Plasticity and therapeutic potential of cAMP and cGMP-specific phosphodiesterases in *Toxoplasma gondii*

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**Abstract**

*Toxoplasma gondii* is a common zoonotic protozoan pathogen adapted to intracellular parasitism in many host cells of diverse organisms. Our previous work has identified 18 cyclic nucleotide phosphodiesterase (PDE) proteins encoded by the parasite genome, of which 11 are expressed during the lytic cycle of its acutely-infectious tachyzoite stage in human cells. Here, we show that ten of these enzymes are promiscuous dual-specific phosphodiesterases, hydrolyzing cAMP and cGMP. *TgPDE1* and *TgPDE9*, with a Km of 18 μM and 31 μM, respectively, are primed to hydrolyze cGMP, whereas *TgPDE2* is highly specific to cAMP (Km 14 μM). Immuno-electron microscopy revealed various subcellular distributions of TgPDE1, 2, and 9, including in the inner membrane complex, apical pole, plasma membrane, cytosol, dense granule, and rhoptry, indicating spatial control of signaling within tachyzoites. Notably, despite shared apical location and dual-catalysis, TgPDE8 and TgPDE9 are fully dispensable for the lytic cycle and show no functional redundancy. In contrast, TgPDE1 and TgPDE2 are individually required for optimal growth, and their collective loss is lethal to the parasite. *In vitro* phenotyping of these mutants revealed the roles of TgPDE1 and TgPDE2 in proliferation, gliding motility, invasion and egress of tachyzoites. Moreover, our enzyme inhibition assays in conjunction with chemogenetic phenotyping underpin TgPDE1 as a target of commonly-used PDE inhibitors, BIPPO and zaprinast. Finally, we identified a retinue of PDE2-interacting kinases and phosphatases, possibly regulating the enzymatic activity. In conclusion, our datasets on the catalytic function, physiological relevance, subcellular localization and drug inhibition of key phosphodiesterases highlight the previously-unanticipated plasticity and therapeutic potential of cyclic nucleotide signaling in *T. gondii*.

**Keywords:** Apicomplexa, Phosphodiesterase, cAMP & cGMP signaling, Lytic cycle, Tachyzoite

1. Introduction

Cyclic nucleotide signaling in apicomplexan parasites has been an active area of research in the last decade. Its phylogenetic divergence, modus operandi, and functional repurposing to enable the specialized lifecycle events in this class of clinically-relevant pathogens has attracted the most attention. *Toxoplasma* and *Plasmodium* are the two standard parasite models deployed to study apicomplexan biology, including cAMP and cGMP signaling. *T. gondii* – the only known species of *Toxoplasma*, is well known for its prominent ability to infect and reproduce in several warmblood organisms without geographic constraints [1]. The parasite undergoes asexual and sexual growth switching between multiple infec-
tious stages, and exhibits exceptional promiscuity and metabolic plasticity, which underlie its widespread infection, inter-host transmission, reproduction, persistence, and pathogenesis. It is, therefore, imperative to examine the molecular mechanisms and determinants of infection and develop efficient anti-parasitic treatment strategies.

This work focuses on the tachyzoite stage of *T. gondii* responsible for the acute infection (tissue necrosis by recurrent lytic cycles). Tachyzoites can infect a broad range of nucleated host cells in humans and animals. The lytic cycle comprises several steps, such as gliding motility, invasion, proliferation, and egress [2]. Besides other known factors, protein kinase-dependent on cAMP (PKA) and protein kinase-dependent on cGMP (PKG) serve as the prime regulators of the lytic cycle events. Cyclic GMP signaling, for example, governs the calcium-dependent micronemal exocytosis needed for the motility-driven invasion and egress by the parasite. It is initiated by an exclusive guanylate cyclase fused to a P4-type ATPase (ATPaseP-GC), and mediated by PKG [3–7].

Given the high enzymatic activity, distinct subcellular locations and the yield of enriched proteins, we focused on the substrate kinetics of *TgPDE1*, *TgPDE2*, *TgPDE7*, and *TgPDE9* (Fig. 2). At first, the enzyme and time dependence of each PDE were assessed under saturating amount of cAMP or cGMP (Fig. S1). Knowing the linearity of individual reactions, we tested the catalytic activity with 1–500 μM of substrates to calculate the Km and Vmax values. All four PDEs displayed the typical Michaelis-Menten kinetics showing a dependence of their catalysis on the substrate concentration (Fig. 2A–E). *TgPDE1*, with a Km of 73 μM for cAMP and 18 μM for cGMP, had a 4-fold higher affinity for the latter (Fig. 2A). *TgPDE2*, exhibiting the lowest Km (14 μM) among all, was functional only with cAMP (Fig. 2B). *TgPDE7* with the Km values of 60 μM and 52 μM for cAMP and cGMP, respectively, displayed a similar affinity for both cyclic nucleotides (Fig. 2C). On the other hand, the hydrolytic activity of *TgPDE9* for cAMP (Km, 118 μM) and cGMP (Km, 31 μM) was analogous to *TgPDE1* (Fig. 2D). As elaborated below, the kinetic parameters of indicated PDEs enabled the interpretation of our mutagenesis and phenotyping datasets besides elucidating their pharmacological relevance in the context of known inhibitors.

