Calcium negatively regulates secretion from dense granules in *Toxoplasma gondii*

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**Abstract**
Apicomplexan parasites including *Toxoplasma gondii* and *Plasmodium* spp. manufacture a complex arsenal of secreted proteins used to interact with and manipulate their host environment. These proteins are organised into three principle exocytotic compartment types according to their functions: micronemes for extracellular attachment and motility, rhoptries for host cell penetration, and dense granules for subsequent manipulation of the host intracellular environment. The order and timing of these events during the parasite’s invasion cycle dictates when exocytosis from each compartment occurs. Tight control of compartment secretion is, therefore, an integral part of apicomplexan biology. Control of microneme exocytosis is best understood, where cytosolic intermediate molecular messengers cGMP and Ca\(^{2+}\) act as positive signals. The mechanisms for controlling secretion from rhoptries and dense granules, however, are virtually unknown. Here, we present evidence that dense granule exocytosis is negatively regulated by cytosolic Ca\(^{2+}\), and we show that this Ca\(^{2+}\)-mediated response is contingent on the function of calcium-dependent protein kinases TgCDPK1 and TgCDPK3. Reciprocal control of micronemes and dense granules provides an elegant solution to the mutually exclusive functions of these exocytotic compartments in parasite invasion cycles and further demonstrates the central role that Ca\(^{2+}\) signalling plays in the invasion biology of apicomplexan parasites.

**KEYWORDS**
Apicomplexa, calcium, dense granules, Protozoa, signalling, Toxoplasma

1 | INTRODUCTION

Apicomplexan parasites comprise a large phylum of primarily obligate intracellular parasites of humans and animals that have a significant impact on human health and livestock production. Notable apicomplexan genera include blood parasites *Plasmodium* (causative agents of malaria), *Babesia* and *Theileria* (common cattle parasites), enteric epithelial parasites *Cryptosporidium* and *Eimeria*, and systemic parasites *Toxoplasma* and *Neospora*. The phylum embraces at least 6,000 species with global distribution infecting animals and even other protists (Adl et al., 2012). Moreover, metagenomic environmental sampling shows that apicomplexans can be dominant components of natural communities, indicating significant roles in ecosystems and the evolutionary success of this group (de Vargas et al., 2015; Mahé et al., 2017). One key to the success of apicomplexans is their efficient infection cycles in which they select their host cell, penetrate it...
nondestructively, feed and multiply within this cell while subduing or deflecting host organism defences, and finally escape from the host cell, releasing multiple progeny (Blader, Coleman, Chen, & Gubbels, 2015). This cycle is largely mediated by co-ordinated release of a number of different exocytotic compartments delivering cargo to a range of extracellular destinations.

Toxoplasma gondii has served as a model for apicomplexan infection cycle events with three categories of secretory compartments identified—micronemes, rhoptries, and dense granules—that facilitate the major events of the invasion cycle (Carruthers & Sibley, 1997). Micronemes are exocytosed when the parasite is searching for a host cell, and secreted microneme proteins (MICS) decorate the parasite cell surface to act as attachment ligands and enable the characteristic gliding motility of the group (Frénal, Dubremetz, Lebrun, & Soldati-Favre, 2017). Upon selection of a cell to invade, proteins from rhoptry organelles are then secreted into the host, forming a "moving junction" entry structure through which the parasite penetrates the host (Guérin et al., 2017). As the parasite enters, a host plasma membrane-derived parasitophorous vacuole (PV) invaginates and surrounds the parasite. PV formation is accompanied by secretion of further rhoptry proteins into the host, some of which actively block host attack of this new internal foreign body (Etheridge et al., 2014; Häkansson, Charron, & Sibley, 2001). Completion of invasion isolates the PV from the plasma membrane, and a third wave of secretion from the dense granules now occurs (Carruthers & Sibley, 1997; Dubremetz, Achbarou, Bermudes, & Joiner, 1993; Mercier & Cesbron-Delauw, 2015; Sibley, Niesman, Parmley, & Cesbron-Delauw, 1995). Dense granule proteins (GRAs) populate and modify the PV membrane for nutrient uptake and help create an elaborate PV-containing membranous nanotubular network (Mercier, Adjigble, Däubener, & Delauw, 2005; Sibley et al., 1995). Other GRAs target the host cytoplasm and nucleus and actively reprogram host cell regulatory pathways and functions to facilitate parasite survival and growth (Hakimi, Olia, & Sibley, 2017). After multiple rounds of parasite division, a new infection cycle begins with the secretion of MICS that disrupt host membranes and reestablish gliding motility for escape, dissemination, and targeting of new host cells (Kafsack et al., 2009).

Broadly, control of secretion from micronemes is critical for the extracellular stages of the Toxoplasma infection cycle, control of rhoptry release for the invasion events, and control of dense granule release for the establishment and maintenance of the host cell environment for the parasite. The coordination of organelle-specific exocytosis is, therefore, a central feature of the parasite's biology.

