\frac{1}{2}$ peak Ca^{2+} as $t = 0$ s, one line represents one vacuole and ceases at the point of egress). This demonstrates that activation of cGMP is linked to Ca^{2+} signalling in activating egress. Given that BIPPO induces a rise in both cGMP and cAMP we further explored the link between cGMP and Ca^{2+} by inhibiting Protein Kinase G (PKG). PKG is a cGMP-dependent protein kinase and in apicomplexan parasites can be specifically inhibited by the trisubstituted pyrrole termed 'Compound 1' (Cmpd1) (Donald and Liberator, 2002). Upon treatment of GCaMP6s expressing tachyzoites with Cmpd1 parasites failed to mount a Ca^{2+} response and could not activate motility or egress, remaining intracellular for the duration of filming, up to 10 min (Figure 2Ci, ii). Indeed, this work is consistent with other studies showing that zaprinast can induce Cmpd-1 dependent increase

Author Manuscript

in Ca^{2+} (Sidik *et al.*, 2016). Together this work supports the role of cGMP in activating Ca^{2+} signalling through the action of PKG during egress of *Toxoplasma*.

Toxoplasma tachyzoites also egress in response to changes in extracellular $[\text{K}^+]$ and pH, which may signal immune attack or a dying host cell (Black *et al.*, 2000; Persson *et al.*, 2007; Roiko *et al.*, 2014). To investigate the impact of these signals on parasite Ca^{2+} fluxes, the membrane of host cells was selectively permeabilized with saponin (Carruthers and Sibley, 1997). When intracellular parasites were treated with saponin, whilst in a low $[\text{K}^+]$ buffer, tachyzoites displayed a rise in cytoplasmic Ca^{2+} , however, as compared to A23187 and BIPPO treated tachyzoites, the increase in cytoplasmic Ca^{2+} was not uniform across the vacuole,. Rather parasites individually activated Ca^{2+} responses over 3-5 s (Figure 2Di arrows) until all intracellular parasites within the same vacuole displayed high Ca^{2+} prior to egress (Figure 2Di,ii). To make sure the saponin was not activating tachyzoite motility by directly acting on the parasite in an unknown fashion extracellular tachyzoites were treated with this detergent and GCaMP6s fluorescence measured. Here, Saponin was unable to induce a rise in GCaMP6s fluorescence not induce parasite motility in the presence of Ca^{2+} (Supplementary figure 1D, E). To define the relationship between extracellular cues, intracellular cGMP and Ca^{2+} during egress under these conditions, we inhibited *Toxoplasma* PKG with Cmpdi before activating egress with saponin, while monitoring the Ca^{2+} responses. Here, in direct contrast to activation with BIPPO, saponin treatment induced a rise in Ca^{2+} although parasites did not activate motility or egress, remaining intracellular for the duration of filming (Figure 2Ei, ii).

To understand in more detail, the effect of stimuli on Ca^{2+} signalling we interrogated specific parameters of the Ca^{2+} response in the different conditions (Figure 2F). In particular we monitored

Ca²⁺ homeostasis, time to egress and peak Ca²⁺ responses. To assess the contribution of PKG to Ca²⁺ homeostasis in intracellular, unstimulated parasites we compared the baseline (basal) Ca²⁺ of WT and Cmpd1 treated cells and observed no difference in GFP/mCherry ratio (Figure 2G), thus suggesting that cGMP is not linked to modulations of basal Ca²⁺ levels. Next, we assessed the time to egress, which we defined as the time-point that the first parasite exited the confines of the parasitophorous vacuole. WT parasites rapidly egressed in response to all three agonists (Figure 2H - grey bars). In contrast Cmpd1 treated cells showed defective egress to all stimuli (A23187, BIPPO and Saponin) (Figure 2H - purple). To probe the maximal Ca²⁺ responses whilst Cmpd1 inhibited PKG, we performed side-by-side comparison of the peak Ca²⁺ reached after agonist stimulation. BIPPO treatment robustly increases cytoplasmic Ca²⁺ levels of WT cells (Figure 2I - BIPPO, grey bar). In contrast, BIPPO failed to induce comparable peak Ca²⁺ responses when PKG was inhibited (Figure 2I - BIPPO, purple bar), reflecting the visual observations seen in Fig. 2Ci. This indicates that cGMP signalling acts upstream of Ca²⁺ flux, as PKG activity is required for the cGMP dependent initiation of Ca²⁺ dynamics and egress. In comparison, Cmpd1 treated tachyzoites display Ca²⁺ flux in response to saponin and A23187 stimulation, achieving Ca²⁺ amplification greater than seen in Cmpd1 - BIPPO (Figure 2I - saponin, A23187). Importantly, however, these apparently normal peak Ca²⁺ responses were rarely followed by egress when PKG is inhibited with Cmpd1 (Figure 2H, I). This suggests that while Ca²⁺ signalling is essential for egress, and can be stimulated by multiple pathways (here either BIPPO or saponin), it still requires PKG to activate motility. Overall, this work has provided new insights into the hierarchical nature of signal transduction suggesting that cGMP precede Ca²⁺ signalling during activation of egress and motility.

Author Manuscript

***TgCDPK3* is not involved in steady state or amplification rate of Ca^{2+} during *Toxoplasma* egress**

Previously, it has been shown that *TgCDPK3* is important for A23187-induced egress but the role of *TgCDPK3* can be bypassed by changes in $[\text{K}^+]$ or activation of cGMP signalling (McCoy *et al.*, 2012; Lourido *et al.*, 2012). Whilst *TgCDPK3* could be considered an effector of Ca^{2+} signalling, published work suggests that it might act in Ca^{2+} homeostasis or contribute to Ca^{2+} amplification in egress (McCoy *et al.*, 2012; Garrison *et al.*, 2012). To investigate the influence of *TgCDPK3* on Ca^{2+} we introduced GCaMP6s/mCherry into $\Delta TgCDPK3$ tachyzoites. When treated with A23187, $\Delta TgCDPK3$ parasites responded with a uniform increase in cytoplasmic Ca^{2+} but did not reliably egress, in line with previously published work (Lourido *et al.* 2012; Garrison *et al.* 2012; McCoy *et al.* 2012) (Figure 3Ai). Ratiometric analysis illustrates the initial peak in Ca^{2+} signal followed by a return to approximate basal levels over the 10 min filming period (Figure 3Aii). Activation of cGMP with BIPPO induced a rapid Ca^{2+} amplification and here egress was seen in all tachyzoites tested (Figure 3Bi,ii). Similarly, saponin treatment induced a Ca^{2+} increase followed by egress (Figure 3Ci,ii). To test whether *TgCDPK3* is required to maintain basal Ca^{2+} levels as suggested by Garrison *et al.* (2012) (Garrison *et al.*, 2012) we compared the basal Ca^{2+} levels of resting, intracellular $\Delta TgCDPK3$ tachyzoites to WT. Here, we saw no difference between $\Delta TgCDPK3$ and WT lines suggesting no role for *TgCDPK3* in Ca^{2+} homeostasis (Figure 3E). Further, when Ca^{2+} potential was probed via measurement of peak Ca^{2+} levels after stimulation, $\Delta TgCDPK3$ tachyzoites were able to upregulate Ca^{2+} to the same extent as WT cells, ~2-4 fold increase (Figure 3F). McCoy *et al.* (2012) hypothesised that *TgCDPK3* could be involved in Ca^{2+} amplification by modulating the rate of Ca^{2+} release (McCoy *et al.*, 2012). We therefore measured the rate of change of Ca^{2+} during the amplification stage (Figure 3G – $\Delta \text{Ca}^{2+}/\text{s}$). Between WT and

$\Delta TgCDPK3$ tachyzoites we did not detect a difference in BIPPO and Saponin induced Ca^{2+} amplification rates (Figure 3H - BIPPO, Saponin). A23187 stimulation saw Ca^{2+} amplification rates of $\Delta TgCDPK3$ only marginally slower than WT (Figure 3H- A23187) suggesting that $\Delta TgCDPK3$ largely does not affect the strength or rate in which A23187 induced Ca^{2+} flux occurs. We then monitored time to egress of $\Delta TgCDPK3$ and noticed that despite there being no difference in intracellular Ca^{2+} levels in response to a stimulus, these parasites still had significant delays in egress. From this data we can surmise that $TgCDPK3$ does not have a role in basal Ca^{2+} levels or rate of response to an egress stimuli. Furthermore, our data suggests that whilst stimulation of cGMP can overcome the loss of $TgCDPK3$ it still results in retarded activation of egress and motility, suggesting that this kinase activates another unknown process that is required for rapid egress.

TgCDPK1 acts downstream of Ca^{2+} to activate *Toxoplasma* egress

Previously it has been demonstrated that $TgCDPK1$ plays a pivotal role in microneme secretion, egress, invasion and motility. Lourido *et al.* (2010) showed $TgCDPK1$ knock down lines fail to respond to A23187 egress stimulation demonstrating a block in Ca^{2+} signal transduction (Lourido *et al.*, 2010). To investigate further the role of $TgCDPK1$ in the Ca^{2+} signalling hierarchy we used the ATP analogue, 3MB-PP1, which specifically targets $TgCDPK1_{WT}$ during egress and motility (Lourido *et al.* 2010). To create a control, a 3MB-PP1-refractory line was made where the $TgCDPK1$ gatekeeper amino acid was modified from glycine to methionine ($TgCDPK1_M$) (Lourido *et al.*, 2010). 3MB-PP1 inhibition of $TgCDPK1_{WT}$ did not alter the Ca^{2+} amplification dynamics when parasites were stimulated to egress with A23187 (Figure 4A - Amplification, Ci) and, as expected, inhibition of $TgCDPK1_{WT}$ prevented egress (Figure 4A - no egress). It is important to

note that, in these circumstances, egress was scored at the point at which the first egressing parasite breached the parasitophorous vacuole. While A23187 induced egress was scored for over half the 3MB-PP1 treated tachyzoites, this egress was clearly defective as the majority of parasites were unable to disseminate away from the parasitophorous vacuole following vacuole breach. A similar response was seen when 3MB-PP1 treated parasites were stimulated with BIPPO where cytoplasmic Ca^{2+} rapidly increased (Figure 4B - amplification, peak, 4Ci) followed by parasitophorous vacuole breach (Figure 4B - *egress, arrows). Parasites could not successfully engage motility or disseminate across host cells and were thus incapable of reinvasion. 3MB-PP1 refractory lines (*TgCDPK1_M*), demonstrated normal egress, demonstrating the specificity this compound to *TgCDPK1* in this experimental setup (Figure 4Di-iii). Basal Ca^{2+} showed no differences between 3MB-PP1 treatment of WT and 3MB-PP1 treatment of control line *TgCDPK1_M* (Figure 4E). Ratiometric quantitation of peak Ca^{2+} responses confirmed visual observations where *TgCDPK1_{WT}* inhibition did not alter peak Ca^{2+} ratios (Figure 4F). When 3MB-PP1-treated tachyzoites were able to breach the parasitophorous vacuole, this event was delayed in comparison to normal egress (Figure 4G). Given that loss of *TgCDPK1* cannot be rescued by cGMP pathway induction nor saponin this data provides further validation of differential roles between *TgCDPK1* and *TgCDPK3* and reinforces the pivotal role of *TgCDPK1* as an essential effector of Ca^{2+} signal transduction during egress.

