Characterization of the ATP4 ion pump in *Toxoplasma gondii*

The *Plasmodium falciparum* ATPase PfATP4 is the target of a diverse range of antimalarial compounds, including the clinical drug candidate cipargamin. PfATP4 was originally annotated as a Ca^{2+} transporter, but recent evidence suggests that it is a Na^{+} efflux pump, extruding Na^{+} in exchange for H^{+}. Here we demonstrate that ATP4 proteins belong to a clade of P-type ATPases that are restricted to apicomplexans and their closest relatives. We employed a variety of genetic and physiological approaches to investigate the ATP4 protein of the apicomplexan *Toxoplasma gondii*, TgATP4. We show that TgATP4 is a plasma membrane protein. Knockdown of TgATP4 had no effect on resting pH or Ca^{2+} but rendered parasites unable to regulate their cytosolic Na^{+} concentration ([Na^{+}]_{cyt}). PfATP4 inhibitors caused an increase in [Na^{+}]_{cyt} and a cytosolic alkalinization in WT but not TgATP4 knockdown parasites. Parasites in which TgATP4 was knocked down or disrupted exhibited a growth defect, attributable to reduced viability of extracellular parasites. Parasites in which TgATP4 had been disrupted showed reduced virulence in mice. These results provide evidence for ATP4 proteins playing a key conserved role in Na^{+} regulation in apicomplexan parasites.

The Apicomplexa are a phylum of unicellular parasitic protists that impose enormous medical, veterinary, and socioeconomic burdens. Members of this phylum include *Plasmodium*, multiple species of which cause malaria in humans, and *Toxoplasma gondii*, the causative agent of toxoplasmosis. Apicomplexan parasites have complex life cycles that involve intracellular and extracellular stages, often in at least two different host organisms, in which they encounter vastly different external environments. To survive, they must regulate their cytosolic Na^{+} concentration ([Na^{+}]_{cyt}) and pH (pH_{cyt}) in the face of widely varying external ionic conditions. How they accomplish this is not well understood.

Cells typically control their [Na^{+}]_{cyt} and pH_{cyt} tightly, using a variety of membrane transporters and channels to do so. Differences between extracellular and intracellular Na^{+} and H^{+} concentrations (i.e. ion gradients) are exploited for essential processes such as nutrient acquisition and cell volume regulation. In most cell types, the cytosol is typically slightly alkaline (pH ~7.2–7.3), and the [Na^{+}]_{cyt} is typically around 10 mM (1, 2).

Most studies of Na^{+} and pH regulation in apicomplexan parasites to date have been performed with the blood-stage form of the most virulent human malaria parasite, *Plasmodium falciparum*. This parasite has, on its plasma membrane, a H^{+}-extruding V-type ATPase that plays a key role in pH regulation (3, 4) while also generating a large, inwardly negative membrane potential (5). The extracellular tachyzoite form of *T. gondii* has also been shown to have a plasma membrane V-type H^{+}-ATPase that plays roles both in controlling pH_{cyt} and generating and maintaining the membrane potential (6).

Na^{+} regulation in apicomplexan parasites has recently emerged as an area of particular interest. In an attempt to identify the target of a promising new class of antimalarials, the “spiroindolones,” *P. falciparum* parasites were exposed to increasing sublethal doses of these drugs *in vitro* until parasites showing low-level resistance emerged. Resistant parasites were found to have mutations in *PfATP4*, a gene encoding a plasma membrane P-type ATPase (7). PfATP4 was originally annotated as a Ca^{2+} transporter (8); however, there are now multiple lines of evidence that the transporter mediates the efflux of Na^{+} from the parasite. First, antiplasmodial spiroindolones (of which cipargamin, formerly known as NITD609 and KAE609, is the clinical candidate) cause a rapid increase in the parasite’s [Na^{+}]_{cyt} and Na^{+} content (9–11). The same is true of a range of structurally unrelated antiplasmodial agents for which mutations in *PfATP4* confer resistance (12–15). Second, spiroindalone-resistant parasites with mutations in *PfATP4* are less...
sensitive to the disruption of $[Na^+]_{cyt}$ by spiroindolones and also have a higher resting $[Na^+]_{cyt}$, consistent with resistance-conferring mutations in PfATP4 causing some impairment of its Na$^+$ transport function (10, 13). Third, spiroindolones inhibit Na$^+$-dependent ATPase activity in parasite membrane preparations (10, 16, 17).

In addition to dissipating the Na$^+$ gradient across the plasma membrane of the malaria parasite, spiroindolones and other “PfATP4-associated” antimalarials cause an increase in pH$_{cyt}$, increasing the pH gradient across the parasite plasma membrane (10, 13–16, 18). PfATP4 has been postulated to expel Na$^+$ from the parasite in exchange for H$^+$, imposing a significant “acid load” on the parasite (19). Lifting this load by inhibiting PfATP4 results in a cytosolic alkalinization.

The disruption of ion regulation by the PfATP4-associated antimalarials triggers a variety of detrimental events in the parasite (9, 20). It is not known whether ATP4 homologues in other apicomplexan parasites are similarly vulnerable.

Little is known about Na$^+$ regulation in T. gondii. The plasma membrane of T. gondii harbors two candidate Na$^+$/H$^+$ exchangers, TgNHE1 and TgNHE4 (21, 22). pH$_{cyt}$ is unaffected by the removal of extracellular Na$^+$ (6) and is unchanged in mutant parasites lacking a functional TgNHE1 (21), consistent with TgNHE1 not playing a significant role in the maintenance of the parasite’s resting pH$_{cyt}$. TgNHE1 mutant parasites have an elevated $[Ca^{2+}]_{cyt}$, suggesting a role for TgNHE1 in Ca$^{2+}$ regulation, perhaps via an effect on $[Na^+]_{cyt}$ (21). However, the $[Na^+]_{cyt}$ of T. gondii has not been studied directly. The antimalarial spiroindolone cipargamin has been shown to inhibit the growth of T. gondii (23), raising the possibility that the T. gondii homolog of ATP4 (TgATP4) may play an important role in this parasite. However, neither the function of TgATP4 nor the sensitivity of TgATP4 to cipargamin have been investigated.

In this study, we used a combination of genetic and physiological approaches to provide evidence that TgATP4 is a plasma membrane Na$^+$ pump that is important for Na$^+$ homeostasis, particularly in extracellular parasites that encounter high $[Na^+]_{cyt}$, and sensitive to a number of compounds that are believed to target PfATP4.