Only the control of microneme exocytosis has been studied and illuminated in any detail. The elevation of cytosolic calcium ion (Ca^{2+}) levels by release from intracellular stores signals release of MICS to the extracellular environment (Carruthers, Giddings, & Sibley, 1999; Sidik et al., 2016). Ca^{2+} also stimulates other processes, including extrusion of the conoid and activation of motility, so Ca^{2+} signalling is clearly part of a broader signalling network of the extracellular events of the invasion cycle (Billker, Louriño, & Sibley, 2009; Borges-Pereira et al., 2015; Graindorge et al., 2016; Stewart et al., 2017; Tang et al., 2014; Wetzel, Chen, Ruiz, Moreno, & Sibley, 2004). Two Ca^{2+}-dependent protein kinases, TgCDPK1 and TgCDPK3, are major controllers of Ca^{2+}-dependent extracellular processes, including MIC secretion (Louriño et al., 2010; Louriño, Jeschke, Turk, & Sibley, 2013; Louriño, Tang, & Sibley, 2012; McCoy, Whitehead, van Dooren, & Tonkin, 2012; Treeck et al., 2014). Loss of function of either results in changes to Ca^{2+}-induced microneme exocytosis, although changes are not identical, suggesting some level of specialization and/or cooperativity of these kinases (Louriño et al., 2012). Numerous protein substrates have been identified for both TgCDPK1 and TgCDPK3, further evidence for an elaborate signalling network that they control (Louriño et al., 2013; Treeck et al., 2014). Ca^{2+} also has a downstream and direct role for MIC release with exocytosis of micronemes at the apical plasma membrane facilitated by DOC2.1 that recruits the membrane fusion machinery in a Ca^{2+}-dependent manner (Farrell et al., 2012).

Other signalling molecules and stimuli occur upstream of Ca^{2+} and illustrate an even broader network of control processes for parasite sensing of cues for its invasion cycle (Carruthers, Moreno, & Sibley, 1999). Cyclic guanosine monophosphate (cGMP) activates protein kinase G (PKG), and PKG in turn triggers cytosolic Ca^{2+} flux in Toxoplasma and Plasmodium (Brochet et al., 2014; Sidik et al., 2016; Stewart et al., 2017). In Plasmodium berghei, PKG acts upon phosphoinositide metabolism that ultimately releases inositol (1,4,5)-trisphosphate (IP3) from diacylglycerol (DAG), and IP3 releases Ca^{2+} stores in many systems (Brochet et al., 2014; Schloßmann et al., 2000). This phospholipid metabolism, notably DAG to phosphatidic acid (PA) interchange, has also been shown to contribute directly to microneme docking at the plasma membrane in Toxoplasma, further implicating cGMP-controlled events in MIC release (Bullen et al., 2016). The ultimate stimulation mechanism(s) for these cGMP- and Ca^{2+}-dependent events has not been identified; however, in Toxoplasma, both a reduction in extracellular pH and potassium ion levels appear to have important roles as a cues for these processes (Endo, Tokuda, Yagita, & Koyama, 1987; Roiko, Svezhova, & Carruthers, 2014). Upon successful entry of parasites into their host cell, all of this activation for MIC release and motility must then be supressed in order for the rhoptry- and dense granule-mediated intracellular events to progress. Recent work suggests that CAMP-signalling that activates protein kinase A (PKA) is involved in reducing cytosolic Ca^{2+} levels upon host cell entry and, in turn, reversing the processes that led to MIC secretion (Jia et al., 2017; Uboldi et al., 2018).

Whereas Ca^{2+} and cGMP have emerged as central signals for positive control of microneme exocytosis, almost nothing is known about how secretion from rhoptries and dense granules is controlled. Dense granules present a particular conundrum, for although some secretion through these organelles has been characterised as unregulated or constitutive, a strong burst of GRA secretion occurs as a postinvasion event, implying some mechanism for its control (Carruthers & Sibley, 1997; Chaturvedi et al., 1999; Coppons, Andries, Liu, & Cesbron-Delauw, 1999; Dubremetz et al., 1993; Mercier & Cesbron-Delauw, 2015; Sibley et al., 1995). Nevertheless, GRA secretion from extracellular parasites is detectable and has often been used as a presumed invariant secretion control in assays of regulated MIC release. This assumption of GRA behaviour, however, has never been thoroughly tested, and, in fact, a decrease in GRA secretion has been seen (but rarely commented upon) when extracellular parasites are treated with some Ca^{2+} agonists.
(Carruthers, Moreno, & Sibley, 1999; Farrell et al., 2012; Kafsack et al., 2009; Paul et al., 2015).

Here, we have examined the role of Ca\(^{2+}\) and cGMP in the regulation of GRA secretion in extracellular tachyzoites using a range of modulators of both Ca\(^{2+}\) and cGMP levels. We have also tested for GRA secretion behaviour in mutant cell lines of \(\text{TgCDPK1, TgCDPK3, TgRNG2}\), and using kinase inhibitors, all of which have known defects in Ca\(^{2+}\)- or cGMP-dependent secretion (Katris et al., 2014; Lourido et al., 2012; McCoy et al., 2012). Our data consistently indicate that Ca\(^{2+}\) has a role in negatively controlling GRA secretion, providing a reciprocal control mechanism to that of MIC secretion in extracellular parasites.