2.2. Catalytic kinetics of *TgPDE1*, *TgPDE2*, *TgPDE7* and *TgPDE9* with cAMP and/or cGMP

2.3. Effect of PDE inhibitors on the parasite phosphodiesterases

In extended enzyme assays, we examined the inhibition kinetics of the *TgPDE1*, *TgPDE2*, *TgPDE7*, and *TgPDE9* preparations by BIPPO, zaprinast, IBMX, and PF-04957325 (Fig. 3). BIPPO and zaprinast are assumed to inhibit cGMP hydrolysis and widely deployed to induce the PKG-dependent egress in tachyzoites [7,8,13,16,17], whereas IBMX (3-isobutyl-1-methylxanthine) is a nonselective drug inhibiting human phosphodiesterases [18], and PF-04957325 specifically blocks the cAMP-specific human PDE8 [19,20]. In our inhibition assays involving the parasite PDEs (Fig. 3A), hydrolysis of both cyclic nucleotides by *TgPDE1* was strongly inhibited (>90 %) in the presence of BIPPO. However, zaprinast was more potent in blocking cAMP degradation (>80 %) compared to cGMP (~40 %). The effect of IBMX on *TgPDE1* was moderate. Notably, hydrolysis of cAMP by *TgPDE2* was sensitive to partial inhibition by IBMX but to none of the other drugs (Fig. 3B). On the other hand, *TgPDE7*-mediated catalysis of cAMP and cGMP were reduced by > 70 % upon inclusion of only BIPPO (Fig. 3C). Consistent with its differential substrate affinity (Fig. 2E), the activity of *TgPDE9* for cAMP was inhibited (>75 %) by BIPPO, zaprinast as well as IBMX, but its catalytic action on cGMP remained largely unaffected (Fig. 3D).

A remarkably strong effect of BIPPO and zaprinast on *TgPDE1* prompted us to perform the inhibition kinetics using different concentrations of inhibitors. The IC50 of BIPPO for the cAMP and cGMP hydrolysis by *TgPDE1* was 0.31 μM (Fig. 3E) and 0.51 μM (Fig. 3F), respectively. In contrast, zaprinast exhibited an approximately 5x

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Fig. 1. Catalytic activity of the native phosphodiesterases present in tachyzoites of *T. gondii*. (A) Scheme for the α-HA agarose bead-based immunoprecipitation to enrich the smHA-tagged PDE proteins from the cell lysate of transgenic parasites. The parental strain (*RHΔku80Δhgprt*) was used as a negative control. (B-D) Immunoblots confirming adequate precipitation of PDE-smHA proteins. Samples from panel A were probed with the mouse α-HA (green) and rabbit α-TgHsp90 (red) antibodies. Note the presence of TgHsp90 (a cytosolic marker) in cell-free extract (CFE) and its absence in the immunoprecipitated pellet (P). Asterisks, if shown, mark the predicted size of smHA-tagged PDEs. (E) Phosphodiesterase activity of PDE-smHA proteins with cAMP and cGMP. The colorimetric enzyme assays were set up using 6 μg of PDE samples (*TgPDE5, 10 μg*) and 200 μM substrate (1 h, 37°C). The control reactions run alongside lacked the substrate or enzyme. The substrate-free enzyme-only reaction was subtracted from samples to quantify the PDE activity (normalized to the protein amount). The negative controls indicate the precipitated protein of the parental strain (N.D., not detectable). The data show the mean ± SE (n = 3–4 assays).
higher IC\textsubscript{50} value (1.49 μM) for cAMP (Fig. 3G). These data advocate that commonly-used inducers of parasite egress can inhibit cAMP as well as cGMP hydrolysis and indicate TgPDE1 as the primary target of these drugs.

2.4. TgPDE7, TgPDE8, and TgPDE9 are expendable during the lytic cycle

Our preceding work has shown that tachyzoite can survive the genetic deletion of TgPDE9 with no apparent defect in the lytic cycle [14]. Herein, we tested the physiological importance and functional redundancy of other designated PDEs by CRISPR/Cas9-aided mutagenesis (Figs. S2-S4A). The gene-specific knockout constructs with 5′ and 3′ homology arms flanking a DHFR-TS selection cassette were transfected into respective progenitor parasite strains expressing smHA-tagged PDEs. Transgenic tachyzoites were selected by pyrimethamine, and mutants were isolated by limiting dilution. The genomic screening of clonal mutants by PCR revealed the occurrence of 5′ and 3′-crossovers, confirming a successful replacement of the TgPDE7, TgPDE8, and TgPDE9 loci by the selection marker (Figs. S2-S4B). The loss of PDE expression in transgenic parasites was tested by immunofluorescence and immunoblot methods (Figs. S2-S4C). Unlike the matching progenitor strains, no HA signal was observed in the mutants. Plaque assays (Figs. S2-S4D), representing the periodic lytic cycles and thus the overall fitness of tachyzoites, disclosed a normal growth in the parental and progenitor strains, as expected. Surprisingly, however, none of the three mutants (ΔTgPDE7, ΔTgPDE8, or ΔTgPDE9) exhibited a growth defect, as deduced by the number and size of plaques.

We subsequently generated a double mutant to investigate the possible redundancy between the two apically-located dual-specific PDEs, i.e., TgPDE8 and TgPDE9 (Fig. 4). First, a conditional TgPDE9 mutant was made by 3′-genomic tagging with a mini auxin-inducible degron (mAID) using the HXGPRT selection marker (Fig. 4A). The mAID system enables maintaining viable parasites in the absence of indole-3-acetic acid (IAA, a type of auxin) if the gene is essential [4]. A plasmid expressing Cas9 nuclease and gene-specific sgRNA targeting the TgPDE9-3′UTR was transfected with a donor amplicon (5′ and 3′-homology arms flanking mAID-3HA and HXGPRT selection cassette) into tachyzoites.
Immunostaining confirmed the apical localization and protein integrity (Fig. 4B). The HA signal was not detectable within 1 h of auxin treatment, ratifying a fast and efficient conditional knockdown of TgPDE9 in tachyzoites (see immunoblot). In the second step, we deleted TgPDE8 in the TgPDE9-mAID-3HA strain by double homologous crossover and pyrimethamine selection (Fig. 4A). The eventual mutant was screened by genomic PCR (Fig. 4C). Compared to the parental parasites, neither the TgPDE9-mAID-3HA nor ΔTgPDE8/TgPDE9-mAID-3HA strain was affected in plaque assays irrespective of auxin supplementation (Fig. 4D-F), suggesting no apparent functional overlap between the two enzymes during the lytic cycle of T. gondii.
2.5. TgPDE1 and TgPDE2 are partly redundant but individually needed for the parasite growth