***TgCDPK1* and *TgCDPK3* contribute to Ca^{2+} dynamics immediately prior to egress**

It is still not understood how CDPKs function to promote egress and motility. Previous data has suggested that both *TgCDPK1* and *TgCDPK3* function in controlling microneme secretion (Lourido *et al.*, 2010; McCoy *et al.*, 2012; Lourido *et al.*, 2012), but whether *TgCDPK1* and *TgCDPK3* act

Author Manuscript

directly to promote secretion of these organelles or whether they work in an upstream signalling event is unknown. During analysis of CDPK mutants we noticed differences in the decay of the Ca^{2+} response from peak level to egress. We found that after stimulation of egress, intracellular Ca^{2+} rose to peak levels and then decreased (~25%) before egress began, suggesting that there may be feedback mechanisms to quench Ca^{2+} flux required for egress to proceed. To analyse if CDPKs could be involved in the Ca^{2+} decay, and thus potentially explain a function of these kinases, we probed the kinetics of Ca^{2+} decay during the period between peak Ca^{2+} and egress. Saponin was used to assay this as it activates egress in the absence of continued pharmacological pressure (as is the case with A23187 or BIPPO). For further dissection of peak to egress Ca^{2+} kinetics, traces were averaged, normalised to peak ratio (0) and analysed as a two-step response (Figure 5A - two phase split at $\frac{1}{2}$ Ca^{2+} decay between peak and egress). Where *Tg*CDPK1 is inhibited with 3MB-PP1, and in control *Tg*CDPK1_M lines, the point when the $\frac{1}{2}$ Ca^{2+} decay was reached was around 10 s post peak ratio with continuation of Ca^{2+} decay rate until egress at around 25 s post peak ratio. In 3MB-PP1 treated or ΔTg CDPK3 the halfway point ($\frac{1}{2}$ Ca^{2+} decay) was slightly delayed to between 15 and 20 s (Figure 5B, inset). Here the rate of decay for 3MB-PP1 treated or ΔTg CDPK3 shows a distinct change slowing between halfway Ca^{2+} decay to egress. For comparison we generated a *GCaMP6s* line where microneme protein Perforin-Like Protein 1 was knocked out (ΔTg PLP1), which causes egress defects due to the inability of parasites to enzymatically breakdown membranes during escape of parasitophorous vacuoles (Kafsack *et al.* 2009). Our analysis revealed normal intracellular Ca^{2+} signalling in ΔTg PLP1 egress, with peak and Ca^{2+} amplification comparable to WT (Supp. Figure 2A, B, C). As expected, ΔTg PLP1 egress was defective in response to A23187 however ΔTg PLP1 parasites exhibited delayed egress to BIPPO and Saponin treatment, most likely due to other means of membrane destruction (Supp. Figure 2Ciii). When

peak to egress Ca^{2+} decay was quantitated for $\Delta TgPLP1$ decay displays continual rate across the two phases, in contrast to the changes observed upon 3MB-PP1 treatment or *TgCDPK3* deletion.

When quantitated as a rate of decay ($\Delta\text{Ca}^{2+}/\text{s}$) from Ca^{2+} peak to $1/2 \Delta\text{Ca}^{2+}$ decay (1st half decay) there were comparable rates between all conditions including 3MB-PP1 and $\Delta TgCDPK3$ (Figure 5C). In comparison, analysis of the second half of Ca^{2+} dampening before egress (Ca^{2+} decay halfway to egress) demonstrated a specific and statistically significant defect where either *TgCDPK1* or *TgCDPK3* were defective (approx. $-0.01 \Delta\text{Ca}^{2+}/\text{s}$) as compared to WT or *TgCDPK1_M* (approx. $-0.06 \Delta\text{Ca}^{2+}/\text{s}$), or $\Delta TgPLP1$ (approx. $-0.04 \Delta\text{Ca}^{2+}/\text{s}$) (Figure 5C). Overall, our data shows modified Ca^{2+} decay specific to *TgCDPK1* and *TgCDPK3* suggesting these kinases play roles in modulating Ca^{2+} levels prior to egress and suggest a possible mechanism by which these kinases work to regulate motility during cell exit.

cGMP and Ca^{2+} signalling are interconnected during *Toxoplasma* motility.

Upon egress, extracellular tachyzoites require gliding motility for movement across the extracellular substrate. We sought to understand the Ca^{2+} signalling dynamics during motility through observations of Ca^{2+} flux using our GCaMP6s approach. Tachyzoites were freshly harvested in King's medium (with Ca^{2+}) and allowed to settle on a substrate surface, before stimulation with motility agonists for a total analysis time of 4 min (2 min pre-stimulation, 2 min post-stimulation). Pre-stimulation, the majority of tachyzoites exhibit low motility and low Ca^{2+} (Figure 6Ai, Bi). Where parasites do engage motility there is a coincident increase in Ca^{2+} activity (Figure 6Ai, Bi arrows). To validate the detection of Ca^{2+} signalling in our model, we first

stimulated these cells with A23187 and detected a rapid increase in tachyzoite movement and Ca^{2+} signalling (Figure 6Aii, representative 60 s time-lapse). Next, to assess the participation of cGMP signalling in motility tachyzoites were stimulated with BIPPO. Similar to egress, tachyzoites rapidly engaged Ca^{2+} signalling and initiated gliding motility in response to BIPPO, indicating a role for cGMP in signalling for movement (Figure 6Bii, representative 60 s time-lapse). During motility, parasites display multiple forms of movement defined as productive; helical (corkscrew-like motion) and circular, or unproductive; twirling and short movements. Quantitation of motility between A23187 and BIPPO showed both compounds induced all forms of movement at comparable levels, with around 70% of tachyzoites exhibiting some form of movement (Figure 6C, WT- A23187 vs. BIPPO). To analyse further the role of cGMP signalling in motility we pre-treated tachyzoites with Cmpd1, inhibiting PKG, before activation with BIPPO. Here, we saw only a small proportion of tachyzoites engage motility, predominantly with non-productive twirling or short movements. To examine the link between Ca^{2+} flux and movement seen in our visual data (Figure 6A, B), and to assess the contribution of cGMP signal disruption in Cmpd1 defective motility, we quantitated the tachyzoite population average of the Ca^{2+} signal during motility assays. Prior to stimulation, WT populations maintained a stable 'basal' Ca^{2+} profile, which rapidly increased upon the addition of agonists A23187 and BIPPO, coincident with movement (Figure 6D, A23187 - Black line, BIPPO - Brown line). In contrast, Cmpd1 pre-treated cells displayed a lowered basal Ca^{2+} level with minimal increases after A23187 or BIPPO stimulation (Figure 6D Cmpd1, A23187- Blue line, BIPPO-Purple line). This demonstrates that, like signalling pathways in egress (Figure 2), cGMP signalling through PKG is an important signalling module driving tachyzoite movement.

Author Manuscript

Level of Ca²⁺ signalling correlates with the quality of motility in *Toxoplasma*

Apicomplexan motility can often alternate between bursts of movement and inertness (Münter *et al.*, 2009; Williams *et al.*, 2015). To determine if modulation of cytoplasmic Ca²⁺ levels was responsible for these motility bursts we tracked the displacement and Ca²⁺ flux of individual tachyzoites. We followed productively motile tachyzoites engaged in circular or helical movement after drug stimulation and compared these with parasites that remained non-motile throughout the assay. Prior to stimulation parasites were non-motile travelling no more than 1 µm at a time (Figure 7A, B – thin black lines). During this non-motile period Ca²⁺ flux remained consistently low (Figure 7A, B – thick grey lines). Upon stimulation with A23187, helical movement drove bursts of increased displacement with tachyzoites travelling up to 5 µm at a time (Figure 7Ai - thin black line). Here, dynamic Ca²⁺ flux (rapid, high amplitude Ca²⁺ transients) paralleled tachyzoite movement during circular movements. In the case of the individual tachyzoite plotted, two circular revolutions were completed before a switch to twirling motility (Figure 7Ai thick lines - gold = circle 1, pink = circle 2, twirling = blue). Displacement versus Ca²⁺ level showed a moderate correlation of $r^2 = 0.6703$ (Figure 7Ai, right plot – correlation quantitation taken from data highlighted in grey). A similar moderate correlation between displacement and Ca²⁺ was seen for helical motility where Ca²⁺ flux mirrored parasite displacement in bursts of activity (Figure 7Aii – maroon - helical). In contrast non-motile parasites, by definition, did not travel more than 1µm at a time, and although Ca²⁺ release was evident with a slight increase in the Ca²⁺ signal, dynamic Ca²⁺ flux was not seen and did not correlate with displacement (Figure Aiii). This indicates that dynamic and higher amplitude Ca²⁺ fluxes are important for motility and lower amplitude and sustained rises in cytoplasmic Ca²⁺ were not sufficient to drive motility. Assessment of BIPPO induced motility

showed similar results to A23187 treatment, with dynamic Ca^{2+} flux moderately correlated with displacement in circular and helical movement but not in non-motile parasites (Figure 7Bi-iii). Quantitative comparison of the motility parameters demonstrated the average parasite velocity of 1-1.5 $\mu\text{m/s}$ for both helical and circular motility in response to A23187 or BIPPO (Figure 7Ci). Parasites travelled an average of 8 μm over an average of 8 s between Ca^{2+} peaks in helical movement (Figure 7Cii, iii - helical). In response to BIPPO treatment, parasites travelled slightly further ($\sim 12 \mu\text{m}$) over a longer Ca^{2+} flux period (~ 11 s) when engaged in circular motility (Figure 7Cii, iii - circular). To scrutinise further the dynamics in non-motile parasites we examined the Ca^{2+} profiles of non-motile cells pre-treated with Cmpd1. Similar to WT, a slight rise in Ca^{2+} was seen but neither correlation nor significant Ca^{2+} flux was evident in response to either A23187 or BIPPO stimulation (Figure 7Di, ii). Here, we see that Ca^{2+} flux did not occur in the absence of PKG activity outlining a similar signalling hierarchy between motility and egress where cGMP precedes Ca^{2+} .

4.2 Discussion

In this work we investigate the interactions between signal transduction pathways that drive egress and motility in *Toxoplasma*. Using the Ca^{2+} biosensor GCaMP6s we confirm and extend previous work on Ca^{2+} signalling in *Toxoplasma* and other apicomplexan parasites (Lovett, 2003; Brochet *et al.*, 2014; Carey *et al.*, 2014; Brown *et al.*, 2016; Sidik *et al.*, 2016). Key parameters of cytosolic Ca^{2+} fluxes, such as signal amplitude, frequency and decay, were monitored, which in other systems can determine the type of cellular response that is invoked by these signalling events. Furthermore, we use specific inhibitors and activators as well as several mutants known to play a role in Ca^{2+} signalling to determine the hierarchy of events that take place that ultimately leads to egress and motility – a key event in the pathogenicity of all apicomplexan parasites.

Our work suggests that cGMP and the activity of PKG acts upstream of Ca^{2+} release to activate *Toxoplasma* motility. This work builds on recent findings in *P. berghei* ookinetes where Brochet *et al.* (2014) identified a potential link between cGMP and Ca^{2+} signal transduction (Brochet *et al.*, 2014). In this study they identify PKG-dependent phosphorylation of components involved in phosphoinositide metabolism, a pathway that in mammalian systems leads to the production of the second messenger IP_3 , which in turn activates Ca^{2+} release channels at the ER (Berridge *et al.*, 2003). Furthermore, two very recent studies have shown that cGMP and Ca^{2+} signalling are also linked in *Toxoplasma*. In this regard our work corroborates these similar findings but uses the more potent apicomplexan-specific PDE inhibitor BIPPO (Howard *et al.*, 2015). Overall, this combined work now strongly supports a mechanism where Ca^{2+} and cGMP pathways are linked across apicomplexan species.

This work also highlights the complexities and redundancies of signal transduction networks in apicomplexan species. We show that a rise in intracellular Ca^{2+} , but not egress, can occur independently of cGMP signalling through *TgPKG*. This is demonstrated by altering host cell and vacuolar milieus (i.e $[\text{K}^+]$ and pH levels) with saponin treatment showing that this causes a rapid induction of Ca^{2+} signalling independent of *TgPKG* activity. This suggests the presence of redundant mechanism(s) complementary to the cGMP/PKG activation that can also feed into a rise in Ca^{2+} signalling for subsequent motility and egress. One such candidate for alternate activation of Ca^{2+} signalling is cAMP, which we show is produced when BIPPO is administered. Other possibilities include cADPR signal transduction pathway (Chini *et al.*, 2005) and Abscisic Acid driven pathways (Nagamune *et al.*, 2008). However, there is little understood about these pathways and no specific genes nor inhibitors are known, so assessing their interaction with Ca^{2+} signalling is currently challenging. What is clear, as presented in this study and in support of the current literature, is that intracellular tachyzoites can sense and respond to multiple stimuli (Moudy *et al.*, 2001; Persson *et al.*, 2007; Niedelman *et al.*, 2013; Roiko *et al.*, 2014; Borges-Pereira *et al.*, 2015). What is unclear, is the identity of physiological stimulators of egress and motility. Brown *et al.* recently shown that serum albumin can stimulate microneme secretion in a Ca^{2+} independent fashion but no other natural triggers are known (Brown *et al.*, 2016).