2 | RESULTS

2.1 | Agonists and antagonists of cytosolic Ca\(^{2+}\) inversely modulated microneme and dense granule exocytosis

To test for any Ca\(^{2+}\)-dependent responses in dense granule exocytosis from extracellular tachyzoites, we applied a range of concentrations of two commonly used Ca\(^{2+}\) ionophores, ionomycin and A23187, which induce a range in levels of MIC secretion response. A gradual increase in MIC2 secretion (above constitutive levels) was observed with increasing concentrations of both ionomycin and A23187 from 1 to 5 \(\mu\)M (Figure 1a). We simultaneously assayed for secretion of the dense GRAs GRA1, GRA2, and GRA5. In all cases, we saw an inverse response to the ionophore treatment, with decreased secretion of dense GRAs observed with increasing ionophore concentration (Figure 1a). We also assayed for MIC5 secretion, a protein that is proteolytically processed before sorting to the micronemes. The shorter, processed form is secreted from the micronemes, whereas the longer pro-form of the protein (proMIC5) is believed to take an alternative route, and its secretion has been described as constitutive (Brydges, Harper, Parussini, Coppens, & Carruthers, 2008). We saw reciprocal responses of MIC5 and proMIC5 secretion with Ca\(^{2+}\) ionophore treatment: MIC5 release responded positively to Ca\(^{2+}\), as for MIC2; whereas proMIC5 showed reduced secretion with Ca\(^{2+}\), similar to the GRA protein responses (Figure 1a).

Cytosolic Ca\(^{2+}\) levels can also be indirectly elevated by activating the PKG signalling pathway with cGMP (Sidik et al., 2016; Stewart et al., 2017). Two phosphodiesterase inhibitors have been widely used to increase cGMP levels: zaprinast, and a more potent analogue 5-benzyl-3-iso-propyl-1H-pyrazolo[4.3-d]pyrimidin-7(6H)-one (BIPPO; Howard et al., 2015). Increasing concentrations of BIPPO and zaprinast over a range shown to stimulate MIC secretion (0–5 \(\mu\)M and 0–500 \(\mu\)M, respectively) were applied to tachyzoites. MIC2 secretion was far more responsive to BIPPO than zaprinast, with approximately equivalent MIC2 secretion seen with 0.5 \(\mu\)M BIPPO and 200–500 \(\mu\)M zaprinast (Figure 1b). At these concentrations, only minor reduction in GRA1 secretion was seen; however, as BIPPO concentrations were further increased through 1–5 \(\mu\)M, a clear decrease in secreted GRA1 was observed (Figure 1b). We note that phosphodiesterase inhibitors have been reported to elevate cAMP in addition to cGMP in Plasmodium (Howard et al., 2015). In Toxoplasma, cAMP is implicated in reducing cytosolic Ca\(^{2+}\) and, although we do not know if any similar elevation of cAMP occurs with BIPPO/zaprinast...
treatment, the observed increase in cytosolic Ca\textsuperscript{2+} in Toxoplasma with these agents suggest that cAMP is unlikely to be a major contributor to this response (Jia et al., 2017; Stewart et al., 2017; Uboldi et al., 2018).

We tested that the changes to MIC and GRA secretion observed with these Ca\textsuperscript{2+} and cGMP agonists were not due to adverse secondary effects on the cell, including cell lysis or premature cell death during the secretion assay. To test for cell lysis or loss of plasma membrane integrity, we assayed for the release of the soluble cytosolic protein profilin under secretion assay conditions using 5\mu M of either A23187 or BIPPO. No release of this marker was seen with either treatment (Figure 1c). To test for cell death, cells were pelleted after the secretion assays, washed in growth medium, and equal volumes used to inoculate host cell monolayers for each of the 5\mu M A23187, 5\mu M BIPPO, or vehicle (DMSO) control treatments. After 8 days of growth, plaque density in the host monolayer reported the relative number of cells that were invasion-competent after the secretion assay and able to generate an ongoing lytic infection cycle. No difference was seen between agonist treatments and the control (Figure 1d). Thus, tachyzoites evidently remain intact and viable throughout the secretion assay.

We further tested for the effect on GRA secretion of modulators of cytosolic Ca\textsuperscript{2+} by treating cells with either BAPTA-AM or thapsigargin. BAPTA-AM is a membrane-permeable Ca\textsuperscript{2+}-chelator so treatment reduces available Ca\textsuperscript{2+}, whereas thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca\textsuperscript{2+}ATPase (SERCA), which is believed responsible for recharging sequestered Ca\textsuperscript{2+} pools. Thapsigargin treatment, thus, leads to cytosolic accumulation of Ca\textsuperscript{2+}. BAPTA-AM treatment resulted in loss of constitutive secretion from micronemes, and no change to GRA secretion, consistent with a role of elevated Ca\textsuperscript{2+} in both of these processes (Figure 2a). Thapsigargin treatment resulted in elevated MIC secretion, but no change in GRA secretion when applied at 10\mu M (Figure 2a).

In summary, we observe an inverse correlation between MIC and GRA secretion in response to changes in cytosolic Ca\textsuperscript{2+}. Further, there is evidence of separation between the manner of eliciting Ca\textsuperscript{2+} signalling and the proportional responses of MIC and GRA secretion. Ionomophore treatment to directly release Ca\textsuperscript{2+} stores results in marked increase in MIC secretion and concomitant decrease in GRA secretion (Figures 1 and 2). Conversely, indirect methods for elevating cytosolic Ca\textsuperscript{2+}—moderate cGMP stimulus and thapsigargin—result in strong MIC secretion, but proportionately less inhibition of GRA secretion (Figures 1 and 2).