**Results**

**The apicomplexan ATP4 proteins form a distinct subfamily of type II P-type ATPases**

TgATP4 (TGM49_278660) shares significant sequence similarity with PfATP4 (Fig. S1). TgATP4 and PfATP4 both contain the eight conserved regions that have been described as comprising the “core of the P-type ATPase superfamily” (24), as well as the PROSITE consensus sequence for the phosphorylation site of P-type ATPases, D-K-T-G-T-[LIVM]-[TI] (Fig. S1). PfATP4 belongs to the type II branch of P-type ATPases (24), which includes ATP-driven Ca$^{2+}$, Na$^+$, K$^+$, and H$^+$ transporters. Type II P-type ATPases have been previously divided into four subgroups (A–D) based on a phylogenetic analysis of their 265-residue “core” region (24). Type IIA and type IIB P-type ATPases transport Ca$^{2+}$, whereas type IIC P-type ATPases include Na$^+$/K$^+$ transporters and H$^+$/K$^+$ transporters (24). Type IID P-type ATPases are “exitus natruses” (ENA)$^6$ transporters that have been proposed to extrude Na$^+$ (24).

We performed a phylogenetic analysis of type II P-type ATPases with a dataset consisting of TgATP4, PfATP4, other type II P-type ATPases from T. gondii, P. falciparum, and other apicomplexans; and type II P-type ATPases from a range of other eukaryotes, including other members of the Alveolata (ciliates, dinoflagellates, and chromerids), the superphylum of eukaryotes to which apicomplexans belong. Our analysis revealed four previously defined subgroups of type II P-type ATPases (24) plus a fifth subgroup of proteins, referred to here as “ATP4-type ATPases” (Fig. 1). The ATP4-type ATPase subgroup has strong bootstrap support and includes TgATP4, PfATP4, and proteins from all other apicomplexans we examined. We also identified ATP4-type ATPases in the chromerids Vittrella brassiciformis and Chromera velia and in the dinoflagellate Symbiodinium minutum. Chromerids and dinoflagellates are the closest extant relatives of apicomplexans (25, 26), and the presence of ATP4-type ATPases encoded in the genomes of these organisms suggests that ATP4-type ATPases evolved before these three lineages diverged. The absence of ATP4-type ATPases in other eukaryotes suggests that this family of proteins is restricted to apicomplexans and closely related organisms.

TgATP4 localizes to the T. gondii plasma membrane and is important for parasite growth

To examine the expression and localization of TgATP4, we fused a HA tag to the 3’ end of the ORF of the gene. Western blotting revealed that the resultant TgATP4-HA protein had an approximate mass of 160 kDa (Fig. 2A), close to the predicted mass of 147 kDa for HA-tagged TgATP4. Immunofluorescence assays revealed that TgATP4 co-localized with a marker for the plasma membrane (P30; 27) (Fig. 2B).

To determine whether TgATP4 is important for T. gondii growth, we generated a TgATP4 inducible knockdown line in which the native TgATP4 promoter was replaced with an anhydrotetracycline (ATc)-regulated promoter, and the resultant protein was fused with an N-terminal HA tag (Fig. S2, A and B). We refer to this strain as “iHA-TgATP4.” We confirmed localization of the HA-TgATP4 protein to the plasma membrane in this strain (Fig. S2C).

Exposure of iHA-TgATP4 parasites to ATc led to down-regulation of HA-TgATP4 expression, with the protein becoming nearly undetectable by Western blotting (Fig. 2C) and in immunofluorescence assays (Fig. S2C) 2 days after the addition of ATc. To determine whether TgATP4 is important for parasite growth and survival, we introduced the bright tandem dimeric Tomato (tdTomato) red fluorescent protein into the iHA-TgATP4 strain (creating a strain we termed “iHA-TgATP4/Tomato”), allowing us to monitor parasite growth fluorometrically using an assay described previously (28). Addition of ATc at the start of the assay resulted in a decrease in the growth of iHA-

$^6$The abbreviations used are: ENA, exitus natruses; ATc, anhydrotetracycline; ANOVA, analysis of variance; gRNA, guide RNA; SBFI, sodium-binding benzo[ furan isophthalate; BCECF, 2’,7’-bis-(2-carboxyethyl)-5’-(and-6)-carboxyfluorescein; IFA, immunofluorescence assay.
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TgATP4/Tomato parasites (p < 0.05, one-way ANOVA; Fig. 2D) but not in that of Tomato-expressing WT (“WT/Tomato”) parasites (p > 0.05, one-way ANOVA; Fig. 2D). Addition of ATc to iHA-TgATP4/Tomato parasites 3 days before commencing the assay (and maintaining its presence throughout the assay) further exacerbated the growth defect (p < 0.05, one-way ANOVA; Fig. 2D). To determine whether these growth defects were specifically the result of TgATP4 knockdown, we complemented iHA-TgATP4/Tomato parasites with constitutively expressed Ty1-tagged TgATP4 (generating a strain we termed “iHA-TgATP4/Tomato/cTy1-TgATP4”). This restored parasite growth in the presence of ATc (Fig. 2D), indicating that the growth phenotype observed in the iHA-TgATP4/Tomato strain resulted exclusively from the lack of expression of TgATP4.

The requirement for TgATP4 expression at different stages of the lytic cycle of T. gondii was investigated using a series of assays. First we investigated whether TgATP4 expression was important for the growth of intracellular parasites. iHA-TgATP4/Tomato parasites and WT/Tomato parasites were incubated with or without ATc for 2 days, extracted from their host cells, and allowed to invade new host cells for 24 h. The number of parasites per host cell vacuole was then determined. Most vacuoles contained either eight or 16 parasites at this time point (Fig. 3A), and there were no significant differences in the percentage of host cell vacuoles having any given number of parasites between WT/Tomato and iHA-TgATP4/Tomato parasites or between ATc-treated and untreated parasites (p > 0.05, one-way ANOVA). These data suggest that TgATP4 expression is not required for the intracellular growth or division of T. gondii.
We next investigated whether knocking down TgATP4 impaired the ability of parasites to egress from their host cells. The egress of parasites from their host cells was stimulated by addition of the Ca\(^{2+}\) ionophore A23187, and “percent egress” (the percentage of individual parasitophorous vacuoles from which parasites were observed to escape) was calculated as described previously (29). No significant difference in the percent egress was observed between WT and iHA-TgATP4 parasites or between parasites maintained for 30 h in the presence or absence of ATc (Fig. 3B; \(p > 0.05\), one-way ANOVA).

To investigate whether TgATP4 is important for invasion, WT/Tomato and iHA-TgATP4/Tomato parasites were grown in the absence or presence of ATc for 2 days and then allowed to invade host cells for 10 min. There was a small apparent decrease in invasion of iHA-TgATP4/Tomato parasites grown in the presence of ATc (Fig. 3C), but this decrease was not statistically significant (\(p > 0.05\), one-way ANOVA). We conclude that the defect in overall parasite growth observed upon TgATP4 knockdown (Fig. 2D) cannot be explained by defects in invasion, egress, or intracellular growth (Fig. 3, A–C).