To examine the relevance of TgPDE1 and TgPDE2, we made their conditional mutants by mAID-3HA tagging (Fig. 5A, S5A). The parasite strains were verified for localization and regulation by fluorescence imaging and western blotting. Both proteins were not detectable after incubation with IAA (Fig. 5B, S5B). Depleting TgPDE1 and TgPDE2 compromised the growth of mutants by ~56 % and ~88 % compared to the respective controls (Fig. S5C-D). A moderate but significant decline in plaque numbers was also observed (Fig. S5E), confirming a requirement of each enzyme for the optimal reproduction of tachyzoites. Nonetheless, the residual growth of the conditional strains encouraged us to make a double mutant of TgPDE1 and TgPDE2, as described above for TgPDE8 and TgPDE9 (Fig. 4), and test their physiological cooperativity (Fig. 5A). Our attempts to ablate the TgPDE1 locus in the TgPDE1-mAID-3HA strain were futile; however, we could delete the TgPDE1 gene in the TgPDE2-mAID-3HA mutant (Fig. 5C). As anticipated, in plaque assays (Fig. 5D-F), the parental strain was not affected. In contrast, the TgPDE2-mAID-3HA mutant showed strongly reduced growth (~80 %) upon IAA exposure. Equally, deletion of TgPDE1 in the TgPDE2-mAID-3HA strain impaired the ΔTgPDE1/TgPDE2-mAID-3HA mutant (~IAA sample, Fig. 5D-F). Importantly, a collective loss of both proteins aborted the parasite growth, as judged by the absence of plaques in the auxin-exposed double mutant (~IAA, Fig. 5D-F).

We next performed detailed phenotyping of the ΔTgPDE1/TgPDE2-mAID-3HA strain to evaluate the lytic cycle events, such as gliding motility, invasion, replication, and egress (Fig. 6). In contrast to the parental and TgPDE2-depleted strains, the double mutant’s motile fraction, trail length, and invasion efficiency were strongly reduced after auxin treatment (Fig. 6A-C). Notably, the deletion of TgPDE1 (~IAA sample) exerted no evident defect across these features. In addition, knockdown of TgPDE2 alone only moderately attenuated the parasite replication; however, the double mutant treated with auxin showed an extreme decline in the number of proliferating parasites, as scored by the size of parasitophorous vacuoles (Fig. 6D). Intriguingly, the depletion of TgPDE2 in the ΔTgPDE1/TgPDE2-mAID-3HA strain exerted opposing effects on the parasite egress in early and late cultures (Fig. 6E), which was increased 36 h post-infection but declined after 60 h. No egress defect was observed in the TgPDE2-mAID-3HA strain at any tested time points. These phenotypic assays underpin the singular and collective significance of TgPDE1 and TgPDE2 enzymes and highlight their mutual interplay during the lytic cycle.

2.6. The ΔTgPDE1/TgPDE2-mAID-3HA strain is refractory to BIPPO-induced egress

To further understand the role of signaling during egress, we deployed a chemogenetic approach utilizing a calcium ionophore (A23187) and inhibitors of “cGMP-specific PDE” (BIPPO) as well as PKG (C2 or Compound 2), all of which are widely used to understand the biology of T. gondii. We tested their impact on egress of the parental, TgPDE2-mAID-3HA and ΔTgPDE1/TgPDE2-mAID-3HA strains cultured without or with auxin (Fig. 6F-G). As envisaged, A23187, an ionophore activating calcium signaling downstream of cyclic nucleotides [21–24], triggered a complete egress of the three strains irrespective of the IAA treatment (Fig. 6F). Equally, BIPPO induced almost total lysis of all samples but the auxin-treated double mutant, which responded by only 20–25 % egress (Fig. 6G). In light of enzyme inhibition assays (Fig. 3A), these data entail TgPDE1 as a primary target of BIPPO. The resistance of auxin-treated double mutant to this drug appeared to be a common outcome of TgPDE1 deletion (i.e., the absence of drug target) and rise in cAMP after knockdown of TgPDE2, leading to suboptimal activation of PKG and hyper-activation of PKA. We, therefore, examined the effect of PKG inhibitor, Compound 2 [25] on BIPPO-induced egress (Fig. 6G). The residual egress of the auxin-treated double mutant was indeed completely blocked by Compound 2, suggesting that the process is mediated by PKG.