### 2.2 Mutants in Ca\textsuperscript{2+} signalling disrupt both MIC and GRA secretion responses

Ca\textsuperscript{2+}-dependent protein kinases (TgCDPKs) 1 and 3 in Toxoplasma are involved in controlling cell processes relevant to invasion and egress, including microneme exocytosis, where elevated Ca\textsuperscript{2+} triggers activation of these processes (Lourido et al., 2010; Lourido et al., 2012; McCoy et al., 2012). Given our data that GRA secretion negatively correlates with cytosolic Ca\textsuperscript{2+} level increase, we tested if TgCDPK1 and/or 3 might be involved in regulating dense granule exocytosis. To test for a role of TgCDPK1, we used an inducible knockdown cell line (iΔHA-TgCDPK1) (Lourido et al., 2010) in which TgCDPK1 levels were strongly depleted after 72 hr of anhydrotetracycline (ATc) treatment (Figure 3a). Untreated (−ATc) iΔHA-TgCDPK1 cells showed typical constitutive MIC and GRA secretion, and both A23187-responsive MIC secretion and coincident inhibition of GRA secretion (Figure 3a–c), consistent with wildtype cells (Figures 1 and 2). When TgCDPK1 was depleted (+ATc), levels of MIC secretion were strongly reduced in both constitutive and A23187-treated states compared with the −ATc controls. The suppression of GRA secretion with A23187 treatment was also reduced in the TgCDPK1-depleted cells (Figure 3a,c). Therefore, depletion of TgCDPK1 simultaneously results in reductions in both the Ca\textsuperscript{2+}-induced increase in microneme exocytosis and inhibition of dense granule exocytosis.

We also used a chemical inhibition strategy to test for the role of TgCDPK1 in the control of secretion from dense granules. The kinase inhibitor 3-methyl-benzyl pyrazolo [3,4-d] pyrimidine (3-MB-PP1) is specific to TgCDPK1 in T. gondii (Lourido et al., 2010; Lourido et al., 2012), and cells treated with this inhibitor were concurrently assayed for changes of microneme and dense granule exocytosis. Extracellular parasites were treated with 3-MB-PP1 for 5 min post harvesting, and then assayed for protein secretion. Ca\textsuperscript{2+}-induced (A23187 treatment) MIC secretion was lost with 3-MB-PP1 treatment, consistent with inhibition of TgCDPK1 (Figure 4a(i)). Interestingly, constitutive levels...

![FIGURE 2](image-url) Relative effects of modulators of cytosolic Ca\textsuperscript{2+} on microneme protein (MIC) and granule protein (GRA) secretion. (a) Ca\textsuperscript{2+} ionophores A23187 and ionomycin; Ca\textsuperscript{2+} sequestration inhibitor thapsigargin; cGMP agonists zaprinast (zap.) and BIPPO; and Ca\textsuperscript{2+} chelator BAPTA-AM; all affect MIC secretion and have varying effects on GRA secretion. (b) Relative secretion of MIC2 and GRA1 shown over eight biological replicates. ESA; extracellular secretion assay; TOM40 used as a cell equivalents loading control. Error bars = SEM.
of microneme secretion were unaffected, unlike the TgCDPK1 KD. In these experimental conditions, dense granule exocytosis was also found to be unaffected in that increasing \([\text{Ca}^{2+}]\) could inhibit levels of GRA secretion, equivalent to the 3-MB-PP1 untreated cells (Figure 4a(i), b(ii)). Absence of CDPK3 is seen by CDPK3 immuno-detection in the cell pellet (d). TOM40 in ESA serves a control for cell lysis and controls for cell equivalent loading in the pellet. Error bars = SEM.

**FIGURE 3** Effect of loss of CDPK1 or CDPK3 on \(\text{Ca}^{2+}\)-induced microneme protein (MIC) and granule protein (GRA) secretion. (a–c) Extracellular secretion assays (ESA) with and without ATc-induced CDPK1 depletion in \(\Delta \text{HA}-\text{CDPK1}\) cells. Depletion of HACDPK1 is seen by immuno-detection of HA in the cell pellet. A23187 (5μM) is used for \(\text{Ca}^{2+}\) stimulation. Relative secretion of MIC2 (b) and GRA1 (c) is shown \((n = 8)\). (d–f) ESA and relative secretion \((n = 7)\) measurements for wildtype (+CDPK3) versus CDPK3 knockout (−CDPK3) cells. Absence of CDPK3 is seen by CDPK3 immuno-detection in the cell pellet (d). TOM40 in ESA serves a control for cell lysis and controls for cell equivalent loading in the pellet. Error bars = SEM.