In other systems, mechanisms of cytosolic Ca^{2+} concentration are controlled by Ca^{2+} pumps and channels, which in turn alter the frequency, location and amplitude of Ca^{2+} transients to drive distinct biological functions (Berridge *et al.*, 2003). Here, we identified amplification and dampening Ca^{2+} dynamics in *Toxoplasma* where, prior to egress cytoplasmic Ca^{2+} release is followed by a ~20 s period of Ca^{2+} decay. This data showed that while *TgCDPK1* and *TgCDPK3*

are not required for Ca^{2+} peak levels, their attenuation slowed Ca^{2+} decay immediately prior to egress. Whilst the importance or function of this Ca^{2+} dampening is unknown the fact that *TgCDPK1* or *TgCDPK3* mutants show this Ca^{2+} attenuation, and do not egress efficiently, suggests that these kinases may hold some important role during this process. What is clear from this analysis is that Ca^{2+} fluctuations are dynamic and must require mechanisms for both Ca^{2+} release and re-uptake into an internal store or efflux to the extracellular environment. In *Toxoplasma*, a thapsigargin-sensitive sarcoplasmic-ER Ca^{2+} ATPase (SERCA) is present at the ER while a $\text{Ca}^{2+}/\text{H}^{+}$ -ATPase (*TgA1*) at the acidocalcisome is required for Ca^{2+} homeostasis (Luo *et al.*, 2004; Nagamune *et al.*, 2007). However to date, the identity and regulation of Ca^{2+} release channels, nor the contribution of *TgA1* and *TgSERCA* in egress and motility has not been described.

Our data shows that each Ca^{2+} oscillation is accompanied by approximately 8 μm of tachyzoite movement. Given that *Toxoplasma* tachyzoites are $\sim 7 \mu\text{m}$ in length this suggests that each Ca^{2+} flux event allows for parasites to move one body length. Similarly, in *P. berghei* sporozoites weak correlations were observed between Ca^{2+} flux and sporozoite speed, although quantitation of distance travelled per Ca^{2+} peaks were not performed (Carey *et al.*, 2014). Indeed, the current model of motility suggests that adhesins released from the micronemes link host substrate to the parasite glideosome and action of the motor pulls adhesins rearward to drive for forward movement (Münter *et al.*, 2009; Heintzelman, 2015). Data presented here therefore suggests that Ca^{2+} flux may induce a wave of adhesins to be secreted from the micronemes onto the surface, which then engage with the glideosome and drive another parasite length of motility before another Ca^{2+} dependent release is required. Indeed, *Toxoplasma* is able to secrete successive rounds of micronemal proteins upon stimulation further adding weight to this hypothesis (Bullen *et al.*, 2016).

Overall, this work aims to incorporate apicomplexan signalling pathways as a unified - albeit complex - network, to produce a hierarchical model of pathway activation from external stimuli to internal signal transduction pathways. Importantly, we have built on work defining a link between cGMP and Ca²⁺ signalling pathways while also detecting potential feedback mechanisms between CDPKs and Ca²⁺. Further, we show a correlation between Ca²⁺ flux and parasite movement that strengthens the current model for Ca²⁺-mediated, adhesin-based gliding motility. This work also highlights the utility of biosensor technology in dissecting components of signal transduction and demonstrates the potential of this application for further analysis of Ca²⁺ dynamics in *Toxoplasma*. Of particular interest would be the targeting of biosensors to subcellular domains to identify more subtle, localised instances of signal initiation or transduction.

Author Manuscript

Materials and Methods

Parasite culture and harvest

Primary human foreskin fibroblasts (HFFs) cells were maintained in culture at 37°C/10% CO₂ with DMEM supplemented with 10% cosmic calf serum. Prior to inoculation HFFs were refreshed with D1 -DMEM supplemented with 1% FCS and 1% GlutamaxTM (Invitrogen). Parasites were inoculated onto HFFs and maintained at 37°C/10% CO₂.

Generation of transgenic parasites

pGP-CMV-GCaMP6s was a gift from Douglas Kim (Addgene plasmid #40753). For our work GCaMP6s was amplified and Gibson assembled with mCherry, driven by *TgTUB1* and *TgGRA1* promoters, respectively. Plasmids were transfected for homologous recombination into the *uprt* locus using FUDR selection, which was subsequently confirmed by fluorescent microscopy.

Tachyzoite preparation for analysis

Ca²⁺ source assessment: Fresh tachyzoites were harvested and inoculated onto confluent host cells and grown for 24-30 h in D1. Wells were washed once with PBS before addition of modified Ringer's -EGTA Buffer (155 mM NaCl, 3 mM KCl, 5 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, 0.1 mM EGTA)(Lovett, 2003). Tachyzoites were harvested prior to vacuole rupture and analysed as per motility (see below).

Egress: Fresh tachyzoites were harvested and inoculated onto confluent host cells grown on IBIDI tissue treat 8-well chamber slides and allowed to grow for 24-30 h in D1. Ringer's media (155 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose) replaced D1 prior to imaging. Where applicable inhibitor drug treatments (Cmpd1- 5 μM,

≥25 min @37°C. 3MB-PP1- 5 μM, ≥20 min @37°C) were performed in Ringer's media prior to egress analysis. Imaging was restricted to vacuoles containing 8-16 tachyzoites where host cells were only infected with a single vacuole. Agonist drugs (A23187 – 4 nM, BIPPO – 55 μM, saponin - 0.05% w/v) were added into chamber via catheter tubing 15 s after initiation of image acquisition. ≥8 vacuoles were analysed for each condition.

Author Manuscript

Motility: Fresh tachyzoites were harvested and inoculated onto confluent host cells and grown for 24-30 h in D1. Where applicable wells were pre-treated with inhibitors. Wells were washed once with Ringer's media and tachyzoites were harvested prior to vacuole rupture via scraping, needle passing through 27-gauge needle and filtration of cell debris (3 μm filter). IBIDI 8-well chambers slides were pre-treated with Poly-L-Lysine for 20 min at RT and allowed to dry. Free tachyzoites were inoculated onto Poly-L-Lysine surface and allowed to settle for 3 min. Tachyzoite motility was captured for 2 min prior to addition of compounds (A23187 and BIPPO) via catheter tubing for a total of 4 min image acquisition. Assays were performed in duplicate. Parasite movement was scored by eye from acquired videos in with ~100 parasites analysed per repeat, n = 3.

Imaging platform, acquisition settings and analysis

Egress: Imaging was performed on the Leica SP8 Confocal system, 60x/1.4 NA Oil immersion objective, in humidified environmental chamber with temperature maintained at 37°C. Image capture was managed by Leica software with acquisition across 5 x 1.25 μm Z-stacks, 1 frame/3 s until egress was detected or up to a maximum of 10 min. Image analysis was performed using FIJI software with Macros designed by Dr. Lachlan Whitehead specifically for use during this project (available upon request). Fluorescent readout was converted from a visual to digital signal for both

mCherry (control) and Ca²⁺ sensitive GCaMP6s signals. Signal was normalised to 0 (zero) GCaMP6s/mCherry ratio at t = 0 s on the y axis using $\frac{1}{2} \text{Ca}^{2+} \text{ Max}$:

$$\frac{(\text{Ratio}_{\text{Peak}} - \text{Ratio}_{\text{Basal}})}{2}$$

Egress is defined as first instance of parasite movement from the vacuole (pinching through host membrane or movement away from vacuole). Peak Ca²⁺ represents the maximum GCaMP6s/mCherry ratio reached per vacuole (n = 8-13 vacuoles, mean +/- s.e.m.). Rate increase was determined as:

$$\frac{(\text{Ratio}_{\text{Peak}} - \text{Ratio}_{\text{Basal}})}{t_{\text{Peak}} - t_{2\%}}$$

where Ratio_{Peak} is the peak GCaMP6s/mCherry ratio per vacuole, Ratio_{Basal} is the GCaMP6s/mCherry ratio averaged across time-points prior to Ca²⁺ induction, t_{Peak} is time peak ratio reached (s) and t_{2%} is first instance of Ca²⁺ induction above 2% peak Ca²⁺ (s).

For Ca²⁺ decrease from peak analysis plots were normalised so 0 = t_{Peak} (x) and Ratio_{Peak} (y). Vacuole Ca²⁺ traces were averaged. 50% Ca²⁺ decay from peak to egress was plotted as:

$$y = \frac{\text{Ratio}_{\text{Peak}} - \text{Ratio}_{\text{Egress}}}{2} \quad x = t \text{ from peak (s)}$$

100% decay from peak to egress was plotted as:

$$y = \text{Ratio}_{\text{Peak}} - \text{Ratio}_{\text{Egress}} \quad x = t \text{ from peak (s)}$$

Rate decay from peak to egress was determined as:

$$1^{\text{st}} \text{ Half: } \frac{\text{Ratio}_{50\% \text{ Ca}^{2+} \text{ decay}}}{t_{50\% \text{ Ca}^{2+} \text{ decay}} - t_{\text{peak}}} \quad 2^{\text{nd}} \text{ Half: } \frac{\text{Ratio}_{100\% \text{ Ca}^{2+} \text{ decay}} - \text{Ratio}_{50\% \text{ Ca}^{2+} \text{ decay}}}{t_{100\% \text{ Ca}^{2+} \text{ decay}} - t_{50\% \text{ Ca}^{2+} \text{ decay}}}$$

Author Manuscript

Motility: Imaging was performed as per egress with a 40x/0.8 1.4NA Oil immersion objective, acquisition across 3 x 1.25 μm in z direction, 1 frame/2 s. Whole population changes were determined using FIJI intensity plot feature and normalised to 0 (zero) GCaMP6s/mCherry ratio where 0 = average of WT population over 60 s of non-stimulated parasite filming. Tracking of individual parasites was performed using FIJI software with Macros designed by Dr. Lachlan Whitehead specifically for motility and Ca^{2+} tracking during motility within this project - adapted from TrackMate plugin. Data was processed in Excel. Types of movement for individual parasites were scored by eye and aligned with ratiometric readouts. For each parasite, Ca^{2+} was internally normalised to zero based on the average of a period of immobility extended across 60s. Parasites were selected for individual tracking if they displayed predominant helical or circular movement, were in frame for both non-motile and motile periods and did not cross paths with other tachyzoites. Representative plots shown are derived from independent experiments, n = 5 – 8.

Motility parameters: Ca^{2+} and displacement traces from above were analysed to determine velocity: total distance travelled (μm) / time (s); Flux distance: average distance travelled for each Ca^{2+} peak in an individual parasite, one point = one parasite track (multiple Ca^{2+} peaks); Time between peaks: average time between each Ca^{2+} peak for an individual parasite, one point = one parasite track (multiple Ca^{2+} peaks). n = 8-20 parasites for each condition and movement type.

Compound synthesis

Compound 1 and 3MBP-PP1 were prepared according to literature procedures (Biftu *et al.*, 2005; Lourido *et al.*, 2010; Towle *et al.*, 2013)

LC-MS analysis of cyclic nucleotide levels

Intracellular tachyzoites were recovered from host cells by needle passage and filtering through a 0.45µm membrane. Purified tachyzoites (3×10^8) were resuspended in DME supplemented with 10mM HEPES, pH 7.2, prior to the addition of either BIPPO (1µM) or A23187 (2µM). Tachyzoite suspensions were incubated for 1 min in a 37°C water bath, then rapidly transferred to ice cold PBS to quench metabolism and centrifuged (20,000rpm, 5min). Tachyzoite cell pellets were extracted in 10-volumes of ice cold acetonitrile containing 2µM $^{13}\text{C},^{15}\text{N}$ -AMP and $^{13}\text{C},^{15}\text{N}$ -UMP and extracts vortex mixed (1 min), then sonicated in an ice-water bath (5 mins), before centrifugation (20,000rpm, 10 mins, 4°C). The supernatants were transferred into HPLC vials, and cyclic nucleotides detected using LC-QTOF as previously described Hortle et al 2016 with the following modifications (Hortle *et al.*, 2016). LC analysis was performed on an Agilent 1260 HPLC system using a ZIC®-pHILIC column ((5 µm particle size, 150 x 4.6 mm, Merck SeQuant®), a 23 minute gradient (containing a 6 min washout and equilibration) with mobile phases A (20mM ammonium carbonate pH 9) and B (100% acetonitrile) eluted at a constant flow rate of 300 µL/min (25°C) and a sample injection of 5 µL. MS was performed on an Agilent 6545 QTOF instrument and ionisation promoted with a capillary voltage of 2.5kV, drying gas flow of 10.0 L/min (N₂) and temperature of 225°C, nebulizer pressure of 20 psi, fragmentor and skimmer cap voltage of -125V and -45V, respectively. Ions were analysed in negative mode with a full scan range of 85 to 1200 m/z at a rate of 0.8 spectra/sec and peaks identified using MassHunter Quantitative Analysis B.07.00 (Agilent).