In preliminary experiments, we observed that extracellular iHA-TgATP4 parasites treated with ATc exhibited an altered, rounded morphology and frequently contained a large intracellular vacuole (Fig. S3). We considered whether iHA-TgATP4 knockdown resulted in decreased viability of extracellular parasites, as has been observed in mutants of a gene involved in T. gondii stress responses (30). Parasites were grown in the absence or presence of ATc for 2 days, mechanically released from host cells by passage through a 26-gauge needle, and incubated in standard growth medium (which has a high [Na\(^+\)]) for 0–27 h. Parasites were then labeled for 20 min in propidium iodide (a membrane-impermeant DNA dye frequently used to assess cell viability; 31) and analyzed by flow cytometry. In all cases, parasite viability declined over the 27-h incubation; however, the viability of iHA-TgATP4 parasites grown in the presence of ATc decreased more rapidly than that of iHA-TgATP4 parasites grown in the absence of ATc or of WT controls, with statistical significance reached after a 20-h incubation in the growth medium (Fig. 3D; \(n = 4, p \leq 0.003\), one-way ANOVA). In summary, TgATP4 is important for the growth of T. gondii parasites over multiple lytic cycles and is not required for intracellular growth, egress or invasion but is important for maintaining the viability of extracellular parasites.

**TgATP4-disrupted parasites show reduced virulence in mice**

To investigate the impact of TgATP4 on parasite virulence in vivo, we generated parasites in which the ORF of TgATP4 was disrupted using CRISPR-Cas9 targeted genome editing. We designed a single guide RNA (gRNA) that targeted the first exon of the TgATP4 ORF (Fig. S4A) and transferred this into WT RH\(\Delta\)hxgprt/Tomato parasites (32). We identified a clonal parasite strain with a 113-bp insertion in the TgATP4 locus, resulting in incorporation of a premature stop codon in the TgATP4...
ORF (Fig. S4B). This strain will synthesize a truncated, and likely nonfunctional, \textit{Tg}ATP4 protein that lacks residues 34 to 1338. We termed this strain \textit{atp4}\textsubscript{H9004}34–1338. Plaque assays revealed that \textit{atp4}\textsubscript{H9004}34–1338 parasites exhibited a growth defect \textit{in vitro} (Fig. S4C). Complementation of \textit{atp4}\textsubscript{H9004}34–1338 parasites with a constitutively expressed copy of \textit{Tg}ATP4 (yielding the strain “\textit{atp4}\textsubscript{H9004}34–1338/cTy1-\textit{Tg}ATP4”) rescued the \textit{in vitro} growth phenotype (Fig. S4C).

To determine whether \textit{Tg}ATP4 influences parasite virulence \textit{in vivo}, we infected mice with either WT, \textit{atp4}\textsubscript{34–1338}, or \textit{atp4}\textsubscript{34–1338/cTy1-\textit{Tg}ATP4} parasites. In each case, five BALB/c mice were infected intraperitoneally with 10\textsuperscript{5} parasites. All mice infected with WT or \textit{atp4}\textsubscript{34–1338/cTy1-\textit{Tg}ATP4} parasites exhibited symptoms of toxoplasmosis and were euthanized on Day 6 or 7 post-infection (Fig. 4). In contrast, mice infected with \textit{atp4}\textsubscript{34–1338} parasites did not display disease symptoms until Day 9 or 10 post-infection (Fig. 4). Thus, parasites lacking \textit{Tg}ATP4 can cause disease \textit{in vivo}, but their virulence is reduced.

Figure 3. Investigation of the importance of \textit{Tg}ATP4 for intracellular growth, egress, and invasion. \textbf{A}, to measure intracellular parasite growth, WT/Tomato and iHA-\textit{Tg}ATP4/Tomato parasites were grown in the presence and absence of ATc for 2 days. Parasites were allowed to invade host cells and then cultured for 24 h before fixing the cells and counting the number of parasites per vacuole. \textbf{B}, the ability of WT and iHA-\textit{Tg}ATP4 parasites (maintained for 30 h in the presence or absence of ATc) to egress from their host cells after a 3-min exposure to the Ca\textsuperscript{2+} ionophore A23187 (2 \textmu M). \textbf{C}, WT/Tomato and iHA-\textit{Tg}ATP4/Tomato parasites were grown in the absence and presence of ATc for 2 days and then allowed to invade host cells for 10 min. \textbf{D}, to measure extracellular parasite viability, WT and iHA-\textit{Tg}ATP4 parasites were grown in the absence or presence of ATc for 2 days. Intracellular parasites were then mechanically egressed from host cells and incubated as extracellular parasites in (high-[Na\textsuperscript{+}]) growth medium for 0–27 h. The viability of parasites was monitored at predetermined time points by labeling with propidium iodide. In A–C, the bars show the mean values (with the error bars showing S.D.) averaged from three independent experiments. The data from individual experiments are shown with circles. In D, the data shown are the mean values (±S.D.) averaged from four independent experiments (asterisks denote statistically significant differences between iHA-\textit{Tg}ATP4 parasites incubated in the presence of ATc and all other parasites and conditions: **, \textit{p} < 0.01; ***, \textit{p} < 0.001; one-way ANOVA). Where not shown, error bars fall within the symbols.

Figure 4. Virulence of \textit{Tg}ATP4-expressing and \textit{Tg}ATP4-disrupted parasites in mice. Five BALB/c mice were infected with 10\textsuperscript{5} WT (black), \textit{atp4}\textsubscript{34–1338} (red), or \textit{atp4}\textsubscript{34–1338/cTy1-\textit{Tg}ATP4} (blue) parasites and monitored for progression of disease. The data are from a single experiment.
**TgATP4 is important for Na\(^+\) regulation in *T. gondii***

The addition of antimalarial spiroindolones or other compounds believed to inhibit PfATP4 to isolated *P. falciparum* parasites results in an immediate-onset, gradual increase in both [Na\(^+\)]\(_{\text{cyt}}\) and pH\(_{\text{cyt}}\) (10, 12–16, 18) but no change in [Ca\(^{2+}\)]\(_{\text{cyt}}\). We investigated the role of TgATP4 in regulating [Na\(^+\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{cyt}}\) in *T. gondii* using the Na\(^+\)-sensitive fluorescent indicator SBFI, the pH-sensitive fluorescent indicator BCECF, and the Ca\(^{2+}\)-sensitive fluorescent indicator Fura-2, respectively. In each case, cultures containing iHA-TgATP4 parasites were exposed to either ATc or 0.025% (v/v) ethanol (solvent control) for 2 days leading up to the experiment. On the day of the experiment, the appropriate fluorescent indicator was loaded into *T. gondii* tachyzoites that had either recently emerged from their host cells or that had been released from their host cells by passage of the culture through a 26-gauge needle. The dyes are all ratiometric, and an increase in the measured fluorescence ratio corresponds to an increase in [Na\(^+\)]\(_{\text{cyt}}\) (SBFI), pH\(_{\text{cyt}}\) (BCECF), or [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fura-2).