**FIGURE 4** Effect of CDPK1-inhibitor 3-MB-PP1 on \(\text{Ca}^{2+}\)-induced microneme protein (MIC) and granule protein (GRA) secretion. (a) Parasites were treated with 3-MB-PP1 either (i) after mechanical egress from host cells, or (ii) before egress, and then extracellular secretion assays (ESA) of MIC2 and GRA1 without or with A23187 (5μM)-induced \(\text{Ca}^{2+}\) release. (b) Relative GRA1 secretion was measured for DMSO controls \((n = 4)\), postharvest 3-MB-PP1 \((n = 4)\) and preharvest 3-MB-PP1 \((n = 3)\). Error bars = SEM.

and \(\text{Ca}^{2+}\)-induced MIC secretion (Figure 4a(ii)). Furthermore, \(\text{Ca}^{2+}\)-induced inhibition of dense GRA secretion was also substantially lost (Figure 4a(ii),b(iii)). These results mimic the effects of TgCDPK1 depletion and suggest that in the first 3-MB-PP1 experiment, some TgCDPK1 targets were phosphorylated after egress and persisted in this state despite subsequent 3-MB-PP1 inhibition.

We also tested for a role of TgCDPK3 in GRA secretion regulation using a cell line with the cdpk3 gene knocked out (ΔTgCDPK3; McCoy et al., 2012). Unlike the TgCDPK1 KD, TgCDPK3 absence did not affect constitutive MIC secretion (Figure 3d,e). When treated with A23187, Δ TgCDPK3 cells showed neither an increase in MIC secretion nor a decrease in GRA secretion, suggesting that TgCDPK3 is required for \(\text{Ca}^{2+}\)-mediated control of both of these processes (Figure 3d–f).

TgRNG2 is an apical complex protein involved in relaying a cGMP signal through to MIC secretion (Katris et al., 2014). Depletion of TgRNG2 interrupts the relay of this signal, although microneme secretion can be rescued with direct \(\text{Ca}^{2+}\) stimulation by A23187 (Figure 5a(i),b(i)). These data suggest that TgRNG2 operates between cGMP sensing and cytosolic \(\text{Ca}^{2+}\) elevation. We therefore used a
TgRNG2 inducible knockdown cell line (iΔHA-TgRNG2) to independently test if dense granule exocytosis control is regulated directly by Ca²⁺ rather than cGMP. TgRNG2-depleted cells showed normal levels of Ca²⁺-induced dense granule exocytosis inhibition and concurrent elevation of microneme secretion (Figure 5a(i),b(i),c(i)). When TgRNG2-depleted cells were activated via cGMP (2.5μM BIPPO), no change in dense granule secretion was seen compared with untreated cells (Figure 5a(ii),c(ii)). These data are consistent with dense granule regulation responding directly to Ca²⁺ and not cGMP.

3 | DISCUSSION

We have tested for changes to rates of dense granule protein secretion from extracellular tachyzoites in response to stimuli known to elicit changes in microneme exocytosis, namely, treatments that elevate cytosolic Ca²⁺ (summarised in Figure 6). We consistently see evidence of reduced GRA secretion in conditions that raise cytosolic Ca²⁺, including both ionophore treatment that allows discharge of Ca²⁺ stores into the cytoplasm, and cGMP treatment that indirectly raises cytosolic Ca²⁺ through activation of PKG (Brochet et al., 2014; Sidik et al., 2016; Stewart et al., 2017). Both treatment types increased MIC secretion. Mutant cell lines for TgCDPK1, TgCDPK3, and TgRNG2 with known phenotypes in microneme secretion control (Katris et al., 2014; Lourido et al., 2010; McCoy et al., 2012) all similarly displayed reciprocal GRA control phenotypes, further supporting a role for cytosolic Ca²⁺ levels in downregulation of GRA secretion. We also note that several published reports show evidence of Ca²⁺-mediated suppression of secretion of dense GRAs, although in these cases, the effects seen were either not commented on or explored (Carruthers, Moreno, & Sibley, 1999; Farrell et al., 2012; Kafsack et al., 2009; Paul et al., 2015). Chaturvedi et al. (1999) explicitly tested for increase of GRA secretion with Ca²⁺ stimulation by supplying exogenous Ca²⁺ to streptolysin O-permeabilized cells. They saw no change in GRA secretion, but it is not known what other cell processes might be perturbed by this permeabilization treatment, and a range of Ca²⁺ concentrations was not tested.

Whereas a reciprocal response of MIC and GRA secretion to Ca²⁺-based signalling is consistently evident, differences in their relative responses depends on the type of treatment, suggesting that different Ca²⁺-signalling events might drive these processes, as is also
the case for other infection-cycle events (Lourodo et al., 2012). For instance, thapsigargin elevates cytosolic Ca2+, but although this does lead to microneme secretion, it does not lead to increased motility or conoid extrusion without increased extracellular Ca2+ (Pace, Mcknight, Liu, Jimenez, & Moreno, 2014). We also observed no change in GRA secretion with thapsigargin, although MIC secretion does increase. Similarly, cGMP agonists BIPPO and zaprinast both resulted in strong MIC secretion increase, but much more subdued GRA secretion inhibition compared with the Ca2+ ionophores. These data indicate that the regulation of microneme exocytosis is more sensitive to Ca2+ than the regulation of dense granule exocytosis. Alternatively, cGMP-based signalling might contribute to some MIC secretion that is independent of Ca2+, such as lipid-mediated control of microneme exocytosis (Figure 6; Bullen et al., 2016).