2.7. Ultrastructural imaging of intracellular tachyzoites

We performed the transmission electron microscopy of the ΔTgPDE1/TgPDE2-mAID-3HA strain to gain ultrastructural insight into the phenotype (Fig. 7). The double mutant cultured without auxin had a normal morphology with intact organelles, excluding a detrimental effect of TgPDE1 deletion (Fig. 7A). In contrast, a knockdown of TgPDE2 resulted in an aberrant/distorted shape of tachyzoites (Fig. 7B). Besides a much lower number of parasites per vacuole, we noted enlarged vacuolar space and impaired budding of progeny. The endodyogeny was arrested in auxin-treated cultures (Fig. 7C). We also observed a population of abnormal-shaped tachyzoites with constricted terminal regions (see red arrow in Fig. 7D). In further assays, we utilized immunogold labeling of TgPDE1 and TgPDE2 to decipher their spatial distribution. Given the distinct apical presence of TgPDE9, we included it as a control for potential sample processing artifacts that may cause mislocalization of PDEs (Fig. S6A-C). The quantification of images disclosed that a majority of parasites (>60 %) expressed TgPDE9-smHA in the conoid region at the apical pole near the plasmalemma (Fig. S6A). TgPDE1-smHA was detected mainly at the cytotoxic periphery (38 %) and inner membrane complex (27 %), whereas TgPDE2-smHA was expressed in the cytosol (45 %), dense granules (24 %) and rhoptries (15 %).

2.8. TgPDE1 and TgPDE2 may be regulated by a specific kinase and phosphatase network

Our final assays explored the interaction network of TgPDE1 and TgPDE2 in T. gondii (Fig. S7). We precipitated PDEs and their protein binding partners using α-HA agarose beads and subjected them to liquid chromatography-mass spectrometric analysis. The parental strain was included as a negative control. The principal component analysis endorsed the proteomic dataset’s technical and biological reproducibility, as illustrated by the grouping of samples in each cohort (Fig. S7A). The heatmap also displayed evident clusters of proteins binding to TgPDE1 and TgPDE2 but absent in the control sample (Fig. S7B). We detected 143 and 22 unique interactors of TgPDE1 and TgPDE2, respectively (p ≤ 0.01, at least twofold enriched compared to the parental control). In total, 38 proteins were bound to both phosphodiesterases (Fig. S7C). Importantly, no other PDE except the bait was present in immunoprecipitated samples (Table S1), validating the quality of enzyme assays involving TgPDE1 and TgPDE2. Based on our current understanding of PDEs in other model organisms and their contextual relevance to signaling in apicomplexan parasites, we shortlisted interacting proteins, including a group of kinases and serine/threonine phosphatases (Fig. S7D). We suspect that some of the proteins identified herein as potential interaction partners may be involved in regulating TgPDE1 and TgPDE2 catalysis (for a complete list, see Table S1).

3. Discussion

Toxoplasma gondii has evolved an expanded panel of highly divergent phosphodiesterases to counter-regulate the cyclic nucleotide signaling. All 18 PDEs encoded by its genome belong to the class I phosphodiesterases [13,14]. Here, we characterized...
Fig. 4. TgPDE8 and TgPDE9 are dispensable individually and do not complement each other during the lytic cycle. (A) The primary structures of TgPDE8 and TgPDE9 as well as schematics for making a double mutant (ΔTgPDE8 in TgPDE9-mAID-3HA strain) by CRISPR/Cas9-assisted homologous crossover. In the first step, an auxin-regulated mutant of TgPDE9 was generated, which also served as a progenitor strain for deleting the TgPDE8 gene. (B) Immunofluorescence and immunoblot showing the dependence of TgPDE9 expression on indole-3-acetic acid (IAA). For immunofluorescence, parasites were cultured for 24 h without or with IAA before staining the HA-tag (green) and TgGap45 (inner membrane complex, red). Immunoblots were generated after IAA treatment for the indicated periods. TgHsp90 served as a loading control. (C) Genomic PCR with recombination-specific primers, confirming the 5' and 3' recombination events at the TgPDE8 locus in the double mutant. For the primer binding sites, see panel A. (D-F) Plaque assays to test the growth of the TgPDE9-mAID-3HA and ΔTgPDE8/TgPDE9-mAID-3HA mutants in comparison to the RHku80/kxgprt-TIR1 (parental) strain. The white speckles on a blue background signify the parasite plaques formed in a host-cell monolayer. Graphs show the area (E) and number (F) of plaques, manifesting the fitness of tachyzoites in the absence or presence of IAA. For panel E, the size of 150–200 plaques of each strain from 3 replicates was scored in arbitrary units (a. u.).
the substrate specificity, physiological relevance, functional redundancy and spatial distribution of PDEs during the lytic cycle of *T. gondii*. This study reports the catalytic activity of 11 enzymes expressed in the tachyzoite stage. Except for *Tg*PDE2 hydrolyzing only cAMP, others are promiscuous dual-specific proteins degrading cAMP and cGMP. Therefore, unlike its mammalian host [26,27] and related parasite *P. falciparum* [28,29], *T. gondii* harbors a much larger set of dual-specific enzymes. We also found that *Tg*PDE1 exhibits a 4x higher affinity for cGMP than cAMP. The mutagenesis, phenotyping and localization datasets reveal func-
tional cooperation of TgPDE1 and TgPDE2 during the lytic cycle. Some of the findings above echo a recent study [15] overlapping with this and our earlier work [14]. The Km values of TgPDEs range from 14 to 118 lM, comparable to most class I phosphodiesterases. For example, of the 11 human PDE families, hPDE3 to hPDE11 display Km of 0.04–9 lM, whereas hPDE1 and hPDE2 are within 10 to 100 lM [30]. Our work also sheds light on the modus operandi of PDE inhibitors frequently used in parasitology research. We observed that BIPPO could potently inhibit cGMP hydrolysis by TgPDE1 and TgPDE7; however, it is the former enzyme that underlies the drug’s effect during the lytic cycle. Moreover, another physiologically-critical enzyme, TgPDE2, is refractory to inhibition by BIPPO and zaprinast. Both drugs can inhibit cAMP catalysis by TgPDE9, although the dispensability of this protein excludes it as a drug target in tachyzoites. Potent inhibition of cAMP hydrolysis compared to cGMP by dual-specific TgPDE1 and TgPDE9 in BIPPO and zaprinast-treated samples can be explained by their differential affinity (Km) for these substrates. Consistent with homology modeling of the Toxoplasma PDEs [13,14] and phenotypic studies performed in Plasmodium PDEs [29], our enzyme assays suggest that the two alleged cGMP-specific PDE inhibitors also perturb the cAMP pathway.