Metabolite identification of purines of interest (ATP, GTP and cAMP) was based on accurate mass, predicted retention time and authentic chemical standards (level 1 standard of identification according to the Metabolomics Standards Initiative). cGMP was identified based on accurate mass and predicted retention time (level 2 standard of identification). All samples were randomized prior to analysis and pooled biological quality control samples (pbQCs) were prepared by pooling 10 μ l of all sample lysate and run every 5 samples to monitor instrument reproducibility

Author Manuscript

Acknowledgements

We would like to thank Professor Silvia N.J Moreno, University of Georgia, USA for useful and generous discussions and Professor Vern Carruthers for sharing the Δ PLP1 line. We also thank C. Bradin for assistance in construct preparation.

Funding

RJS is supported by an Australian Postgraduate Award and CJT by a National Health and Medical Research Council (NHMRC) Career Development Fellowship (Level 1) and an Australian Research Council (ARC) Future Fellowship. MJM is an NHMRC Principal Research Fellow. This work was supported by NHMRC project grants GNT1022559, GNT1047806 and GNT1017059 and institutional support from the Victorian State Government Operational Infrastructure Support, the Australian Cancer Research Foundation and the Australian Government NHMRC IRIISS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Manuscript

Author Manuscript

References

- Bansal, A., Singh, S., More, K.R., Hans, D., Nangalia, K., Yogavel, M., *et al.* (2013) Characterization of *Plasmodium falciparum* calcium-dependent protein kinase 1 (PfCDPK1) and its role in microneme secretion during erythrocyte invasion. *J Biol Chem* **288**: 1590–1602.
- Berridge, M.J. (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* **1793**: 933–940.
- Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* **4**: 517–529.
- Biftu, T., Feng, D., Ponpipom, M., Girotra, N., Liang, G.B., Qian, X.X., *et al.* (2005) Synthesis and SAR of 2,3-diarylpyrrole inhibitors of parasite cGMP-dependent protein kinase as novel anticoccidial agents. *Bioorg Med Chem Lett* **15**: 3296–3301.
- Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* **117**: 503–514.
- Billker, O., Lourido, S., and Sibley, L.D. (2009) Calcium-dependent signaling and kinases in apicomplexan parasites. *Cell Host Microbe* **5**: 612–622.
- Black, M.W., Arrizabalaga, G., and Boothroyd, J.C. (2000) Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Mol Cell Biol* **20**: 9399–9408.
- Blodgett, J., Coleman, B.I., Chen, C.-T., and Gubbels, M.-J. (2015) Lytic Cycle of *Toxoplasma gondii*: 15 Years Later. *Annu Rev Microbiol* **69**: 463–485.
- Borges-Pereira, L., Budu, A., McKnight, C.A., Moore, C.A., Vella, S.A., Hortua Triana, M.A., *et al.* (2015) Calcium Signaling Throughout the *Toxoplasma gondii* Lytic Cycle. A Study Using Genetically Encoded Calcium Indicators. *J Biol Chem* **290**: 26914–26926.
- Brockett, M., Collins, M.O., Smith, T.K., Thompson, E., Sebastian, S., Volkmann, K., *et al.* (2014) Phosphoinositide Metabolism Links cGMP-Dependent Protein Kinase G to Essential Ca²⁺ Signals at Key Decision Points in the Life Cycle of Malaria Parasites. *PLoS Biol* **12**: e1001800.
- Brown, K.M., Lourido, S., and Sibley, L.D. (2016) Serum Albumin Stimulates Protein Kinase G-dependent Microneme Secretion in *Toxoplasma gondii*. *J Biol Chem* **291**: 9554–9565.
- Bullen, H.E., Jia, Y., Yamaryo-Botté, Y., Bisio, H., Zhang, O., Jemelin, N.K., *et al.* (2016) Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in *Toxoplasma*. *Cell Host Microbe* **19**: 349–360.
- Carey, A.F., Singer, M., Bargieri, D., Thiberge, S., Frischknecht, F., Ménard, R., and Amino, R. (2014) Calcium dynamics of *Plasmodium berghei* sporozoite motility. *Cell Microbiol* **16**:

768–783.

Carruthers, V.B., and Sibley, L.D. (1997) Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. *European Journal of Cell Biology* **73**: 114–123.

Carruthers, V.B., and Sibley, L.D. (1999) Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol* **31**: 421–428.

Chen, T.-W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., *et al.* (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**: 295–300.

Chini, E.N., Nagamune, K., Wetzel, D.M., and Sibley, L.D. (2005) Evidence that the cADPR signalling pathway controls calcium-mediated microneme secretion in *Toxoplasma gondii*. *Biochem J* **389**: 269–277.

Collins, C.R., Hackett, F., Strath, M., Penzo, M., Withers-Martinez, C., Baker, D.A., and Blackman, M.J. (2013) Malaria Parasite cGMP-dependent Protein Kinase Regulates Blood Stage Merozoite Secretory Organelle Discharge and Egress. *PLoS Pathog* **9**: e1003344.

Dawn, A., Singh, S., More, K.R., Siddiqui, F.A., Pachikara, N., Ramdani, G., *et al.* (2014) The Central Role of cAMP in Regulating *Plasmodium falciparum* Merozoite Invasion of Human Erythrocytes. *PLoS Pathog* **10**: e1004520.

Donald, R.G.K., and Liberator, P.A. (2002) Molecular characterization of a coccidian parasite cGMP dependent protein kinase. *Mol Biochem Parasitol* **120**: 165–175.

Donald, R.G.K., Zhong, T., Wiersma, H., Nare, B., Yao, D., Lee, A., *et al.* (2006) Anticoccidial kinase inhibitors: Identification of protein kinase targets secondary to cGMP-dependent protein kinase. *Mol Biochem Parasitol* **149**: 86–98.

Dvornik, J.D., Martyn, D.C., Patel, S.D., Grimley, J.S., Collins, C.R., Hopp, C.S., *et al.* (2010) A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science* **328**: 910–912.

Eaton, M.S., Weiss, L.M., and Kim, K. (2006) Cyclic nucleotide kinases and tachyzoite-bradyzoite transition in *Toxoplasma gondii*. *Int J Parasitol* **36**: 107–114.

Flemer, J., Prandota, J., Sovičková, M., and Israili, Z.H. (2014) Toxoplasmosis—a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS ONE* **9**: e90203.

Garrison, E., Treeck, M., Ehret, E., Butz, H., Garbuz, T., Oswald, B.P., *et al.* (2012) A Forward Genetic Screen Reveals that Calcium-dependent Protein Kinase 3 Regulates Egress in *Toxoplasma*. *PLoS Pathog* **8**: e1003049.

Heintzelman, M.B. (2015) Gliding motility in apicomplexan parasites. *Semin Cell Dev Biol* **46**: 135–142.

Hortle, E., Nijagal, B., Bauer, D.C., Jensen, L.M., Ahn, S.B., Cockburn, I.A., Lampkin, S., Tull, D., McConville, M.J., McMorrán, B.J., Foote, S.J., Burgio, G. (2016) Adenosine monophosphate deaminase 3 activation shortens erythrocyte half-life and provides malaria resistance in mice. *Blood* **128**(9):1290-301.

Howard, B.L., Harvey, K.L., Stewart, R., Azevedo, M.F., Crabb, B.S., Jennings, I.G., *et al.* (2015) Identification of Potent Phosphodiesterase Inhibitors that Demonstrate Cyclic Nucleotide-Dependent Functions in Apicomplexan Parasites. *ACS Chem Biol* **10**: 1145–1154.

Kafsack, B.F.C., Pena, J.D.O., Coppen, I., Ravindran, S., Boothroyd, J.C., Carruthers, V.B. (2009) Rapid membrane disruption by a Perforin-Like Protein facilitates parasite exit from host cells. *Science* **323**: 530-533

Lourido, S., and Moreno, S.N.J. (2014) The calcium signaling toolkit of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium* **57**: 186-193.

Lourido, S., Shuman, J., Zhang, C., Shokat, K.M., Hui, R., and Sibley, L.D. (2010) Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. *Nature* **465**: 359–362.

Lourido, S., Tang, K., and Sibley, L.D. (2012) Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. *EMBO J* **31**: 4524–4534.

Lovett, J.L. (2003) Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells. *J Cell Sci* **116**: 3009–3016.

Lovett, J.L., Marchesini, N., Moreno, S.N.J., and Sibley, L.D. (2002) *Toxoplasma gondii* microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP3)/ryanodine-sensitive stores. *J Biol Chem* **277**: 25870–25876.

Luo, S., Ruiz, F.A., and Moreno, S.N.J. (2004) The acidocalcisome Ca²⁺-ATPase (TgA1) of *Toxoplasma gondii* is required for polyphosphate storage, intracellular calcium homeostasis and virulence. *Mol Microbiol* **55**: 1034–1045.

McCoy, J.M., Whitehead, L., van Dooren, G.G., and Tonkin, C.J. (2012) TgCDPK3 regulates calcium dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog* **8**: e1003066.

Meissner, M., Schluter, D., and Soldati, D. (2002) Role of *Toxoplasma gondii* myosin A in governing parasite gliding and host cell invasion. *Science* **298**: 837–840.

Mouly, B., Manning, T.J., and Beckers, C.J. (2001) The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J Biol Chem* **276**: 41492–41501.

Münter, S., Sabass, B., Selhuber-Unkel, C., Kudryashev, M., Hegge, S., Engel, U., *et al.* (2009) *Plasmodium* sporozoite motility is modulated by the turnover of discrete adhesion sites. *Cell Host Microbe* **6**: 551–562.

Nagamune, K., Beatty, W.L., and Sibley, L.D. (2007) Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. *Eukaryotic Cell* **6**: 2147–2156.

Nagamune, K., Hicks, L.M., Fux, B., Brossier, F., Chini, E.N., and Sibley, L.D. (2008) Abscisic acid controls calcium-dependent egress and development in *Toxoplasma gondii*. *Nature* **451**: 207–210.

Niedelman, W., Sprockholt, J.K., Clough, B., Frickel, E.-M., and Saeij, J.P.J. (2013) Cell death of gamma interferon-stimulated human fibroblasts upon *Toxoplasma gondii* infection induces early parasite egress and limits parasite replication. *Infect Immun* **81**: 4341–4349.

Pappas, G., Roussos, N., and Falagas, M.E. (2009) Toxoplasmosis snapshots: Global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *Int J Parasitol* **39**: 1385–1394.

Persson, E.K., Agnarson, A.M., Lambert, H., Hitziger, N., Yagita, H., Chambers, B.J., *et al.* (2007) Death receptor ligation or exposure to perforin trigger rapid egress of the intracellular parasite *Toxoplasma gondii*. *J Immunol* **179**: 8357–8365.

Roiko, M.S., Svezhova, N., and Carruthers, V.B. (2014) Acidification Activates *Toxoplasma gondii* Motility and Egress by Enhancing Protein Secretion and Cytolytic Activity. *PLoS Pathog* **10**: e1004488.

Sebastian, S., Brochet, M., Collins, M.O., Schwach, F., Jones, M.L., Goulding, D., *et al.* (2012) A *Plasmodium* Calcium-Dependent Protein Kinase Controls Zygote Development and Transmission by Translationally Activating Repressed mRNAs. *Cell Host Microbe* **12**: 9–19.

Siddh, S.M., Hortua Triana, M.A., Paul, A.S., Bakkouri, El, M., Hackett, C.G., Tran, F., *et al.* (2016) Using a Genetically Encoded Sensor to Identify Inhibitors of *Toxoplasma gondii* Ca²⁺ Signaling. *J Biol Chem* **291**:9566-9580

Taylor, G.J., McRobert, L., and Baker, D.A. (2008) Disruption of a *Plasmodium falciparum* cyclic nucleotide phosphodiesterase gene causes aberrant gametogenesis. *Mol Microbiol* **69**: 110–118.