The comparison of the TgCDPK1 and TgCDPK3 mutants provides further evidence of a separation between the mechanisms of Ca2+-mediated control of microneme versus dense granule secretion. Depletion of either of these kinases results in loss of Ca2+-dependent inhibition of GRA secretion, suggesting that substrates of both are required for this process. However, the MIC secretion response is quite different between TgCDPK1 and TgCDPK3 depletion: TgCDPK1 loss maintains a Ca2+-dependent MIC response although greatly reduced in magnitude, whereas TgCDPK3 loss results in Ca2+ insensitivity. This is consistent with some cooperativity between these two kinases where TgCDPK3 might activate the responsiveness of TgCDPK1 for MIC secretion, as others have suggested (Treeck et al., 2014). The complexity of Ca2+ signalling targets and their dynamics is further indicated by 3-MB-PP1 treatment either before or after egress, which also result in differences to constitutive MIC secretion as well as GRA control. Post-egress 3-MB-PP1 treatment leaves GRA secretion Ca2+-responsive, yet pre-egress treatment blocks this. This suggests that some stable, necessary TgCDPK1 phosphorylation of substrates occurs at the point of egress, and that TgCDPK3 substrates are then sufficient for the responsiveness of dense granules to Ca2+.

The location of GRA secretion from Toxoplasma tachyzoites has not been unambiguously determined; however, it has been suggested that dense granules fuse laterally at the parasite cell surface, rather than apically as micronemes and rhoptries do (Dubremetz et al., 1993). In any case, it is unlikely that suppression of dense granule release is a direct effect of increased microneme secretion and competition for space at the site of secretion. Even if they were to share the same exit point, our data show instances of strongly elevated microneme secretion with no change to dense granule secretion (e.g., zaprinast 250μM and thapsigargin 10μM).

The mechanism for Ca2+-mediated control of GRA secretion is currently unclear. Some GRA proteins that bear transmembrane domains and are membrane-associated after release into the host cell environment are known to be maintained as soluble high-molecular mass protein aggregates within the dense granules prior to release (Labruyere, Lingnau, Mercier, & Sibley, 1999; Lecordier, Mercier, Sibley, & Cesbron-Delauw, 1999; Sibley et al., 1995). GRA1, a highly abundant GRA with two Ca2+ binding domains, has been speculated to potentially play a role in control of this aggregated state (Lebrun, Carruthers, & Cesbron-Delauw, 2014). It is conceivable that switching from aggregated to disaggregated state of GRAs has a role in secretion regulation and that Ca2+ could modulate this. If so, dense granule luminal Ca2+ levels would be relevant and must also be controlled. Irrespective of such an internal control process, cytosolic factors are likely to be implicated given evidence of both TgCDPK1 and TgCDPK3 substrates participating in GRA regulation. These substrates might control trafficking of the dense granules or derived vesicles to the relevant location for exocytosis, and/or mediate fusion with the plasma membrane. Several proteins implicated in vesicular trafficking have been identified as CDPK targets in Plasmodium (Brochet et al., 2014). If a lateral site of dense granule fusion occurs, some reorganisation of the IMC including membrane cisternae and subpellicular filamentous network would likely be required for vesicular contact with the plasma membrane, and this might require further direction from CDPK1/3-mediated processes.

Although the mechanism for Ca2+-controlled secretion of dense granules is currently unknown, reciprocal control of micronemes and dense granules using the same signals has a clear biological logic. The protein cargos are mutually exclusive in terms of function—one for the extracellular processes, the other for the intracellular processes. After successful parasite invasion of a host cell, the suppression of MIC secretion by cAMP-mediated depletion of cytosolic Ca2+ would concomitantly allow for relaxation of GRA secretion suppression. Furthermore, the coupling of these signalling networks reinforces the potential for signal disruption as a druggable therapeutic strategy. Dysregulation of both MIC and GRA secretion could both interrupt control of the lytic invasion cycle and promote immune-recognition of a wide suite of GRA proteins that might otherwise only be released in the intracellular context.

4 | EXPERIMENTAL PROCEDURES

4.1 | Parasites cultures

*T. gondii* tachyzoites were grown by serial passage in human foreskin fibroblast (HFF) cells as previously described (Jacot, Meissner, Sheiner, Soldati-Favre, & Striepen, 2014). Briefly, Toxoplasma RH strain parasites were serially passaged in confluent HFF cells containing in ED1 media (Dulbecco’s modified Eagle’s medium [DMEM] supplemented with 1% foetal bovine serum [FBS], 0.2mM additional L-Glut, 50 Units/ml Penicillin/Streptomycin, and 0.25 μg/ml of amphotericin-B).