Fig. 6. A collective loss of TgPDE1 and TgPDE2 disrupts the lytic cycle. (A-B) Gliding motility of the TgPDE2-mAID-3HA and ΔTgPDE1/TgPDE2-mAID-3HA mutants compared to the parental (RHAKu80Moxgpt-TR1) strain. The motile fraction (700 parasites) and trail length (150–250 trails) of each parasite strain (+/- IAA) were scored by the ImageJ program. (C) Invasion efficiency of the shown strains in the absence or presence of IAA. 1500–2000 parasites of each strain were imaged to compute the invasion rates. (D) Intracellular replication (40 h infection). Cell division was scored by counting tachyzoites within their parasitophorous vacuoles (600–700). (E) Natural egress from the host cells. The phenotype was measured by calculating the fraction of ruptured vacuoles (500–600) 36 h and 60 h post-infection. (F, G) Effect of A23187 (Ca** ionophore, 2 lM), BIPPO (PDE inhibitor, 10 lM) and Compound 2 (C2, PKG inhibitor, 2 lM) on the egress of tachyzoites (Mot; 3; 24 h infection). Treatment with IAA, as applicable, was initiated 24 h before setting up the motility and invasion assays, whereas it was added 2 h post-infection during the replication and egress experiments to alleviate the effect of motility and invasion defects. Graphs display the mean ± SE (n = 5 for A-C, n = 6 for D, n = 3 for E-G; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).
besides cGMP signaling. Thus, the enzyme kinetics and chemoge-
netic phenotyping presented herein offer a renewed prospect for
developing novel PDE inhibitors and parasite-specific therapeutics.

This work uncovers remarkable plasticity in the counter-
regulation of cyclic nucleotide signaling, as exemplified by cataly-
sis and mutagenesis of \( \text{TgPDE1-2} \) and \( \text{TgPDE7-9} \) proteins. Tachy-
zoites can survive the absence of \( \text{TgPDE7} \), \( \text{TgPDE8} \) and \( \text{TgPDE9} \) enzymes, whereas they depend on the cooperation of \( \text{TgPDE1} \) and \( \text{TgPDE2} \) for specific events during their asexual growth. Nota-
ably, neither the knockout of \( \text{TgPDE1} \) nor the knockdown of \( \text{TgPDE2} \) affects the motility and invasion, but a loss of both compromises these features. Surprisingly, the \( \Delta\text{TgPDE1/TgPDE2-mAID-3HA} \) mutant showed a higher (premature) egress at 36 h, which reversed into a moderate impairment at 60 h, implying pro-
grammed crosstalk of cAMP and cGMP signaling as the parasite
nears the end of its lytic cycle. Regarding parasite replication,
mutagenesis of \( \text{TgPDE1} \) and \( \text{TgPDE2} \) exerted a negligible effect,
though the simultaneous loss of both phosphodiesterases deliv-
ered a potent phenotype. Unlike \( \text{TgPDE1} \), we could not knockout
\( \text{TgPDE2} \), suggesting that any other orthologs cannot fully compen-
sate for the physiological role of the latter enzyme. A dominant
expression of \( \text{TgPDE1} \) and \( \text{TgPDE2} \) in the parasite cytosol may
account for their partial functional redundancy. Varied subcellular
localization of PDEs in tachyzoites also reflects a

Fig. 7. Ultrastructural imaging of the \( \Delta\text{TgPDE1/TgPDE2-mAID-3HA} \) mutant by transmission electron microscopy. (A) Intracellular tachyzoites cultured without IAA (control). (B-D) Tachyzoites of the double mutant after treatment with IAA (24 h). Scale bar: 500 nm. Abbreviations: PV: parasitophorous vacuoles; IMC: inner membrane complex; PM: plasma membrane; Nu: Nucleus.
compartmentalized control of cyclic nucleotide signaling, warranting further studies. Last but not least, we demonstrate TgPDE1 and TgPDE2 as potential drug targets to control the acute infection of T. gondii.

4. Materials and methods

4.1. Biological reagents

The RHΔku80Δhxgprt [31] and RHΔku80Δhxgprt-TIR1 [4] strains of T. gondii were offered by Vern Carruthers (University of Michigan, MI) and David Sibley (Washington University, St. Louis, MO), respectively. The pUG6-Universal and pSag1-Cas9-U6-sgUPRT vectors for expression of Cas9 and single guide RNA (sgRNA), and the pTUB1-YFP-mAID-3HA-HXGPRT plasmid, were provided by David Sibley (Washington University, St. Louis, USA). The antibodies recognizing TgGap45 [32] and TgHsp90 [33] were donated by Dominique Soldati-Favre (University of Geneva, Switzerland) and Sergio Angel (JIB-INTECH, Buenos Aires, Argentina), respectively. Other antibodies against the HA epitope and TgSag1 were purchased from Takara-Taka (Japan) and Sigma-Aldrich (Germany). The secondary antibodies (Alexa488, Alexa594; IRDye 680RD, 800CW) and oligonucleotides (Table S2) were obtained from Sigma-Aldrich and Carl Roth (Germany). The kits for isolation, cloning and purification of nucleic acids were acquired from Ana- lytik Jena and Life Technologies (Germany). The PDE assay kits (colorimetric) were purchased from Abcam (UK) and Enzo Life Science (USA).