Towle, T., Chang, I., Kerns, R.J., and Bhanot, P. (2013) Chemical probes of a trisubstituted pyrrole to identify its protein target(s) in *Plasmodium* sporozoites (vol 23, pg 1874, 2013). *Bioorg Med Chem Lett* **23**: 2812–2812.

Uhlen, P. and Fritz, N. (2010) Biochemistry of calcium oscillations. *Biochem Biophys Res Commun* **396**: 28–32.

Weinmont, A.K., Artz, J.D., Finerty, P., Lin, Y.-H., Amani, M., Allali-Hassani, A., *et al.* (2010) Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. *Nat Struct Mol Biol* **17**: 596–601.

Wetzel, D.M., Chen, L.A., Ruiz, F.A., Moreno, S.N.J., and Sibley, L.D. (2004) Calcium-mediated protein secretion potentiates motility in *Toxoplasma gondii*. *J Cell Sci* **117**: 5739–5748.

Wiersma, H.I., Galuska, S.E., Tomley, F.M., Sibley, L.D., Liberator, P.A., and Donald, R.G.K. (2004) A role for coccidian cGMP-dependent protein kinase in motility and invasion.

Int J Parasitol **34**: 369–380.

Williams, M.J., Alonso, H., Enciso, M., Egarter, S., Sheiner, L., Meissner, M., *et al.* (2015) Two essential light chains regulate the MyoA lever arm to promote *Toxoplasma* gliding motility. *MBio* **6**: e00845–15.

World Health Organization (2014) report 2014. 1–242.

Author Manuscript

Figure legends

Figure 1. GCaMP6s can detect changes in intracellular Ca^{2+} of *Toxoplasma*.

(A) Genetic strategy for expression GCaMP6s/mCherry integrated as a single copy into *uprt* of tachyzoites. (B) GCaMP6s and mCherry are expressed and localised to the tachyzoite cytoplasm in extracellular and intracellular tachyzoites. GCaMP6s = green, mCherry = red. (C) Validation of GCaMP6s for monitoring release of intracellular Ca^{2+} stores. Tachyzoites were harvested in modified Ringer's buffer (-0.1 mM EGTA) to remove external Ca^{2+} sources and then stimulated with A23187. Ca^{2+} response false coloured where blue = low Ca^{2+} and red = high Ca^{2+} . Scale bar = 10 μm here and throughout. (D) Tracking of Ca^{2+} response before and after A23187 addition shows a 2-3 fold change in Ca^{2+} signalling as determined by ratiometric analysis of ΔCa^{2+} (GCaMP6s/mCherry, normalised to 0). (E) Motility change accompanying Ca^{2+} responses in absence of environmental Ca^{2+} shows an average of 60% of tachyzoites engaging motility after A23187 stimulation -/+s.e.m, n=4. (F) Basal intracellular tachyzoites maintain low Ca^{2+} levels. A23187 treatment stimulates amplification of the Ca^{2+} signal, peaking prior to parasite egress as detected with our GCaMP6s approach.

Figure 2. Protein Kinase G is required for Ca^{2+} signalling and egress in *Toxoplasma*

A. Relative levels of (i) cGMP and (ii) cAMP upon 1 μM BIPPO and 2 μM of A23187. n=2-5, -/+s.e.m (Bi) Induction of cGMP signalling using BIPPO activates

cytoplasmic Ca^{2+} signalling within *Toxoplasma* prior to egress. **(Bii)** Ratiometric analysis of Ca^{2+} responses to BIPPO confirms rapid amplification of Ca^{2+} levels before peaking prior to egress. Here and throughout ratiometric analysis is displayed as: one line = one vacuole, analysis ceases at point of egress, x axis = ΔCa^{2+} as GCaMP6s/mCherry normalised to half max Ca^{2+} , y axis = normalised to GCaMP6s/mCherry = t_0 . n = 12 vacuoles. **(Ci)** PKG inhibition by Cmpd1 blocks cGMP signal transduction preventing BIPPO induced Ca^{2+} response and egress of intracellular tachyzoites. **(Cii)** Ratiometric analysis confirms inability of Cmpd1 treated cells to engage Ca^{2+} signalling in response to BIPPO. n = 12, filmed up to 10 min. **(Di)** Host cell permeabilisation with saponin leads to a rise in intracellular Ca^{2+} and activation of egress. Ca^{2+} activation is not uniform across tachyzoites within individual vacuoles displaying differential Ca^{2+} amplification before reaching peak Ca^{2+} followed by egress (amplification, arrows). **(Dii)** Ratiometric analysis shows rapid amplification of Ca^{2+} signalling in response to saponin treatment, reaching a peak before a short period of Ca^{2+} dampening preceding egress. **(Ei)** Inhibition of PKG by Cmpd1 prevents saponin-induced egress but tachyzoites still show variable Ca^{2+} responses indicating alternate Ca^{2+} activation mechanisms independent of PKG signalling. **(Eii)** Ratiometric analysis demonstrates initial peak of Ca^{2+} levels followed by dynamic Ca^{2+} response to saponin in Cmpd1 treated cells, but egress does not occur. **(F)** Schematic of Ca^{2+} metrics investigated by ratiometric analysis; basal Ca^{2+} , time to egress and peak Ca^{2+} ratio. **(G)** Basal Ca^{2+} levels of resting intracellular vacuoles assessed as GCaMP6s/mCherry ratio, measured over 15 s,

normalised to 1 = WT average. No difference was detected between basal Ca^{2+} of WT and Cmpd1 treated tachyzoites. $n = 12$, \pm s.e.m. **(H)** WT parasites egressed within 100 s of BIPPO, Saponin or A23187 stimulation. Cmpd1 treated tachyzoites show defective activation with no egress seen for BIPPO and Saponin stimulation. **(I)** Analysis of Peak Ca^{2+} ratio derived from ratiometric analysis compared across $-/+$ Cmpd1 stimulated with BIPPO, Saponin and A23187. Quantitation demonstrates a defect in Ca^{2+} induction with BIPPO stimulation of Cmpd1 treated cells. Although Peak Ca^{2+} is retained in Cmpd1 treated cells activated with Saponin and A23187 this Ca^{2+} signalling is not sufficient for egress without cGMP signalling through PKG. $n = 8-14$ vacuoles, \pm s.e.m.

Figure 3. $\Delta TgCDPK3$ egress defects can be overcome with activation of cGMP pathways.

(Ai) Temporal analysis of egress and Ca^{2+} signalling in $\Delta TgCDPK3$ parasites shows A23187 induces Ca^{2+} amplification but not egress. **(Aii)** Ratiometric tracking of Ca^{2+} response demonstrates Ca^{2+} peak prior to slow decline of Ca^{2+} levels over 10 min of filming, parasites do not egress. $n = 11$ vacuoles. **(B)** Visual **(i)** and ratiometric **(ii)** tracking of BIPPO induced Ca^{2+} induction leading to egress of $\Delta TgCDPK3$. $n = 10$ vacuoles. **(C)** Visual **(i)** and ratiometric **(ii)** tracking of Saponin induced Ca^{2+} induction leading to egress of $\Delta TgCDPK3$. $n = 11$ vacuoles. **(E)** Comparison of basal Ca^{2+} of resting intracellular tachyzoites shows no difference between WT v. $\Delta TgCDPK3$ indicating no role for $TgCDPK3$ in Ca^{2+} homeostasis. **(F)** Quantitation of

Peak Ca^{2+} in $\Delta TgCDPK3$ when stimulated with A23187, BIPPO or Saponin show comparable peak Ca^{2+} levels of ~3 fold change indicating no role of $TgCDPK3$ in Ca^{2+} release potentials. **(G)** Schematic outlining assessment of rate of change of Ca^{2+} during amplification period. **(H)** Comparison of WT and $\Delta TgCDPK3$ amplification capacity in response to A23187, BIPPO and saponin. A moderate amplification defect is seen in $\Delta TgCDPK3$ stimulated with A23187; $p=0.029$, unpaired student t-test $-/+$ s.e.m. Amplification rates are comparable between WT and $\Delta TgCDPK3$ for BIPPO and Saponin treatment. **(I)** Egress analysis confirms activation of BIPPO and Saponin treated $\Delta TgCDPK3$ cells, but not with A23187. BIPPO and Saponin egress is significantly delayed in comparison to WT lines ($p<0.0001$, unpaired student t-test). $n = 10-14$ vacuoles, $-/+$ s.e.m.

Figure 4. $TgCDPK1$ is required downstream of Ca^{2+} signalling during *Toxoplasma* egress

(A) Temporal analysis of 3MB-PP1-treated tachyzoites shows intact Ca^{2+} responses to A23187 stimulation but egress does not occur within a 10 min timeframe. **(B)** BIPPO induction of Ca^{2+} signalling for 3MB-PP1 pre-treated parasites. Tachyzoites do engage with parasitophorous membranes (arrows) although do not disseminate away from vacuoles productively. First instances of parasite interaction with parasitophorous membranes is scored as faulty egress (*egress). **(C)** Ratiometric tracking of Ca^{2+} dynamics shows normal amplification and peak Ca^{2+} of 3MB-PP1 treated lines for stimulation with A23187 **(i)**, BIPPO **(ii)**, and Saponin **(iii)** $n = 8-13$ vacuoles. **(D)** A point mutation of the gatekeeper residue at the opening of the

nucleotide-binding pocket (Gly to Met) renders *Tg*CDPK1_M resistant to 3MB-PP1 inhibition. *Tg*CDPK1_M parasites pre-treated with 3MB-PP1 respond to A23187 (i), BIPPO (ii) and Saponin (iii) similar to WT showing no obvious defects in Ca²⁺ or egress responses. (E) Intracellular Ca²⁺ ratios are comparable between 3MB-PP1 sensitive and resistant (*Tg*CDPK1_M) lines, assessed as GCaMP6s/mCherry ratio, measured over 15 s, normalised to 1 = CDPK1_M + 3MB-PP1 average. (F) Both 3MB-PP1 sensitive and resistant (*Tg*CDPK1_M) lines induced similar Ca²⁺ peak ratios prior to *egress for A23187, BIPPO and Saponin stimulation. (G) Egress was morphologically abnormal and delayed in all 3MB-PP1 sensitive *egress stimulations. *Tg*CDPK1_M 3MB-PP1 resistance retains normal kinetics with egress occurring within 100 s for all conditions. -/+s.e.m. unpaired student t-test.

Figure 5. *Tg*CDPK1 and *Tg*CDPK3 play a role in Ca²⁺ dampening prior to egress

(A) Schematic outlining ratiometric analysis of Ca²⁺ sequestration immediately prior to egress, denoted as rate decrease. Analysis was performed in two phases; Ca²⁺ decay peak to halfway and Ca²⁺ decay halfway to egress. Halfway is defined as point at which ΔCa^{2+} is 50 % of $\text{Ca}^{2+}_{\text{peak}} - \text{Ca}^{2+}_{\text{egress}}$. (B) Analysis of saponin traces reveals differences between WT (black), $\Delta\text{TgCDPK3}$ (green) and 3MB-PP1 (blue) treated tachyzoites in the peak to egress phase. Rate of decrease was assessed with Peak Ca²⁺ normalised to 0. Kinetics of Ca²⁺ sequestration in the peak to halfway phase shows similar rates of Ca²⁺ decrease in all cell lines (inset). Halfway to egress analysis shows a dramatic decrease in Ca²⁺ decay and delay to initiation of egress for

$\Delta TgCDPK3$ and 3MB-PP1 treated WT tachyzoites (light blue). Deletion of microneme protein *TgPLP* causes delayed saponin induced egress with a Ca^{2+} decay rate distinct from that of CDPK mutants. (C) Quantitation of Ca^{2+} rate decrease shows comparable rates between all lines for peak to halfway Ca^{2+} decay phase. WT lines maintain the rate of decay in the hallway to egress phase. $\Delta TgCDPK3$ and 3MB-PP1 biphasic response shows clear, significant decreases in Ca^{2+} decay rate in the halfway to egress phase that is not present in WT, $CDPK1_M$ or egress defective $\Delta TgPLP$ tachyzoites. Student t-test \pm s.e.m. n = 7-13 vacuoles.

Figure 6. cGMP and Ca^{2+} signalling are interconnected during *Toxoplasma* motility.