We incubated iHA-TgATP4 parasites grown in the absence of ATc (i.e. expressing TgATP4) in a saline solution containing 130 mM Na\(^+\) and measured the resting [Na\(^+\)]\(_{\text{cyt}}\). We found that the resting [Na\(^+\)]\(_{\text{cyt}}\) was less than 20 mM (Fig. 5A). Upon addition of cipargamin (50 nM; a concentration that has been used in similar experiments with isolated *P. falciparum* parasites (16)), the [Na\(^+\)]\(_{\text{cyt}}\) in these parasites increased (Fig. 5A). The cipargamin-induced increase in Na\(^+\) content in *T. gondii* parasites was confirmed using an alternate, HPLC-based approach (Fig. S5). In contrast, in iHA-TgATP4 parasites that had been exposed to ATc for 2 days to knock down TgATP4 expression, the resting [Na\(^+\)]\(_{\text{cyt}}\) was 130 mM (i.e. close to the [Na\(^+\)] in the medium in which the parasites were suspended; Fig. 5A). Cipargamin had little effect on [Na\(^+\)]\(_{\text{cyt}}\) in these parasites (Fig. 5A).

These data indicate that, as proposed for PfATP4, TgATP4 plays a key role in maintaining a low [Na\(^+\)]\(_{\text{cyt}}\) in the parasite. Our data are consistent with TgATP4 functioning as an Na\(^+\) efflux pump and being inhibited by nanomolar concentrations of cipargamin.

We next measured the effects of cipargamin and TgATP4 knockdown on resting pH\(_{\text{cyt}}\). We found that the resting pH\(_{\text{cyt}}\) of iHA-TgATP4 parasites grown in the absence or presence of ATc was similar (7.33 ± 0.09 and 7.40 ± 0.08, respectively; *p* = 0.10, unpaired *t* test; mean ± S.D.; *n* = 8). However, iHA-TgATP4 parasites expressing TgATP4 (−ATc), but not those with TgATP4 knocked down (+ATc), were found to undergo a cytosolic alkalinization upon addition of 50 nM cipargamin (Fig. 5B). This is consistent with the hypothesis that TgATP4 extrudes Na\(^+\) in exchange for H\(^+\), maintaining a low [Na\(^+\)]\(_{\text{cyt}}\) while at the same time imposing an acid load on the parasite. Inhibition of TgATP4 results in a lifting of this load and consequent cytosolic alkalization.

The V-type H\(^+\)-ATPase inhibitor concanamycin A (100 nM) resulted in a cytosolic aciddification in parasites that had been maintained both with (+) or without (−) ATc (Fig. 5B). This is consistent with previous evidence that the V-type H\(^+\)-ATPase plays a key role in pH regulation in *T. gondii* (6) and, importantly, shows that the parasites that had been maintained in the presence of ATc for 2 days were still actively maintaining their resting pH (and, hence, were metabolically active) at the time point at which all of our fluorometric assays were performed.

The spiroindolone cipargamin is one of a structurally diverse range of antimalarial agents that inhibit *P. falciparum* growth through an effect on ion homeostasis, attributed to inhibition of Na\(^+\) efflux via PfATP4 (10, 13–16). The pyrazoleamide PA21A050 is another such compound, structurally unrelated to the spiroindolones (15). To determine whether PA21A050 can inhibit *T. gondii* growth, we performed fluorescence growth assays. The growth of *T. gondii* parasites was inhibited by PA21A050 with an IC\(_{50}\) of 0.56 ± 0.17 μM (mean ± S.D.; *n* = 3; Fig. 6A). This IC\(_{50}\) value is much higher than the previously reported IC\(_{50}\) value for inhibition by PA21A050 of the proliferation of blood-stage *P. falciparum* parasites (−0.7 nM (15)). We investigated whether PA21A050, like cipargamin, impaired Na\(^+\) homeostasis in *T. gondii*. As seen for cipargamin, addition of PA21A050 (50 nM) to iHA-TgATP4 parasites grown in the absence of ATc resulted in an increase in [Na\(^+\)]\(_{\text{cyt}}\) (Fig. 6B). Addition of PA21A050 to iHA-TgATP4 parasites grown in the presence of ATc had little effect on the (already high) [Na\(^+\)]\(_{\text{cyt}}\) (Fig. 6B).

We next measured the effects of PA21A050 on resting pH\(_{\text{cyt}}\). Again, as seen for cipargamin, addition of PA21A050 to iHA-
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TgATP4 parasites grown in the absence of ATc resulted in an increase in resting pHcyt (Fig. 6C). No such increase was observed in iHA-TgATP4 parasites grown in the presence of ATc (Fig. 6C). The pyrazoleamide PA21A050 therefore affects Na\(^{+}\)/H\(^{+}\) and pH homeostasis in a manner similar to cipargamin.

TgATP4 does not play a direct role in Ca\(^{2+}\) regulation in T. gondii

We next examined the role of TgATP4 in Ca\(^{2+}\) regulation using the Ca\(^{2+}\)-sensitive dye Fura-2. The resting cytosolic [Ca\(^{2+}\)]\(_{cyt}\) ([Ca\(^{2+}\)]\(_{cyt}\)) was similar in TgATP4-expressing and TgATP4 knockdown parasites (Fig. 7A), and addition of cipargamin had little effect on [Ca\(^{2+}\)]\(_{cyt}\) in either case (Fig. 7A). We also compared the ability of TgATP4-expressing and TgATP4 knockdown parasites to regulate their [Ca\(^{2+}\)]\(_{cyt}\) in the presence of increasing external [Ca\(^{2+}\)]. TgATP4-expressing and TgATP4 knockdown parasites were both able to regulate their [Ca\(^{2+}\)]\(_{cyt}\) with [Ca\(^{2+}\)]\(_{cyt}\) maintained at less than 150 nM in the presence of external [Ca\(^{2+}\)] of up to 1 mM (the highest concentration tested; Fig. 7B) for both strains and with no discernible difference between them (p > 0.39, unpaired t tests). Decreased expression of TgATP4 therefore has no significant effect on Ca\(^{2+}\) regulation in these parasites.