4.2. Host cell and parasite cultures

The human foreskin fibroblasts (HFFs; Cell Lines Service, Eppel- heim, Germany) were grown to confluence and harvested for further passageing by trypsin-EDTA treatment. Cells were cultured in Dulbecco’s modified Eagle medium containing glucose (4.5 g/L), 10 % heat-inactivated fetal bovine serum (FBS; PAN Biotech), 2 mM glutamine, 1 mM sodium pyruvate, 1x minimum Eagle’s medium nonessential amino acids, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified incubator (37 °C, 5 % CO2). The tachyzoite stage of T. gondii was maintained by serial culture in confluent HFF monolayers using a multiplicity of infection (MOI) of 3. Parasites for all experiments were prepared by squinting infected cultures through a 27G syringe (2x) unless stated otherwise.

4.3. Making of transgenic parasites

To generate the direct knockout of TgPDE1 and TgPDE8 in the conditional mutants of TgPDE2-mAID-3HA and TgPDE9-mAID-3HA, respectively, a CRISPR-Cas9 vector expressing two sgRNA to target the 5′UTR and 3′UTR regions of each gene was constructed. In the first step, we made two separate CRISPR-Cas9 vectors for TgPDE1 and TgPDE8 knockout by replacing the UPRT sgRNA with gene-specific sgRNAs targeting 5′ or 3′ UTR in the pSAG1-Cas9-U6- sgUPRT plasmid. We then amplified the pUG6-sg3′UTR-TgPDE1/8 region (~678 bp) using sgRNA-Knlf-F and sgRNA-Xhol-R primers (Table S2) and pSAG1-Cas9-U6-5′UTR-TgPDE1/8 as templates. The amplicons were ligated into Knlf-Xhol-digested pSAG1-Cas9-U6-sg5′UTR-TgPDE1 and pSAG1-Cas9-U6-5′UTR-TgPDE8 vectors, as applicable. The subsequent dual CRISPR-Cas9 plasmids were co-transfected with respective donor amplicons. The latter comprised a DHFR-TS selection cassette flanked by 5′- and 3′-homology arms (~70 bp) targeting the upstream and downstream TgPDE1 and TgPDE8 loci. Transgenic parasites were cloned using 1 μM pyrimethamine [35] and screened by genomic PCR (primers in Table S2).

4.4. Immunofluorescence assays

As described elsewhere [36], HFF cultured on glass coverslips were infected with tachyzoites for the indicated periods. Samples were fixed with 4 % paraformaldehyde (15 min) and neutralized by 0.1 M glycine-PBS (5 min). Afterward, cells were permeabilized in 0.2 % Triton X-100/PBS (20 min), followed by blocking with 2 % bovine serum albumin (0.2 % Triton X-100/PBS) for 20 min. Samples were stained with primary antibodies (α-HA, mouse, 1:3000; αTgGap45, rabbit, 1:3000; αTgSag1, mouse, 1:1000) for 1 h. They were washed 3x with 0.2 % Triton X-100/PBS and stained with Alexa488/594-conjugated antibodies for 45 min. After additional PBS washing, samples were finally mounted in Fluoromount G containing DAPI (Southern Biotech, Birmingham, AL) and stored in the dark at 4 °C. Images were acquired by fluorescence microscopy (Zeiss, Germany).

4.5. Immunoblot analysis

Tachyzoites were harvested and pelleted (800 g, 4 °C, 10 min), followed by washing with ice-cold PBS and re-pelletting in a 1.5 mL tube (8000 rpm, 3 min, 4 °C). Cells were lysed in 55 μL buffer (10 mM KHPO4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, pH 7.4; 0.2 % sodium deoxycholate, 1 % Triton X-100) and protease inhibitors (trypsin inhibitor, 20 μg/mL; aprotinin, 10 μg/mL; benzimidazole, 500 μg/mL; PMSF, 0.5 mM; Na3VO4, 0.1 mM; NaF, 50 mM). Samples were incubated on ice for 30 min and then centrifuged (20000 × g, 15 min, 4 °C) to collect the cell-free extract (50 μL), which were mixed with 5x loading buffer (13 μL, no boiling), followed by SDS-PAGE (6–8 %). Proteins were blotted onto a nitrocellulose membrane (85 mA/cm2, 2 h, semi-dry) and stained overnight at 4 °C with the mouse α-HA (1:10000) and rabbit α-TgHsp90 (1:10000) antibodies diluted in 5 % skimmed milk with 0.2 % Tween 20/TBS. Immunoblot was washed 3x with 0.2 % Tween 20/TBS (5 min) and incubated with IRDye-conjugated secondary antibodies (680RD and 800CW with 1:15000 dilution, 1 h). The antibody-stained protein bands were visualized by an Odyssey FC imaging system (LI-COR Biosciences).

4.6. Lytic cycle assay

The impact of genetic manipulation on the lytic cycle of tachyzoites was determined by standard phenotyping methods, as described in our previous works [37,38]. For plaque assay, the confluent HFF monolayers in 6-well plates were infected with 200 parasites/well and incubated for 7 to 8 days without perturbation.
Samples were fixed with ice-cold methanol for 10 min and then stained with crystal violet solution for 15 min. Plaques were imaged and scored for size and number using the ImageJ software (NIH, Bethesda). To quantify the invasion efficiency, the HFF monolayers on coverslips placed in 24-well plates were infected with tachyzoites (Mol: 10) for 30 min at 37 °C, followed by fixation with 4 % paraformaldehyde/PBS and neutralization with 0.1 % glycine/PBS. Before permeabilization, samples were stained with the mouse α-TgSag1 antibody (1:1000) to visualize the non-invaded/extracellular parasites. Cells were washed 3x with PBS, permeabilized with 0.2 % Triton X-100/PBS for 20 min, and stained with the rabbit α-TgGap45 antibody (1:10000) to score the invaded parasites. The fractions of invaded/intracellular parasites were determined to compare the invasion rates across the parasite strains.