(Ai, Bi) Extracellular tachyzoites settled onto substrate maintain low Ca^{2+} and low motility pre-stimulation, time-lapse projection over 60 s. False coloured Ca^{2+} reads, blue = low Ca^{2+} , red = high Ca^{2+} . Where parasites are motile in these conditions Ca^{2+} signalling is also evident (arrows). A23187 (Aii) or BIPPO (Bii) activates Ca^{2+} signalling and motility in extracellular tachyzoites, time lapse projection over 60 s. (C) Tachyzoite movement classification where >80% of WT parasite are non-motile pre-stimulation but show ~80% induction of either circular, helical, twirling or short motility after A23187 or BIPPO stimulation. Cmpd1 pre-treatment prevents motility activation with ~80% of parasites remaining non-motile after BIPPO stimulation. (D) Population averages of Ca^{2+} responses over time demonstrate gross Ca^{2+} induction of the entire population. Cmpd1 inhibition sees tachyzoite populations maintain a lower

resting GCaMP6s/mCherry ratio that fails to induce robust Ca^{2+} changes after A23187 or BIPPO activation (purple line). Normalised average WT GFP/mCHERRY over an unstimulated 60 s period. WT; n = 6, Cmpd1; n = 5, -/+s.e.m.

Figure 7. Level of Ca^{2+} signalling correlates with the quality of motility in *Toxoplasma*

Individual motility profiles tracking parasites in response to A23187 (A) and BIPPO (B) stimulation. In all cases, prior to stimulation displacement remains $<1 \mu\text{m}$ (non-motile – thick grey line, μm = thin black line). Circular (Ai, Bi) and helical (Aii, Bii) motility show a moderate correlation between displacement and Ca^{2+} flux where bursts of motility are paralleled with Ca^{2+} peaks (circles = coloured lines, helical = maroon, twirling = blue). Parasites that remain non-motile post stimulation do not show dynamic Ca^{2+} flux or correlation between Ca^{2+} and displacement (Aiii, Biii) (Greyed out zone represents data incorporated into correlation assessment). Quantitation of motility parameters velocity (Ci), flux distance – distance travelled between Ca^{2+} peaks (Cii), and time elapsed between Ca^{2+} peaks (Ciii). n = 8-20 individual parasites tracked for each condition, displayed as points, -/+ s.e.m. (Di, ii) Cmpd1 pre-treated parasites are not motile and do not display Ca^{2+} flux when activated with A23187 or BIPPO.

Supplementary information

Methods

Drug autofluorescence: Fluorescent measurements of GCaMP6s and mCherry (arbitrary units A.U) were measured in regions devoid of parasites before and after the addition of drugs used in this study in increasing concentrations. Measurements were averaged from 15seconds of filming, n=2.

Selective permeabilisation of membranes using saponin: Fresh tachyzoites were harvested away from host cells in Ringer's medium (containing Ca^{2+}) and settled onto Poly-L-lysine coated glass slide. Tachyzoite movement and Ca^{2+} response was recorded before and after addition of saponin.

Figure legends

Supplementary Figure 1.

(1A) Fluorescent intensity of media and drugs was measured for regions of host cells devoid of tachyzoites. GCaMP and mCherry fluorescence were measured (arbitrary units A.U) before and after addition of a titration of the drugs A23187 (1Ai), BIPPO (1Bi) and Saponin (1Ci) with concentrations as indicated. No appreciable change in fluorescence was detected. GCaMP6s/mCherry ratio was also determined (1Aii-Cii) where no appreciable change was seen before and after addition of drugs. Green = GCaMP6s, red = mCherry, grey = ratio, -/+ s.e.m.

(D) Selective permeabilisation of saponin was determined using extracellular tachyzoites settled onto Poly-L-lysine coated slides (Di). Addition of saponin alone, without changes in pH or $[\text{K}^+]$, did not activate parasite motility or Ca^{2+} signalling (Dii). Average fluorescent intensity was measured for two fields of view (FOV),

FOV1: pre- and post- saponin addition, FOV2: post – saponin addition. Green = GCaMP6s, red = mCherry, grey = ratio.

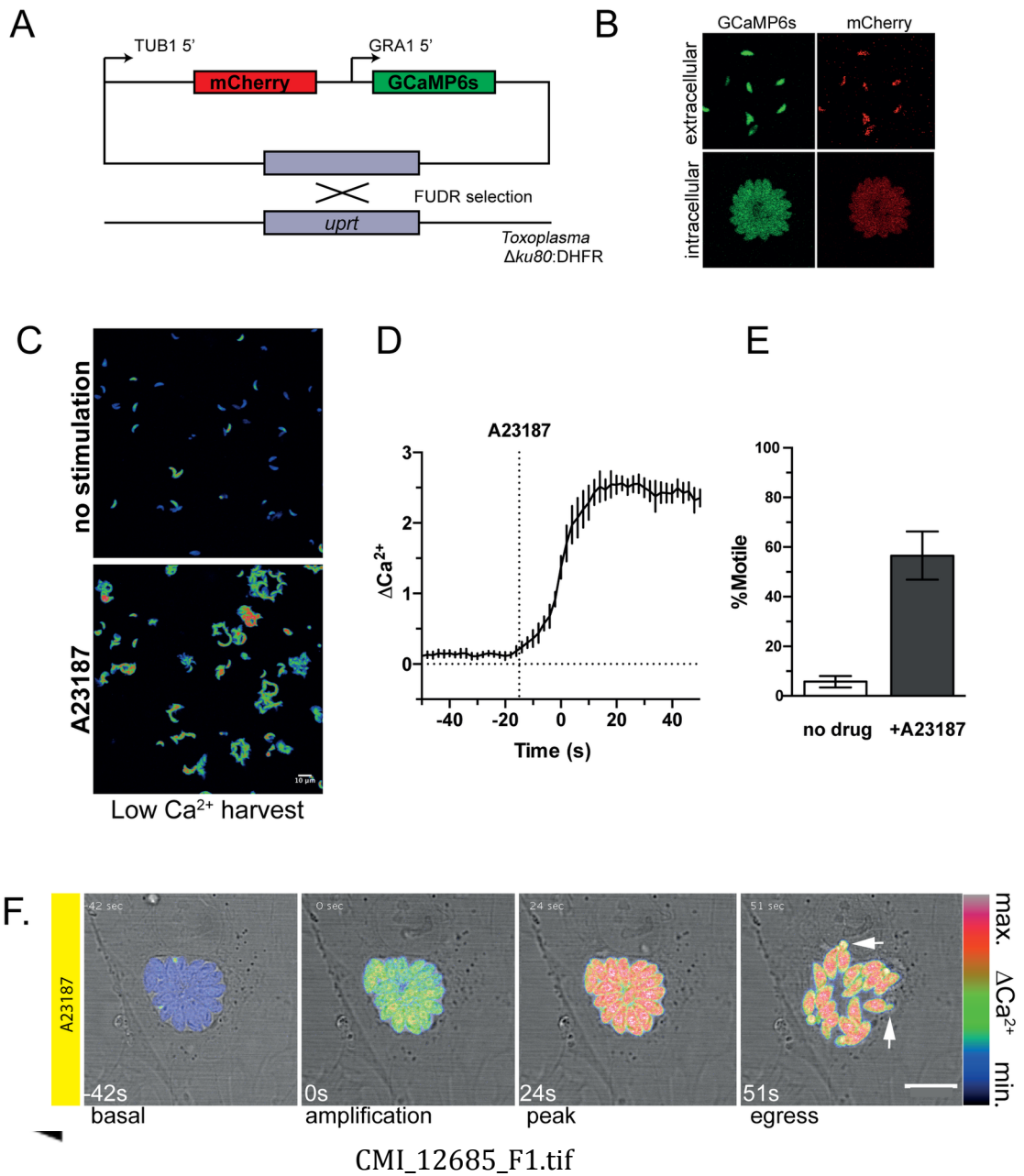
Supplementary Figure 2.

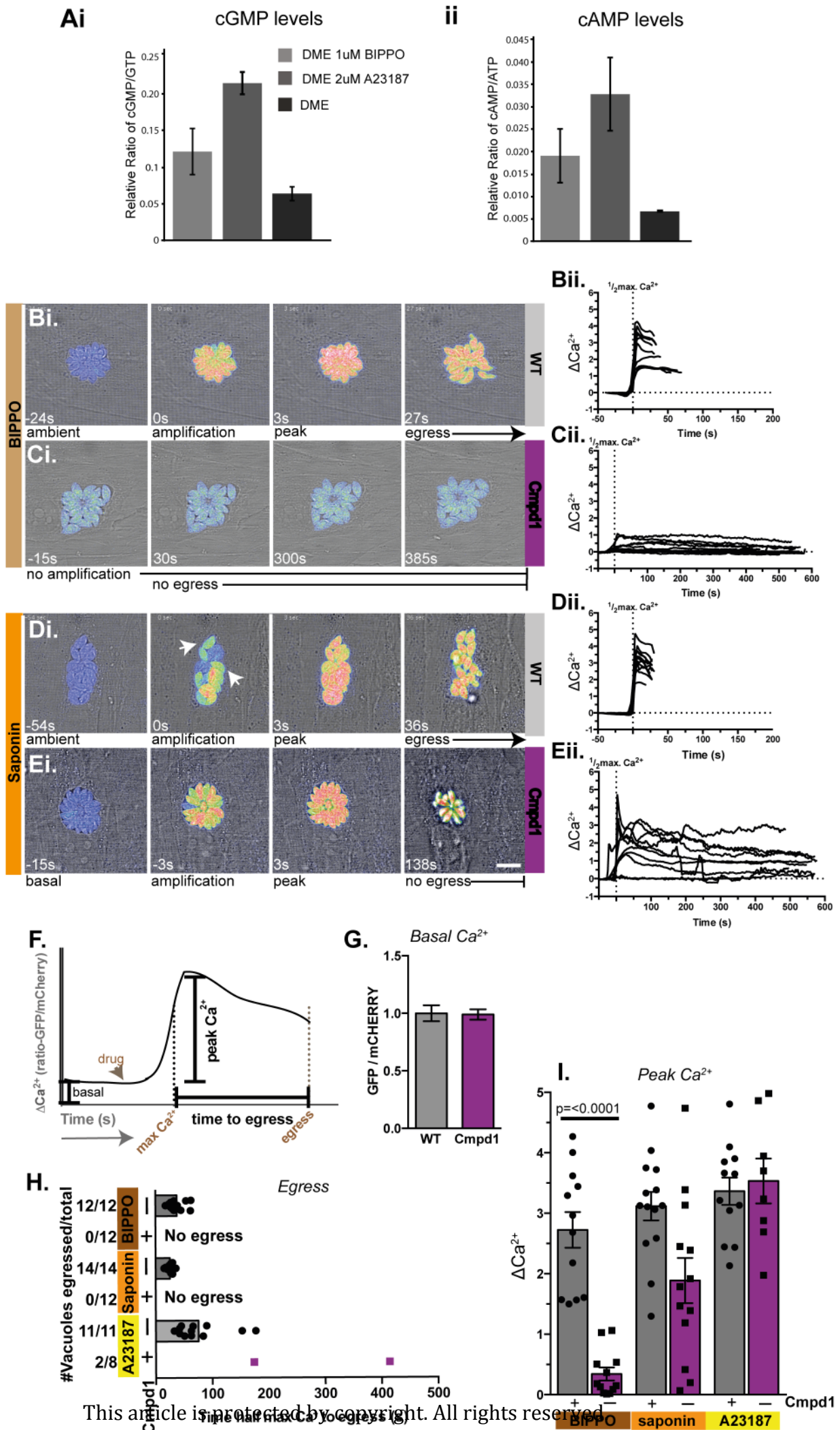
Microneme protein PLP does not attenuate Ca²⁺ metrics during egress

Ai. Following BIPPO stimulation Δ PLP parasites rapidly engage Ca²⁺, amplifying cytosolic Ca²⁺ from basal levels to peak levels prior to intravacuole movement characteristic of Δ PLP. Egress proceeds peak Ca²⁺ intravacuole movement, possibly due to mechanical disruption of membranes in the absence of enzymatic of PLP. **Aii.** Saponin treatment similarly stimulates rapid amplification of Ca²⁺ before intravacuolar movement and egress of Δ PLP parasites. **Bi-Biii.** Ratiometric analysis of Ca²⁺ response to egress agonists confirms visual assessment with rapid amplification of Ca²⁺ prior to egress. **C.** Side by side comparison of egress agonists reveals no defect for Peak Ca²⁺ (Ci), or rate of Ca²⁺ amplification (Cii) in Δ PLP parasites. As expected A23187 stimulated egress was defective in Δ PLP parasites (Cii - A23187). Δ PLP had delayed time to egress when stimulated with BIPPO and Saponin in comparison to WT lines (Ciii – BIPPO, Saponin).