Discussion

The first sequence analyses of PfATP4 led to annotation of this protein as a Ca\(^{2+}\) transporter (8, 33, 34), albeit one that was phylogenetically distinct from other types of Ca\(^{2+}\) ATPases that had been categorized at that time. These studies also identified residues in PfATP4 that were proposed to be involved in Ca\(^{2+}\) binding (8, 33, 34). In a detailed phylogenetic analysis of the 265-residue core region of 159 P-type ATPases, Axelsen and Palmgren (24) placed PfATP4 in the type IIA subgroup of P-type ATPases, most members of which are believed to transport Ca\(^{2+}\). However, few organisms belonging to the Apicomplexa or closely related phyla were included in their analysis. It has also been suggested that PfATP4 might be an ENA (a type IID P-type ATPase) (10). This suggestion was based on an
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Figure 7. Ca\(^{2+}\) regulation in TgATP4-expressing and TgATP4 knockdown parasites and the effect of cipargamin. A, traces showing the effects of cipargamin on [Ca\(^{2+}\)]\(_{\text{cyt}}\) in IHA-TgATP4 parasites expressing TgATP4 (−ATc, black) and in parasites in which TgATP4 is knocked down (+ATc, gray). The traces are representative of those obtained in at least three similar experiments for each condition. The parasites were suspended in physiological saline, and the concentration of cipargamin was 50 μM. Cyclopiazonic acid (CPA) was added at a concentration of 10 μM to release the endoplasmic reticulum Ca\(^{2+}\) store (59) and confirm that the assay enabled the detection of changes in [Ca\(^{2+}\)]\(_{\text{cyt}}\). B, the [Ca\(^{2+}\)]\(_{\text{cyt}}\) in IHA-TgATP4 parasites expressing TgATP4 (−ATc, gray symbols) and in parasites in which TgATP4 was knocked down (+ATc, gray symbols) in the presence of varying external [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{\text{out}}\)). The data are averaged from those obtained in four independent experiments performed on different days and are shown as the mean ± S.D. The [Ca\(^{2+}\)]\(_{\text{out}}\) values for TgATP4-expressing and TgATP4 knockdown parasites were compared at each [Ca\(^{2+}\)]\(_{\text{out}}\) using unpaired t tests. There was no significant difference between the [Ca\(^{2+}\)]\(_{\text{out}}\) in the two strains at any of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) values tested.

eight-residue sequence in PfATP4\(^{8491}\)IVQSLKRK with similarity to a semiconserved motif in ENA ATPases (MIEALHRR in ScTNA1). Included in this motif is a triplet of positively charged residues (KRK in PfATP4) that may be important for Na\(^{+}\) transport (35). However, our analysis using the entire P-type ATPase core sequence and a dataset containing representatives from a variety of organisms, including members of the Apicomplexa, Dinoflagellata, and Chromerida, reveal that ATP4 proteins do not belong to one of the previously defined subgroups of P-type ATPases but, rather, represent a distinct subgroup. One consequence of this is that the function or substrate specificity of the ATP4 proteins cannot be inferred from their sequences and must be determined experimentally.

In this study, we found that cipargamin and PA21A050 induced a gradual, immediate-onset increase in both [Na\(^{+}\)]\(_{\text{cyt}}\) and pH\(_{\text{cyt}}\) in T. gondii parasites expressing TgATP4. This mirrors the results of equivalent experiments in P. falciparum (10, 15). Notably, we observed no increases in [Na\(^{+}\)]\(_{\text{cyt}}\) and pH\(_{\text{cyt}}\) in parasites in which TgATP4 was knocked down. Unlike TgATP4-expressing parasites, which maintained a low [Na\(^{+}\)]\(_{\text{cyt}}\) of less than 20 mM in a solution with a high [Na\(^{+}\)]\(_{\text{cyt}}\) similar to that found in serum and extracellular fluid (130 mM), TgATP4 knockdown parasites were unable to regulate their [Na\(^{+}\)]\(_{\text{cyt}}\) and had an internal [Na\(^{+}\)] similar to that of the extracellular solution. Together, these data are consistent with ATP4 function being conserved between P. falciparum and T. gondii and provide genetic evidence that ATP4 proteins are cipargamin- and PA21A050-sensitive pumps that extrude Na\(^{+}\) while importing H\(^{+}\). However, the possibility that the flux of H\(^{+}\) equivalents that accompanies the transport of Na\(^{+}\) does not occur directly via ATP4 cannot be excluded.

Extracellular parasites lacking TgATP4 lost viability more rapidly than parasites expressing TgATP4. Upon prolonged extracellular incubation (~ 6–12 h), these parasites appeared swollen and frequently contained a large internal vacuole that resembled the previously described plant-like vacuole of the parasite (36). The plant-like vacuole has been associated with parasite tolerance to salt stress (36), and its apparent increase in size in extracellular parasites lacking TgATP4 may represent a parasite response to the presence of high [Na\(^{+}\)]\(_{\text{cyt}}\). Our observation that extracellular T. gondii parasites swell upon depletion of TgATP4 is in line with previous studies reporting that P. falciparum parasites swell on exposure to compounds believed to inhibit PfATP4 (9, 13, 15, 16). The observation that the pH\(_{\text{cyt}}\) of TgATP4-knockdown parasites was not significantly different from that of TgATP4-expressing parasites indicates that although, when present, TgATP4 imposes a significant acid load on the parasite, it does not play a major role in maintaining the normal resting pH\(_{\text{cyt}}\).

In the first experimental investigation of ATP4 function, it was reported that membranes prepared from Xenopus oocytes that had been injected with PfATP4 cRNA displayed greater Ca\(^{2+}\)-ATPase activity than membranes from noninjected oocytes (8). Others have since reported being unable to reproduce these findings (7, 37). Furthermore, addition to isolated P. falciparum parasites of either the spiroindolone NITD246 (10) or the pyrazoleamide PA21A050 (15) (for which resistance is mediated by mutation of PfATP4 (7, 15)) resulted in an increase in [Na\(^{+}\)]\(_{\text{cyt}}\) and pH\(_{\text{cyt}}\) but no change in [Ca\(^{2+}\)]\(_{\text{cyt}}\) (10, 15). Consistent with this, Ca\(^{2+}\) did not stimulate the cipargamin-sensitive ATPase activity measured in P. falciparum membrane preparations (17). Our observations that cipargamin had no significant effect on [Ca\(^{2+}\)]\(_{\text{cyt}}\) in T. gondii and that TgATP4 knockdown parasites were no less effective than TgATP4-expressing parasites in regulating their [Ca\(^{2+}\)]\(_{\text{cyt}}\) in the face of increasing external [Ca\(^{2+}\)] provide further evidence against a role for ATP4 in parasite Ca\(^{2+}\) regulation. Our findings that extracellular TgATP4 knockdown parasites cannot maintain their plasma membrane Na\(^{+}\) gradient but maintain tight control over their [Ca\(^{2+}\)]\(_{\text{cyt}}\) suggest that the parasite is not reliant on a plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger to regulate [Ca\(^{2+}\)]\(_{\text{cyt}}\). Orthologs of Na\(^{+}\)/Ca\(^{2+}\) exchange genes are lacking in apicomplexan parasites, and to the extent of our knowledge, there is no evidence, either from genomic or physiological data, that T. gondii parasites possess a Na\(^{+}\)/Ca\(^{2+}\) exchange system. We conclude that TgATP4 is required for Na\(^{+}\) regulation but
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not for maintaining the normal cytosolic pH or Ca\textsuperscript{2+} concentration in T. gondii parasites.