To gauge the intracellular replication of tachyzoites, HFF cells grown on coverslips were infected (Mol:1, 40 h). Samples were subjected to permeabilization, neutralization, blocking, and staining with the rabbit α-TgGap45 antibody. The tachyzoite proliferation was assessed by enumerating parasitophorous vacuoles harboring a variable number of progeny. For the egress assay, the invasion was assessed by enumerating parasitophorous vacuoles harboring a variable number of progeny. The motile fraction was counted on the microscope, and trail lengths were quantified by the ImageJ program.

4.7. Immunoprecipitation of PDE proteins

The cell-free extract was prepared as described above. To precipitate the native PDEs, 50 μL of anti-HA agarose beads were added to the tachyzoite extract (2 mg protein). The volume was adjusted to 1 mL by a lysis buffer containing protease inhibitors. The pull-down reaction was set with constant rotation (4 °C, 4 h). Afterward, protein-conjugated beads were pelleted (200 g, 30 s), washed 2x with ice-cold lysis buffer with protease inhibitors, and then once with the PDE dilution buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.4) [29]. Samples were given a final wash with 10 mM Tris-HCl buffer (pH 7.4) before using them for the enzyme assays.

4.8. PDE enzyme assay

The experiment was performed using colorimetric kits (Enzo Life Science, Netherlands; Abcam, UK) based on the enzymatic cleavage of 3’5’cAMP/3’5’cGMP to 5’AMP/5’GMP, which are further hydrolyzed by 5’-nucleotidase to their nucleoside and phosphate moieties. The phosphate group is quantified to determine the PDE activity. To set up the assay, immunoprecipitated proteins (1–10 μg) were suspended in the reaction buffer (50 μL), followed by the addition of cAMP or cGMP (200 μM). Samples were incubated at 37 °C for 1 h and mixed with 100 μL of the green reagent (30 min, room temperature) to terminate the reaction. Subsequently, the OD620 was measured to quantify the phosphate group. We also included a cAMP-specific PDE from the bovine brain as a positive control and several negative controls (no protein, no substrate) for validation purposes. The standards with varying phosphate amounts (0.25–4 nmol) provided by the kit were included in all experiments to quantify the enzymatic hydrolysis of cAMP and cGMP.

To determine the kinetic parameters (Km, Vmax, IC50) of selected phosphodiesterases (TgPDE1, TgPDE2, TgPDE7, TgPDE9), assays were standardized for the protein amount and incubation period. The substrate dependence was tested in the linear range of reaction time and amount of each PDE. The Km and Vmax were calculated by the Michaelis-Menten equation using the GraphPad Prism suite. We also attempted to examine the kinetics of TgPDE8 (dual-specific, apical location); however, its low expression and poor catalytic activity prevented us from determining reproducible Km values. Our additional assays tested the PDE inhibition by 3-isobutyl-1-methylxanthine (IBMX, 100 μM), 1,4-Dihydropyridine-5-(2-propanopyphenyl)-7H-1,2,3-triazolo[4,5-d]pyrimidin-7-one (zarinaprin, 300 μM), 5-benzyl-3-isopropyl-1H-pyrazolo[4,3-d] pyrimidin-7(6H)-one (BIPPO, 100 μM) [13] and P04957325 (50 μM, Pfizer Inc). For the IC50 estimation, different concentrations of BIPPO (1–100 μM) and zarinaprin (1–350 μM) were used. The key reaction parameters (protein amount, time, substrate) were optimized prior to the inhibition kinetics of phosphodiesterases.

4.9. Proteolytic digestion for mass spectrometry

As described elsewhere [39,40], samples were processed by a single-pot solid-phase-enhanced preparation method. In brief, anti-HA agarose beads were incubated for 15 min at 60 °C in an SDS-containing buffer (1 % w/v SDS, 50 mM HEPEs, pH 8.0) to release proteins, which were afterward reduced and alkylated by dithiothreitol and iodoacetamide, respectively. They were supplemented with 2 μL of carboxyamide-modified paramagnetic beads (Sera-Mag SpeedBeads, GE Healthcare, 0.5 μg solids/μL water), followed by adding acetonitrile to a final concentration of 70 % (v/v). Beads were allowed to settle for 20 min at room temperature. Subsequently, samples were washed twice with 70 % (v/v) ethanol in water and once with acetonitrile. Beads were suspended in 50 mM NH4HCO3 supplemented with trypsin (Mass Spectrometry Grade, Promega) at an enzyme-to-protein ratio of 1:25 (w/w) and incubated overnight at 37 °C. Acetonitrile was added to the samples to reach a final concentration of 95 % (v/v), followed by incubation at room temperature for 20 min. To maximize the yield, supernatants derived from this initial peptide-binding step were subjected to the peptide purification procedure [40]. Each sample was washed with acetonitrile. Paramagnetic beads from the original reaction and corresponding supernatants were pooled in 2 % (v/v) dimethyl sulfoxide in water and sonicated for 1 min. After centrifugation (12500 rpm, 4 °C), supernatants containing tryptic peptides were transferred into a glass vial for mass spectrometry analysis and acidified with 0.1 % (v/v) formic acid.