Author Manuscript

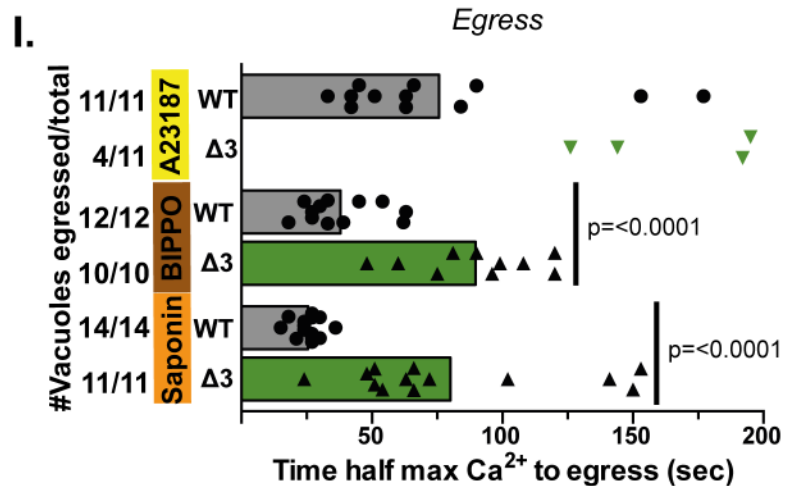
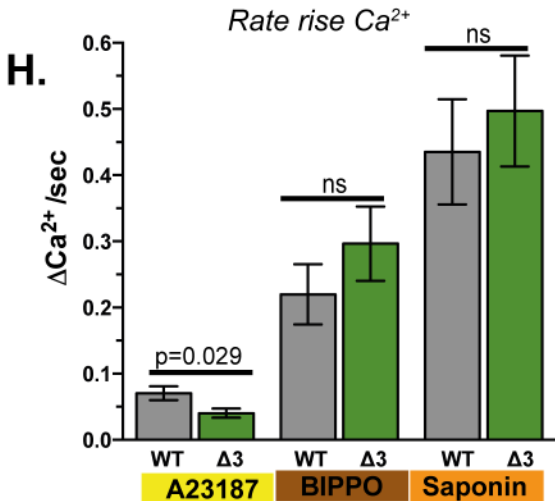
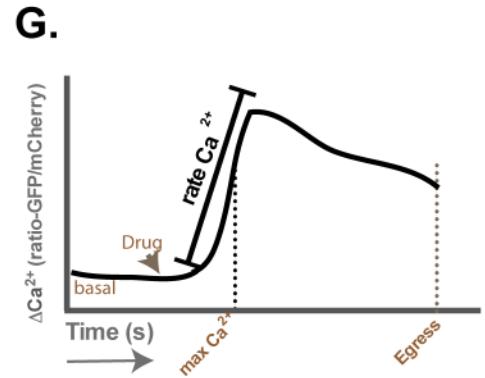
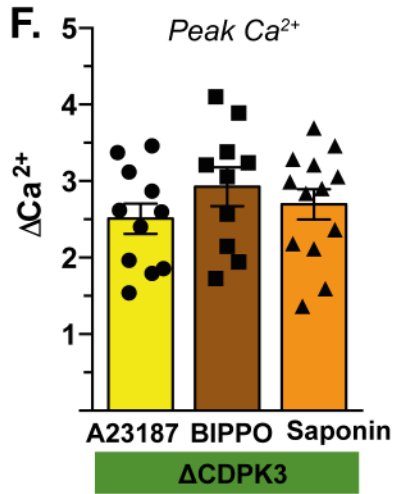
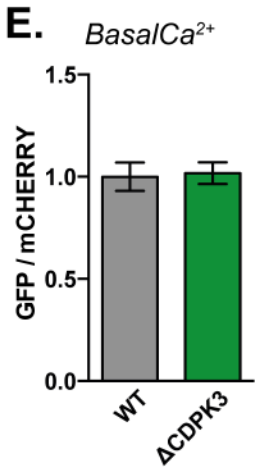
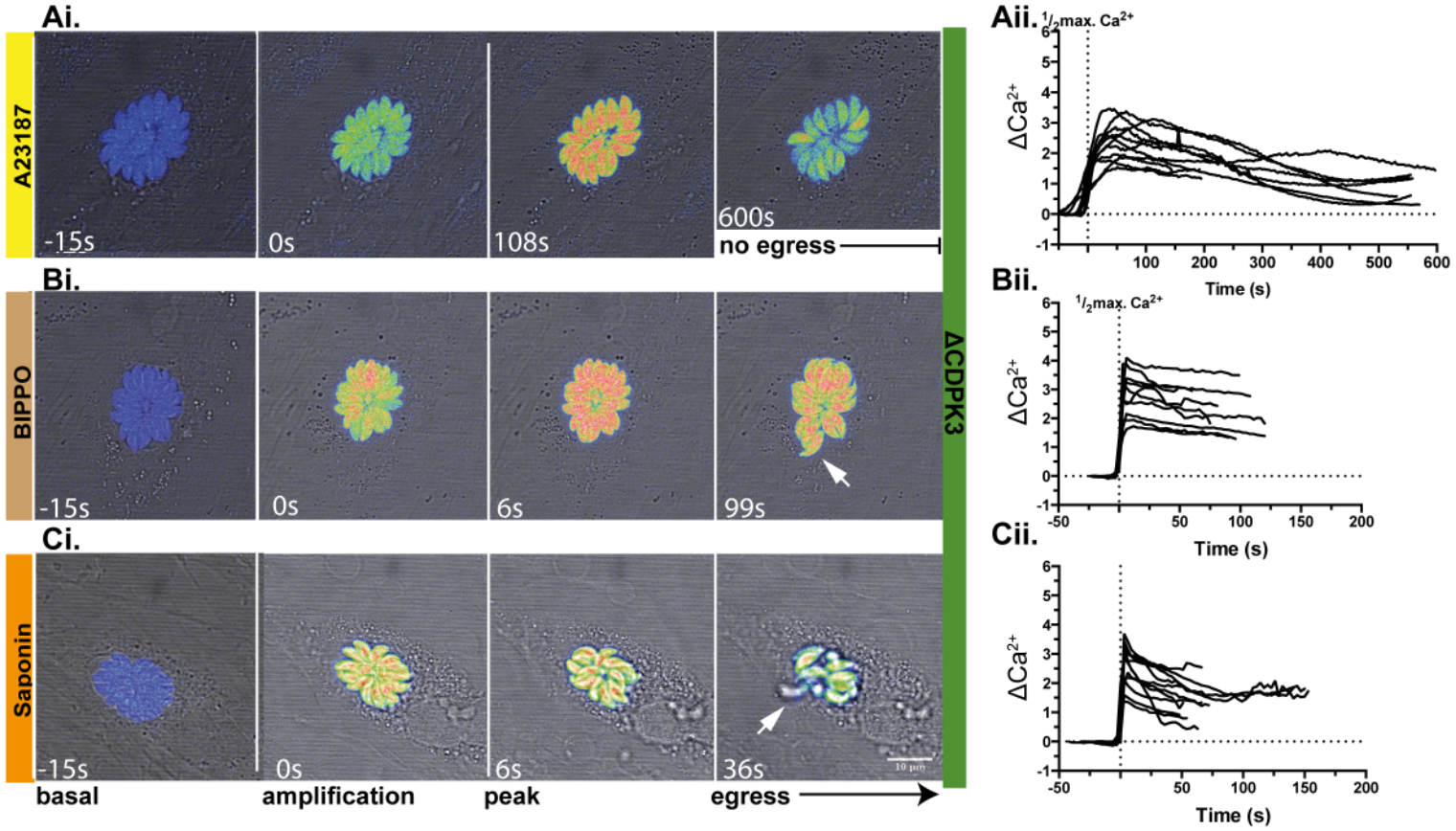
Stewart *et al.* Figure 1.

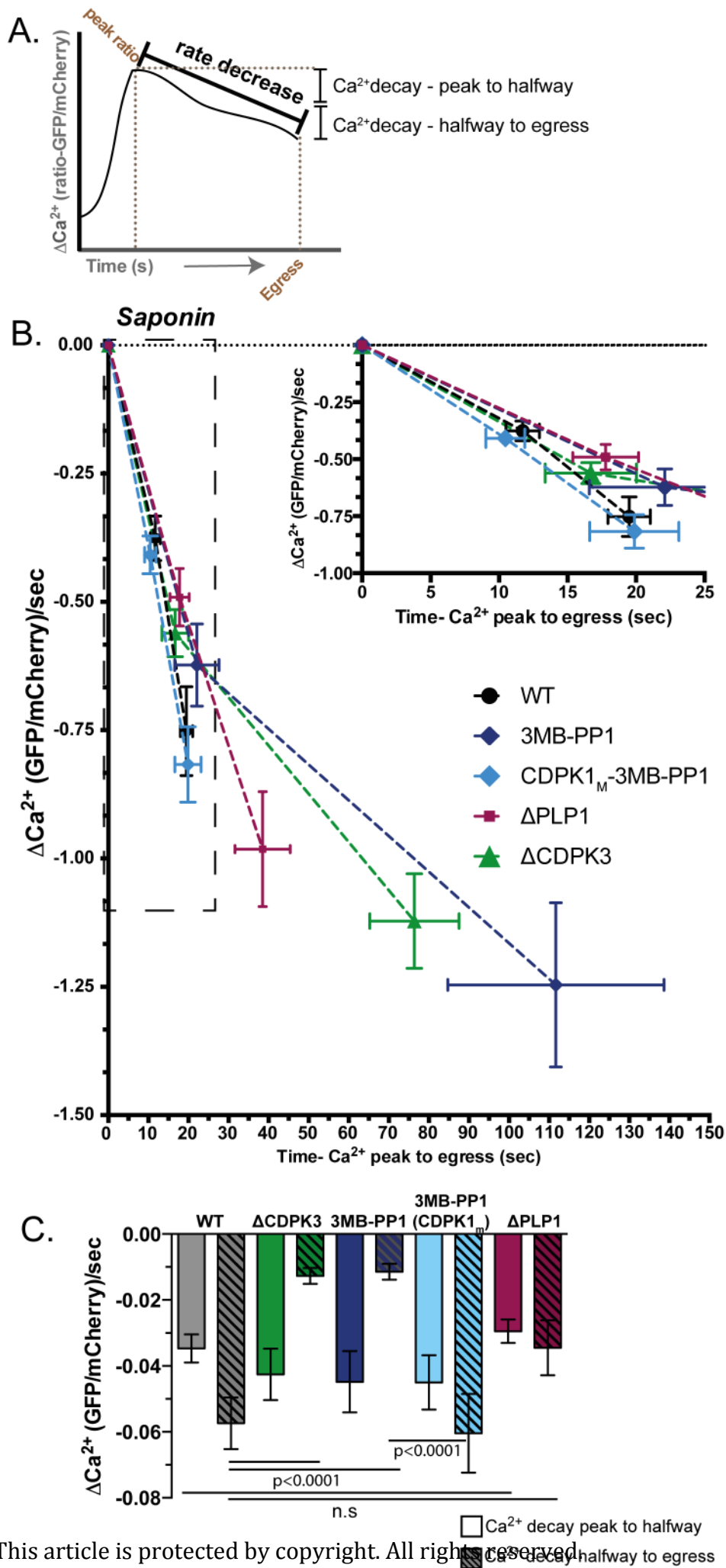




Stewart et al. Figure 3.