Although a 50 nM concentration of either the spiroindolone cipargamin or the pyrazoleamide PA21A050 was sufficient to perturb pH\textsubscript{cyt} and [Na\textsuperscript{+}]\textsubscript{cyt} in extracellular tachyzoites (Figs. 5 and 6), much higher concentrations of each of these compounds were needed to inhibit the proliferation of the parasite. An IC\textsubscript{50} of 1 \muM has been reported for inhibition of the proliferation of T. gondii tachyzoites by cipargamin (23); for P. falciparum, the IC\textsubscript{50} is orders of magnitude lower (~1 nM (7)). Similarly, our testing of PA21A050, a structurally unrelated PfATP4 inhibitor, for its effect on the proliferation of T. gondii revealed an IC\textsubscript{50} value much more than 500-fold higher than that reported for P. falciparum (15). Other antiplasmodial compounds that are believed to inhibit PfATP4 (PfATP4-associated compounds) also lack potency against T. gondii. Of the 28 antiplasmodial PfATP4-associated compounds identified in the 400-compound "Malaria Box" (14), all lacked potency against T. gondii, growth, with IC\textsubscript{50} values greater than 14 \muM in all cases (38). Thus, the available data indicate that, whereas in P. falciparum cipargamin and PA21A050 both perturb ion homeostasis and inhibit parasite proliferation in the nanomolar range, in T. gondii the concentrations required to inhibit parasite proliferation are much higher than those required to perturb ion homeostasis. Given that parasites can continue to proliferate (albeit at a reduced rate) in the absence of TfATP4 expression, it is likely that cipargamin and PA21A050 exert their growth-inhibitory effects on T. gondii via targets other than TfATP4.

The differential susceptibility of P. falciparum and T. gondii to growth inhibition by ATP4 inhibitors might be explained on the basis of the different ionic conditions each of the two parasites experience in the course of their asexual cycles. Both cells spend the majority of their asexual lytic cycles within host cells; for T. gondii, these can be nucleated cells of any type, whereas for P. falciparum (during the disease-causing stage of malaria), the host cells are erythrocytes. Mammalian cells maintain a low resting [Na\textsuperscript{+}]\textsubscript{cyt}. In the case of T. gondii-infected cells, there is no evidence of significant perturbation of host cell ion homeostasis; it is therefore presumed that the intracellular T. gondii parasite is exposed to a low-[Na\textsuperscript{+}] environment, and the parasite itself may therefore have little if any need to expend energy on extruding Na\textsuperscript{+} ions, relying instead on the host cell’s homeostatic mechanisms (Fig. 8). This might explain our finding that the intracellular growth of T. gondii is not affected when TfATP4 is knocked down. By contrast, while inside a human erythrocyte, the asexual-stage P. falciparum parasite induces profound changes to the permeability of the host erythrocyte membrane, through so-called new permeability pathways, such that the membrane can no longer maintain a large Na\textsuperscript{+} gradient, and [Na\textsuperscript{+}]\textsubscript{cyt} is increased to levels comparable with that in the extracellular medium (Fig. 8) (39–42). Consequently, the P. falciparum parasite is exposed to a high-[Na\textsuperscript{+}] environment for much of its 48 h occupancy of the erythrocyte (from ~20 h to 48 h post-invasion) (39). This might be expected to render P. falciparum dependent on its Na\textsuperscript{+} extrusion mechanism for most of its intraerythrocytic cycle, and hence particularly vulnerable to ATP4 inhibition (Fig. 8).

Knockdown and disruption of TgATP4 impaired, but did not fully inhibit, T. gondii proliferation (Fig. 2D) (Fig. S4). We also observed a defect in parasite virulence upon TgATP4 disruption (Fig. 4). A recent genome-wide CRISPR-based screen provided evidence that TgATP4 is important for normal T. gondii growth, with TgATP4 ranked in the top 7% of the most important genes in the parasite (“phenotype score” of ~4.77 (43)). The growth and virulence defects we observed were perhaps less pronounced than what may have been expected from the genome-wide study. We observed no significant decreases in invasion, intracellular growth, or egress upon TgATP4 knockdown (Fig. 3, A–C), consistent with the growth defect being due to an effect of TgATP4 knockdown on the parasite at another part of the lytic cycle. The inability of TgATP4 knockdown parasites to regulate [Na\textsuperscript{+}]\textsubscript{cyt} is likely to make these parasites particularly sensitive to high-[Na\textsuperscript{+}] conditions, as prevail in the extracellular medium (Fig. 8). Consistent with this, we found that the decline in the viability of extracellular parasites over time was more rapid for TgATP4 knockdown parasites than for parasites expressing TgATP4 (Fig. 3D). We conclude that TgATP4 is of particular importance in the extracellular stage of the T. gondii lytic cycle, consistent with its proposed role in mediating Na\textsuperscript{+} homeostasis in the parasite (Fig. 8).

In summary, this study provides genetic evidence that the T. gondii ATP4 protein, TfATP4, plays an important role in the extracellular stage of the lytic cycle of this parasite and functions as a spiroindolone- and pyrazoleamide-sensitive Na\textsuperscript{+}/H\textsuperscript{+} pump, acting to extrude Na\textsuperscript{+} from the parasite and maintain low [Na\textsuperscript{+}]\textsubscript{cyt}. The TfATP4 knockdown and disrupted lines provide tools for future functional studies to elucidate the importance of key residues in the ATP4 protein. For example, complementing the inducible TfATP4 knockdown line described here with mutant versions of TfATP4 (or with ATP4 variants from other organisms) will enable the roles of individual ATP4 residues, both in the function of the protein and in conferring resistance to PfATP4 antagonists, to be established.