4.10. Liquid chromatography-mass spectrometry analysis

Tryptic peptides were separated using an Ultimate 3000 RSLCnano LC system (Thermo Fisher Scientific) equipped with a PepMap100, C18, 5 μm, 0.3 × 5 mm trap (Thermo Fisher Scientific) and an HSS-T3 C18, 1.8 μm, 75 μm × 250 mm analytical reversed-phase column (Waters Corporation). Mobile phase A was water containing 0.1 % (v/v) formic acid and 3 % (v/v) DMSO. Peptides were separated using a gradient of 2–35 % mobile phase B (0.1 % v/v formic acid, 3 % v/v DMSO in acetonitrile) over 40 min at a flow rate of 300 nL/min. The total analysis time was 60 min including the wash and column re-equilibration (temperature, 55 °C). Mass spectrometric analysis of eluting peptides was conducted on an Orbitrap Exploris 480 instrument platform (Thermo Fisher Scientific). The spray voltage was set to 1.8 kV, the funnel RF level to 40, and the capillary temperature was at 275 °C. Data were acquired in data-dependent acquisition mode targeting the 10 most abundant peptides for fragmentation (Top10). Full MS resolution was set to 120,000 at m/z 200, and full MS automated gain
control (AGC) target to 300 % with a maximum injection time of 50 ms. The mass range was adjusted to m/z 350–1500. For MS2 scans, the collection of isolated peptide precursors was limited by an ion target of 1x10^5 (AGC target value of 100 %) and maximum injection times of 25 ms. The fragment ion spectra were acquired at a resolution of 15,000 at m/z 200, and the intensity threshold was kept at 1E4. The isolation window width of the quadrupole was set to 1.6 m/z, and the normalized collision energy was fixed at 30 %. All data were acquired in profile mode using positive polarity.

4.11. Data analysis and label-free quantification

The raw data acquired with the Exploris 480 were processed by MaxQuant (v2.0.1) suite [41,42] using standard settings and label-free quantification (LFQ) enabled for each parameter group, i.e., control and affinity-purified samples (LFQ ratio count 2, stabilize large LFQ ratios disabled, match-between-runs). Data were searched against UP000005641) and common contaminants. For peptide identification, trypsin was set as a protease, allowing for two missed cleavages. Carbamidomethylation was programmed as fixed, and methionine oxidation and acetylation of protein N-termini were set as variable modifications. Only peptides with a length of 7 amino acids or more were considered. Peptide and protein false discovery rates (FDR) were 1 %. In addition, proteins were identified by the presence of at least two peptides. Statistical analysis was conducted using the student’s t-test, which was corrected by the Benjamini–Hochberg method for multiple hypothesis testing. In addition, proteins were considered.

4.12. Transmission electron microscopy

4.12.1. Immunogold labeling

The immunogold analysis was carried out according to Tokuyasu [43,44]. Confluent HFF monolayers infected by parasites (Mol:4, 24 h) were fixed with 2 % paraformaldehyde/0.1 % glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 2 h at room temperature. Samples were scraped and pelleted by centrifugation (6000 g, 1.5 min). The cell pellets were infiltrated gradually in gelatin (1 %, 5 %, 10 % gelatin in 100 mM PB buffer, pH 7.4 at 37 °C). Subsequently, samples were cooled down on the ice to solidify gelatin and cut into small pieces, which were infiltrated overnight at 4 °C in 100 mM PB buffer containing 2.3 M sucrose (pH 7.4). Sample blocks (~700 μm^3) were mounted on aluminum pins and placed into the cryo-chamber, precooled to −110 °C of a cryo-ultramicrotome (UC7, Leica Microsystems, Wetzlar). Ribbons of 60 nm thin sections were picked by a Perfect Loop cryo-ultramicrotome (UC7, Leica Microsystems, Wetzlar). Ribbons were scraped and stained in 2 % uranyl acetate and 3 % lead citrate (Roth, Germany). Samples were washed 5x with anhydrous acetone, stepwise embedded in EPON 812 mixed with acetone (30 %, 60 %, 100 %) and finally polymerized for 48 h at 60 °C. Ultrathin sections of 70 nm were prepared using a Leica UC7 ultramicrotome (Germany) and a 35° Ultra diamond knife (DiATOME, Switzerland). Sections were collected on formvar-coated grids and stained for 30 min with 2 % uranyl acetate and 20 min with 3 % lead citrate (Roth, Germany). Images were collected using the JEOL 2100Plus system (200 kV, JEOL, Japan), equipped with a XAROSA CMOS 20MP camera (Emsis, Germany).

5. Data analysis, availability, and presentation

All assays were executed at least three independent times unless specified otherwise. The mass spectrometry data were processed using proprietary programs associated with each instrument. The datasets have been deposited to the ProteomeXchange Consortium (PXD032173) via the jPOST partner repository (JPST001521) (http://proteomexchange.org, https://doi.org/10.1093/nar/gkw1080). Other results presented herein were analyzed and plotted using the GraphPad Prism v8 software. The error bars in graphs signify means with SE. The p-values were computed by Student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Images of transgenic strains and phenotyping assays (plaque, immunofluorescence, immunoblot, PCR etc.) show only a representative of the three or more biological replicates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Author contributions

NG conceived, designed and supervised the project; KCV standardized and performed the wet-lab assays; LR assisted KCV in cell culture; OEP supervised KCV and RF to conduct the electron microscopy; UD and ST carried out the mass spectrometry analysis; NG, PH and MH contributed reagents and resources; KCV and NG analyzed and performed the wet-lab assays; LR assisted KCV in cell culture; K.C. Vo, L. Ruga, O.E. Psathaki et al. Computational and Structural Biotechnology Journal 20 (2022) 5775–5789

Appendix A. Supplementary data

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