4.2 | Secretion assays

Parasite cultures were preincubated for at least 48 hr with or without ATc. Parasites were harvested after multiple rounds of parasite replication within the PV and with approximately 50–80% of vacuoles intact prior to natural egress. Cultures were scraped, host cells disrupted by syringe passing through a 26 gauge needle, filtered through a 3-μm polycarbonate filter to remove host debris, and parasites pelleted at 1000x g at 15°C for 10 minutes. Pellets were aspirated and washed with 3 ml of invasion buffer (DMEM with 3% FBS and 10mM HEPES, pH 7.4) and pelleted as before. Supernatants were aspirated and pellets resuspended in invasion buffer at 2.5 × 10⁶ cells. ml⁻¹. Fifty microlitres of parasite suspension was then mixed with an
equal volume of invasion buffer containing 2× the final concentration of agonist or vehicle (DMSO) equivalent. Parasite samples were incubated at 37°C for 20 min to allow secretion and quenched on ice for 2 min to stop secretion. Cells were then separated from supernatants by centrifugation (8,000 rpm, 2 min, 4°C), 85 μl of supernatant removed, and this was centrifuged a second time to remove any remaining cells, with a final volume of 75 μl carefully aspirated. The cell pellets were washed with 1× PBS, repelleted as before, and the supernatant was aspirated. Supernant and cell pellet proteins were solubilised in SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and GRA, MIC, or mitochondrial proteins immuno-detected. Western blot detection was performed with horseradish peroxidase conjugated secondary antibodies detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Signal strength was quantified using a BioRad Chemidoc imager and ImageLab software, and replicate assays signals were normalised to their respective cell pellet TOM40 signal to control for any minor cell number variation. Standard error of the means (SEM) were calculated for replicate data as a measure of experimental consistency, however, due to the non-linear nature of chemiluminescent detection, statistical analyses of these data are not appropriate. Plaque assays were performed on cells recovered after the 2-min ice quenching. Cells were pelleted, washed, and then resuspended in 1 ml of growth medium, and finally inoculated into HFFs at equal parasite densities.

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REFERENCES

Adl, S. M., Simpson, A. G. B., Lane, C. E., Lukes, J., Bass, D., Bowser, S. S., ... Heiss, A. (2012). The revised classification of eukaryotes. Journal of Eukaryotic Microbiology, 59(3), 429–493. http://doi.org/10.1111/j.1550-7482.2012.00644.x

Billker, O., Lourido, S., & Sibley, L. D. (2009). Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host & Microbe, 5(6), 612–622. http://doi.org/10.1016/j.chom.2009.05.017

Blader, I. J., Coleman, B. I., Chen, C.-T., & Gubbels, M.-J. (2015). Lytic cycle of Toxoplasma gondii: 15 years later. Annual Review of Microbiology, 69, 463–485. http://doi.org/10.1146/annurev-micro-091014-104100

Borges-Pereira, L., Budu, A., McKnight, C. A., Moore, C. A., Vella, S. A., Horta Triana, M. A., ... Moreno, S. N. (2015). Calcium signaling throughout the Toxoplasma gondii lytic cycle: A study using genetically encoded calcium indicators. Journal of Biological Chemistry, 290(45), 26914–26926. http://doi.org/10.1074/jbc.M115.652511

Brochet, M., Collins, M. O., Smith, T. K., Thompson, E., Sebastian, S., Volkman, K., ... Billker, O. (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 Biology, 12(3), e1001806. http://doi.org/10.1371/journal.pbio.1001806

Brydges, S. D., Harper, J. M., Parussini, F., Coppen, I., & Carruthers, V. B. (2008). A transient forward-targeting element for microneme-regulated secretion in Toxoplasma gondii. Biology of the Cell, 100(4), 253–264. http://doi.org/10.1042/BC20070076

Bullen, H. E., Jia, Y., Yamaryo-Botté, Y., Bisio, H., Zhang, O., Jemelin, N. K., ... Soldati-Favre, D. (2016). Phosphatidic acid-mediated signaling regulates microneme secretion in Toxoplasma. Cell Host & Microbe, 19(3), 349–360. http://doi.org/10.1016/j.chom.2016.02.006

Carruthers, V., Moreno, S., & Sibley, L. (1999). Ethanol and acetalddehyde elevate intracellular [Ca2+] and stimulate microneme discharge in Toxoplasma gondii. The Biochemical Journal, 342, 379–386. https://doi.org/10.1014/bj3420379

Carruthers, V. B., Giddings, O. K., & Sibley, L. D. (1999). Secretion of micronemal proteins is associated with toxoplasma invasion of host cells. Cellular Microbiology, 1(3), 225–235. https://doi.org/10.1046/j.1462-5822.1999.00023.x

Carruthers, V. B., & 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(2), 114–123.

Chaturvedi, S., Qi, H., Coleman, D., Rodriguez, A., Hanso, P. I., Striepen, B., ... Joiner, K. A. (1999). Constitutive calcium-independent release of Toxoplasma gondii dense granules occurs through the NSF/SNARE/Rab machinery. The Journal of Biological Chemistry, 274(4), 2424–2431. http://doi.org/10.1074/jbc.274.4.2424

Coppen, I., Andries, M., Liu, J. L., & Cesbron-Delauw, M.-F. (1999). Intracellular trafficking of dense granule proteins in Toxoplasma gondii and experimental evidences for a regulated exocytosis. European Journal of Cell Biology, 78, 463–472. http://doi.org/10.1016/S0717-9335(99)80073-9

de Vargas, C., Audic, S., Henry, N., Decelle, J., Mahé, F., Logares, R., ... Carmichael, M. (2015). Ocean plankton, Eukaryotic plankton diversity in the sunlit ocean. Science, 348(6237), 1261605. http://doi.org/10.1126/science.1261605