Although Na\textsuperscript{+} regulation in T. gondii and P. falciparum parasites is sensitive to nanomolar concentrations of ATP4 inhibitors, our results suggest key differences in the effects of ATP4 inhibition on the growth of the two parasite species. These differences may be accounted for by the different exposure to extracellular Na\textsuperscript{+} experienced by the two parasites. P. falciparum parasites are exposed to high external Na\textsuperscript{+} levels throughout much of the intracellular phase of their intraerythrocytic cycle, whereas exposure of T. gondii parasites to a high external Na\textsuperscript{+} level is largely restricted to the extracellular phase of their lytic cycle. Together with the observation that parasites lacking TgATP4 have only a slight reduction in virulence, these findings raise important reservations about the utility of TfATP4 as a drug target in T. gondii.

Finally, our phylogenetic analysis indicates that ATP4 proteins constitute a distinct subgroup within the type II P-type ATPases, although proof that TfATP4 has a P-type ATPase mechanism requires additional study. Nevertheless, our data are consistent with ATP4-type ATPases having conserved roles as ATP-dependent Na\textsuperscript{+} pumps in apicomplexans and their relatives. The last common ancestors of apicomplexans, chromerids, and dinoflagellates were free-living organisms that inhab-
edited a marine environment (44), and it is conceivable that ATP4-type ATPases first evolved a role in regulating [Na\(^+\)/H\(^+\)] \(_{\text{cyt}}\) in the high-Na\(^+\)/H\(^+\) (460 mM (45)) marine environment.

**Experimental procedures**

**Ethics statement**

Mouse studies examining *T. gondii* virulence were performed according to procedures outlined in protocol A2016/42, approved by the Australian National University Animal Experimentation Ethics Committee.

**Phylogenetic analysis of TgATP4**

TgATP4 homologs and a representative selection of type II P-type ATPases from a broad range of eukaryotic organisms were aligned using ClustalX (46). The accession numbers for the proteins in the dataset are shown in Fig. 1. The "acces-
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sion.version” sequence identifiers are provided for sequences that are in the NCBI database. S. minitum accession numbers were derived from the S. minitum genome browser (http://marinegenomics.oist.jp/) (47). The Hematodinium sequences are available in BioProject PRJNA317731 (48), and the C. velia sequence Cvel-6710 is available through the Uniprot database. The editing software Jalview was used to trim the alignment to the core sequence of 265 residues for P-type ATPases as defined by Axelsen and Palmgren (24). The dataset was subjected to maximum likelihood phylogenetic analysis using PHYLIP as described previously (49). Bootstrap analysis, which provides a statistical measure of confidence in the nodes of the phylogenetic tree, was performed using 1000 replicates.

Host cell and parasite culture

T. gondii was cultured in human foreskin fibroblasts (a kind gift from Holger Schlüter, Peter MacCallum Cancer Centre) in DMEM supplemented with 1% (v/v) fetal calf serum, 200 μM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml gentamicin, and 0.25 μg/ml amphotericin B. Cells were cultured at 37 °C in a humidified 5% CO2 incubator. Unless stated otherwise, the type I TATi/Δku80 strain was used as the parental strain for the parasites generated in this study (50). Where applicable, ATc (dissolved in ethanol) was added at a final concentration of 0.5 μg/ml, yielding a final ethanol concentration of 0.025% (v/v).

Generation of genetically modified T. gondii lines

To generate a 3’ HA tag replacement in the TgATP4 locus, we amplified the 3’ region of the TgATP4 ORF using the primers 5’-GACTGGATCCGCACCATGCAGTTTATGGAATCC and 5’-CATGCTCTAGGCGGATCAATGCAGGGCGCATTTAGGAAAG. We digested the resultant product with BamHI -CATGCCTAGGGGCCATAATCGCCGGCG- and a 3’ PstI site. We digested the resultant vector with MfeI, transfected into iHA-TgATP4 parasites, and selected on chloramphenicol. All parasite strains were cloned by limiting dilution before characterization.

To generate a parasite strain in which the TgATP4 ORF was disrupted, we adopted a CRISPR/Cas9-based genome editing approach. Using Q5 site-directed mutagenesis (New England Biolabs), we modified the pSAG::Cas9-U6::sgUPRT vector (Addgene plasmid 54467 (54)) to express a gRNA targeting the first exon of the TgATP4 ORF. We PCR-amplified this vector using the primers 5’-ACTTTCCTCAGCCGACAGCGGTGTTTAGAGCTGAAATAGCAAG (the underlined sequence encodes the TgATP4 targeting region) and 5’-AACTTGACACTCCCCATTAC and circularized the PCR product according to the manufacturer’s instructions (New England Biolabs). The resultant vector co-expresses the TgATP4-targeting gRNA and Cas9-GFP. We transfected this vector into type I RH/Δhxgprt/Tomato strain parasites (32) and cloned GFP-positive parasites 3 days after transfection. A mutant clone containing a frameshift mutation (a 113-bp insertion) was identified and further verified by direct Sanger sequencing of the TgATP4 locus. The resultant strain, termed atp4ΔAT5710, was complemented by integration of the pUGCTTy(TgATP4) vector as described above.

Immunofluorescence assays and Western blotting

Immunofluorescence assays (IFAs) and Western blots were performed as described previously (28). For IFAs, we used monoclonal rat anti-HA (1:500 dilution; clone 3F10, Roche) and monoclonal mouse anti-TgP30 (1:1000; clone TP3, Abcam, catalog no. ab8313) as primary antibodies and anti-mouse Alexa Fluor 546 (1:500; Thermo Fisher, catalog no. A-11030) and anti-rat Alexa Fluor 488 (1:200; Thermo Fisher, catalog no. A-11006) as secondary antibodies. Microscopy was performed on a DeltaVision Elite system (GE Healthcare) using an inverted IX71 microscope with a ×100 UPlanSapo oil immersion lens (Olympus). Images were taken using a Photometrics CoolSNAP HQ2 camera and deconvolved and adjusted for contrast and brightness using SoftWoRx Suite 2.0 software. For Western blotting, we used monoclonal rat anti-HA (1:500 dilution; clone 3F10, Roche) and mouse anti-GR8 (1:10,000 dilution; a kind gift from Gary Ward, University of Vermont for contrast and brightness using SoftWoRx Suite 2.0 software.