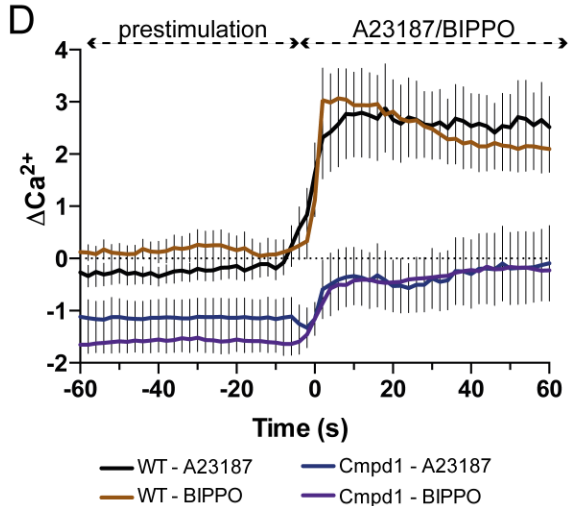
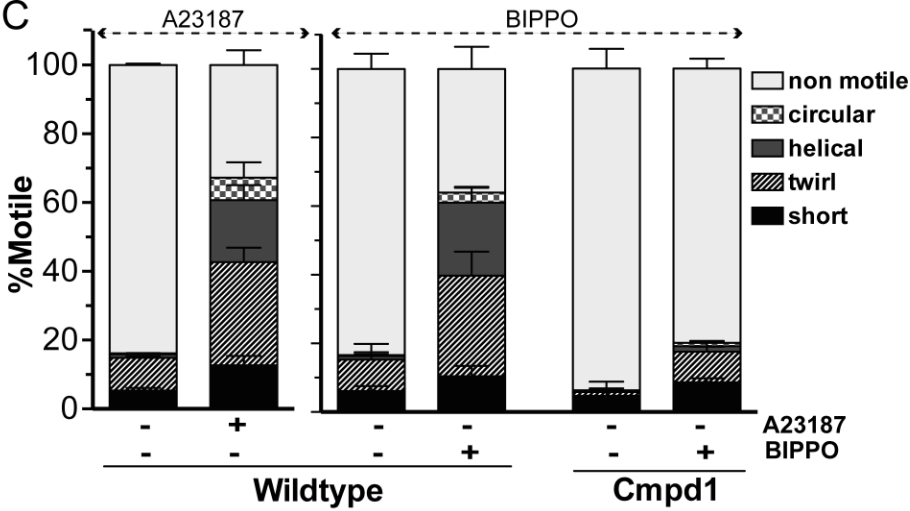
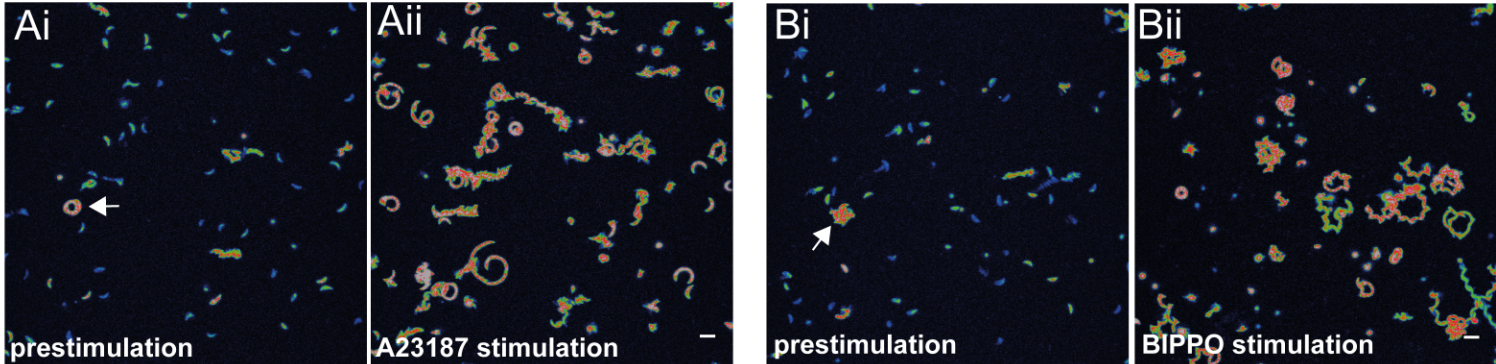
Δ CDPK3





pt

Stewart et al. Figure 6.

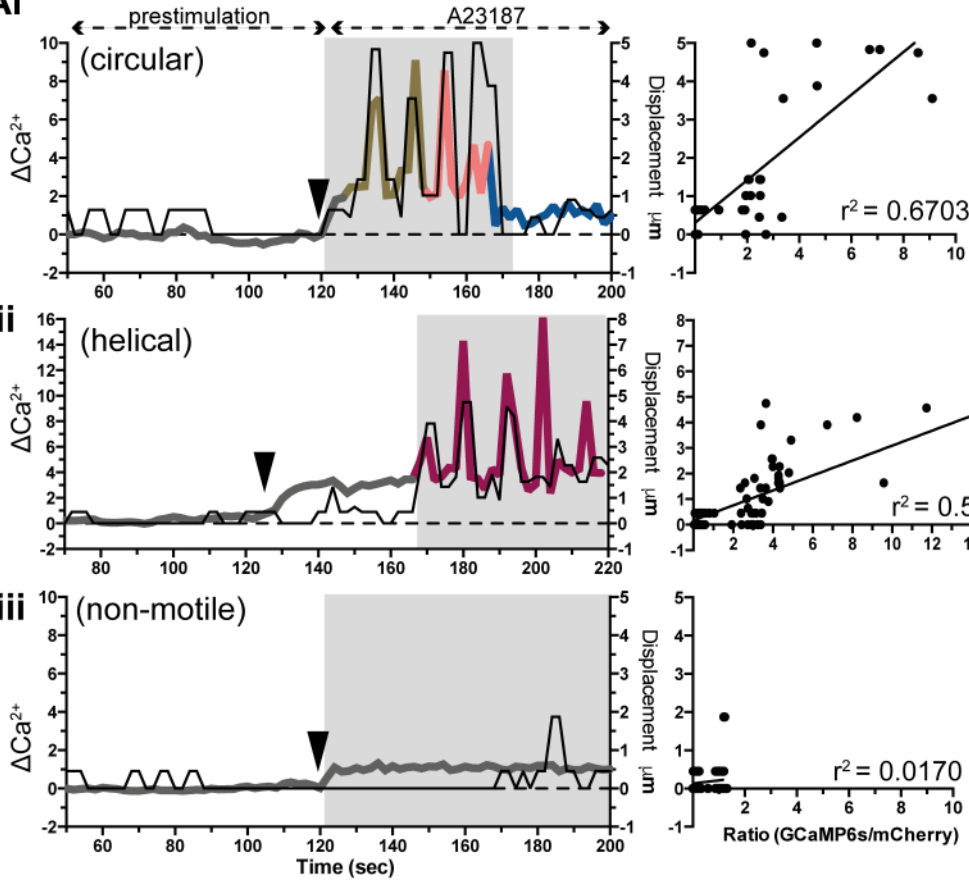


Al

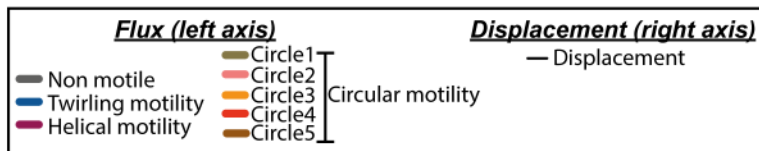
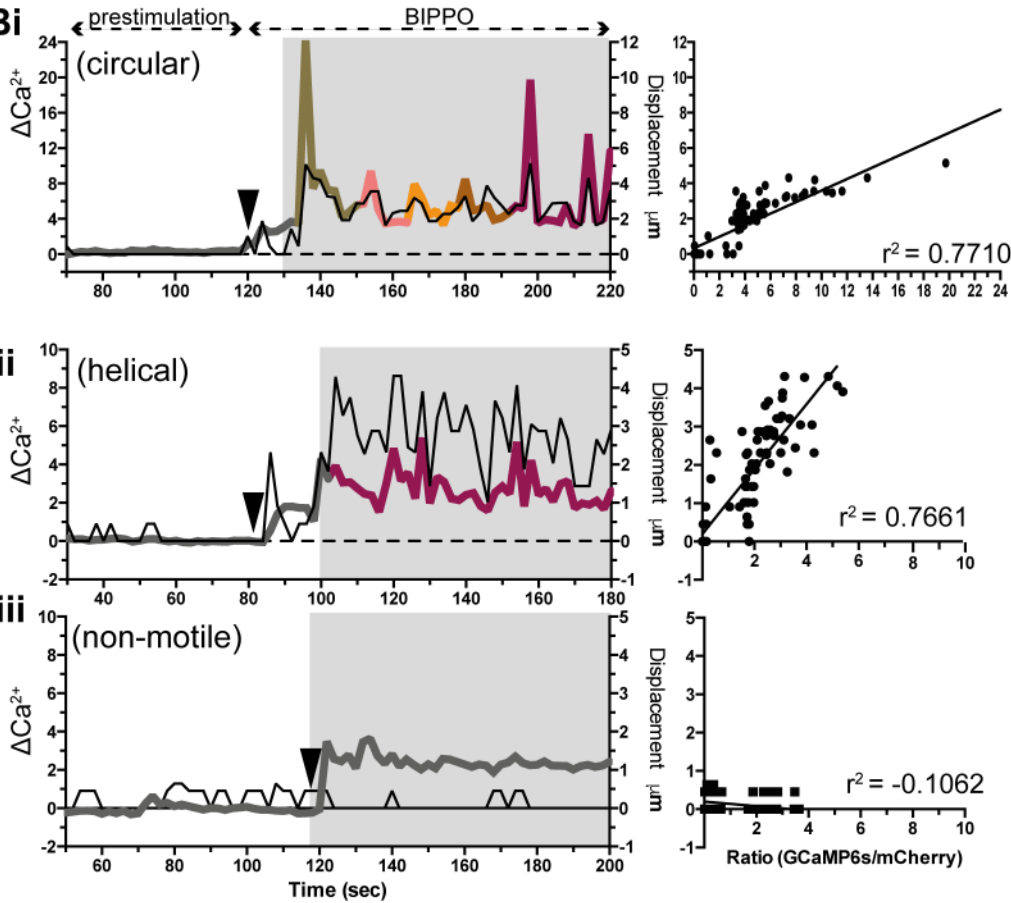
CMI_12685_F6.tif

Stewart et al. Figure 7.

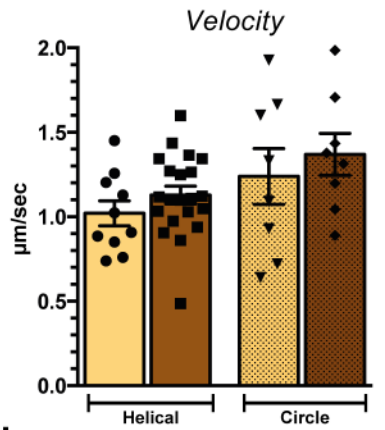
Ai



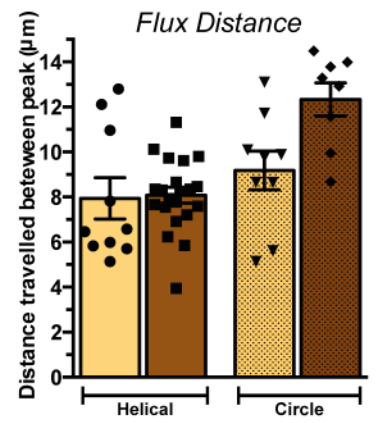
Bi



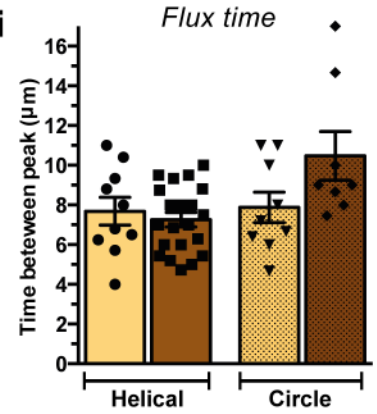
Ci



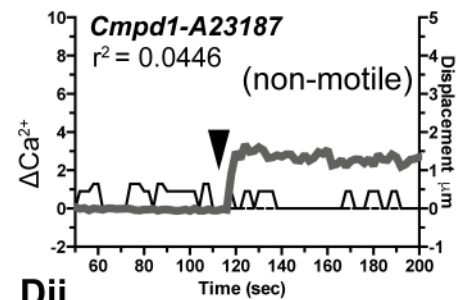
Cii



Ciii



Di



Dii