Dubremetz, J. F., Acharbou, A., Bermudes, D., & Joiner, K. A. (1993). Kinetics and pattern of organelle exocytosis during Toxoplasma gondii host-cell interaction. Parasitology Research, 79(5), 402–408. http://doi.org/10.1007/BF00931830

Endo, T., Tokuda, H., Yagita, K., & Koyama, T. (1987). Effects of extracellular potassium on acid release and motility initiation in Toxoplasma gondii. The Journal of Protozoology, 34(3), 291–295. https://doi.org/10.1111/j.1550-7408.1987.tb01777.x

Etheridge, R. D., Alaganan, A., Tang, K., Lou, H. J., Turk, B. E., & Sibley, L. D. (2014). The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. Cell Host & Microbe, 15(5), 537–550. http://doi.org/10.1016/j.chom.2014.04.002

Farrell, A., Thirunagesan, S., Lorestani, A., Dvorin, D. J., Eidel, K. P., Ferguson, D. J. P., ... Gubbels, M. J. (2012). A DOC2 protein identified by mutational profiling is essential for apicomplexan parasite exocytosis. Science, 333(6065), 218–221. http://doi.org/10.1126/science.1210829

Frénel, K., Dubremetz, J.-F., Lebrun, M., & Soldati-Favre, D. (2017). Gliding motility powers invasion and egress in Apicomplexa. Nature Reviews Microbiology, 15(11), 645–660. http://doi.org/10.1038/nrmicro2017.8.6

Graindorge, A., Frénel, K., Jacot, D., Salumun, J., Marq, J.-B., & Soldati-Favre, D. (2016). The conoid associated motor MyoH is indispensable for Toxoplasma gondii entry and exit from host cells. PLoS Pathogens, 12(1), e1005388. http://doi.org/10.1371/journal.ppat.1005388

Guérin, A., Corrales, R. M., Parker, M. L., Lamarque, M. H., Jacot, D., El Hajj, H., ... Lebrun, M. (2017). Efficient invasion by Toxoplasma depends on the subversion of host protein networks. Nature Microbiology, 2(10), 1358–1366. http://doi.org/10.1038/s41564-017-0018-1
Håkansson, S., Charron, A. J., & Sibley, L. D. (2001). Toxoplasma evolues: A two-step process of secretion and fusion forms the parasitophorous vacuole. The EMBO Journal, 20(12), 3132–3144. http://doi.org/10.1093/emboj/20.12.3132

Hakimi, M.-A., Olias, P., & Sibley, L. D. (2017). Toxoplasma effectors targeting host signaling and transcription. Clinical Microbiology Reviews, 30(3), 615–645. http://doi.org/10.1128/CMR.00005-17

Howard, B. L., Harvey, K. L., Stewart, R. J., Azevedo, M. F., Crabb, B. S., Jennings, I. G., ... & van Dooren, W. R. (2014). Calcium entry in Toxoplasma gondii and its enhancing effect of invasion-linked traits. Journal of Biological Chemistry, 289, 19637–19647. http://doi.org/10.1074/jbc.M114.565390

Mercer, C., Cesbron-Delauw, M.-F. (2015). Toxoplasma secretory granules: One population or more? Trends in Parasitology, 31(2), 60–71. http://doi.org/10.1016/j.pt.2014.12.002

Pace, D. A., Mcknight, C. A., Liu, J., Jimenez, V., & Moreno, S. N. J. (2014). Calcium entry in Toxoplasma gondii and its enhancing effect of invasion-linked traits. Journal of Biological Chemistry, 289, 19637–19647. http://doi.org/10.1074/jbc.M114.565390

Paul, A. S., Saha, S., Engelberg, K., Jiang, R. H. Y., Coleman, B. L., Kosber, A. L., ... Duraisingh, M. T. (2015). Parasite calcineurin regulates host cell recognition and attachment by apicomplexans. Cell Host & Microbe, 18, 1–12. http://doi.org/10.1016/j.chom.2015.06.003

Roiko, M. S., Svezhova, N., & Carruthers, V. B. (2014). Acidification activates Toxoplasma gondii motility and egress by enhancing protein secretion and cytolytic activity. PLoS Pathogens, 10, e1004488. https://doi.org/10.1371/journal.ppat.1004488

Schlossmann, J., Ammundola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., ... & van Dooren, W. R. (2014). Calcium-dependent egress of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by Toxoplasma gondii. Journal of Cell Science, 108(4), 1669–1677.

Stewart, R. J., Whitehead, L., Nijagal, B., Slabbs, B. E., Lessene, G., McConville, M. J., ... & van Dooren, W. R. (2014). Calcium-dependent phosphorylation alters class IVa myosin function in the protozoan parasite Toxoplasma gondii. Molecular Biology of the Cell, 25(17), 2579–2591. http://doi.org/10.1091/mbc.E13-11-0468

Tang, Q., Andennatten, N., Hortua Triana, M. A., Deng, B., Meissner, M., Moreno, S. N. J., ... & van Dooren, W. R. (2014). Calcium-dependent phosphorylation alters class IVa myosin function in the protozoan parasite Toxoplasma gondii. Molecular Biology of the Cell, 25(17), 2579–2591. http://doi.org/10.1091/mbc.E13-11-0468

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