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Characterization of ATP4 in Toxoplasma gondii

To measure the viability of extracellular parasites, iHA-TgATP4 and WT parasites were grown in the absence or presence of ATc for 2 days. Flasks containing infected host cells were washed once in growth medium to remove extracellular parasites, and then parasites were mechanically egressed from host cells by passage through a 26-gauge needle. The released parasites were filtered through a 3-μm filter to remove host cell debris and then incubated for 27 h in growth medium in a humidified 5% CO2 incubator set to 37°C. Parasite samples were taken at 0, 4, 9, 20, and 27 h, pelleted by centrifugation at 12,000 × g for 1 min, and then resuspended in a solution of 1 μg/ml propidium iodide in PBS supplemented with 10 mM D-glucose. Samples were incubated at room temperature in the dark for 20 min and then analyzed by flow cytometry on an LSR II flow cytometer (BD Biosciences). Viable parasites were gated based on forward scatter, side scatter, and propidium iodide fluorescence and analyzed using FlowJo software.

To measure the effects of the disruption of the TgATP4 gene on parasite virulence, we infected five 6- to 8-week-old female BALB/c mice with 10⁴ WT (RHΔhxprt/Tomato), atp4ΔΔΔΔΔ–iHA-TgATP4, or atp4ΔΔΔΔΔ–iHA-TgATP4 parasites. Parasites were intracellular at the time of preparation and were manually egressed by passage through a 26-gauge needle. After centrifugation at 1500 × g for 10 min to sediment the parasites, the parasites were resuspended in PBS and injected into mice within 1 h of egress. Mice were monitored daily for symptoms of toxoplasmosis, and those exhibiting terminal symptoms were euthanized.

Fluorometric ion measurements

iHA-TgATP4 parasites, grown in 175-cm² tissue culture flasks for 2 days in the presence of either ATc (0.5 μg/ml) or solvent (0.025% v/v ethanol), were harvested by passage of cultures through a 26-gauge needle. The parasites were then passed through a 3-μm filter to remove host cell debris and centrifuged at 1500 × g for 10 min. The supernatant medium was removed, and the parasites were resuspended in 1 ml of physiological saline (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 20 mM glucose, and 1 mM MgCl₂ (pH 7.10)) and then centrifuged at 12,000 × g for 1 min. The supernatant solution was removed, and the parasites were resuspended again in physiological saline to which the appropriate fluorescent dye was added.

[Na⁺], [H⁺], and [Ca²⁺] were measured using the Na⁺-sensitive dye SBF1, the pH-sensitive dye BCECF, and the Ca²⁺-sensitive dye Fura-2, respectively. In all cases, parasites were suspended in physiological saline and loaded using the ace- toxymethyl ester forms of the dyes (Molecular Probes) at 37°C. In the case of SBF1, parasites were loaded with 6 μM SBF1 for 30 min in the presence of 0.1% w/v Pluronic F-127. For BCECF, parasites were loaded for 14 min in the absence of Pluronic F-127, and the BCECF concentration was 5 μM. For Fura-2, loading was performed over 30 min using 6 μM Fura-2 and 0.04% (w/v) Pluronic F-127. The parasites were washed twice after dye loading to remove extracellular dye.

In all cases, fluorescence measurements were performed at 37°C on 5–10 × 10⁷ parasites using a PerkinElmer LS 50B fluorescence spectrometer with a dual excitation Fast Filter accessory. Measurements were taken every 0.6 s, and a 6-s roll-

Parasite growth, invasion, egress, viability, and virulence assays

Fluorometric T. gondii growth assays were performed as described previously (32). The experiments used in the experiments shown in Fig. 2D had egressed naturally 2–12 h prior to setting up the assays. Plaque assays were performed as described previously (28), except that 2000 parasites were added per flask. For intracellular growth assays, infected host cells were washed in a high-[K+] “intracellular” buffer (125 mM KCl, 5 mM NaCl, 25 mM HEPES, 20 mM glucose, and 1 mM MgCl₂ (pH 7.4)). Parasites were then mechanically released from host cells by passing infected cells through a 26-gauge needle. Released parasites were filtered through a 3-μm filter and incubated on human foreskin fibroblast–containing coverslips for 1 h. The intracellular buffer was removed by aspiration and replaced with DMEM. Parasites were incubated a further 4 h, during which they were able to invade host cells; then parasites that had failed to invade were removed by washing the host cells three times in DMEM. Infected host cells were cultured for a further 20 h before fixation and counting of the number of parasites in vacuoles. Parasites within at least 100 vacuoles were counted for each condition.

Parasites for invasion assays were prepared as for the intracellular growth assays, except that parasites were incubated in the presence of DMEM for 10 min to allow invasion into host cells. Parasites were fixed in a solution of 3% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in PBS. Parasites were then mechanically released from host cells by passing infected cells through a 26-gauge needle. Released parasites were filtered through a 3-μm filter and incubated on human foreskin fibroblast–containing coverslips for 1 h. The intracellular buffer was removed by aspiration and replaced with DMEM. Parasites were incubated a further 4 h, during which they were able to invade host cells; then parasites that had failed to invade were removed by washing the host cells three times in DMEM. Infected host cells were cultured for a further 20 h before fixation and counting of the number of parasites in vacuoles. Parasites within at least 100 vacuoles were counted for each condition.

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Egress assays were performed as described previously (29). Briefly, parasites were allowed to invade host cells for 1 h, and then parasites that had not invaded were removed by washing the host cells three times with DMEM. Parasites were then grown with or without ATc (0.5 μg/ml) for 30 h. The culture medium was removed, infected host cells were washed three times with DMEM, and stimulation medium (DMEM supplemented with 10 mM HEPES and 2 μM A23187 (Sigma-Aldrich)) was added to induce egress. After 3 min, parasites were fixed using 4% (v/v) paraformaldehyde in PBS and stained for IFAs using the primary antibody rabbit anti-

GAP45 (1:500 dilution; Santa Cruz Biotechnology, catalog no. sc-2006) and goat anti-mouse (1:10,000 dilution; Santa Cruz Biotechnology, catalog no. sc-2005) as secondary antibodies.

(55)) as primary antibodies and horse radish peroxidase–conjugated goat anti-rat (1:5000 dilution; Santa Cruz Biotechnology, catalog no. sc-2006) and goat anti-mouse (1:10,000 dilution; Santa Cruz Biotechnology, catalog no. sc-2005) as secondary antibodies.

Parasite growth, invasion, egress, viability, and virulence assays

Fluorometric T. gondii growth assays were performed as described previously (32). The experiments used in the experiments shown in Fig. 2D had egressed naturally 2–12 h prior to setting up the assays. Plaque assays were performed as described previously (28), except that 2000 parasites were added per flask. For intracellular growth assays, infected host cells were washed in a high-[K+] “intracellular” buffer (125 mM KCl, 5 mM NaCl, 25 mM HEPES, 20 mM glucose, and 1 mM MgCl₂ (pH 7.4)). Parasites were then mechanically released from host cells by passing infected cells through a 26-gauge needle. Released parasites were filtered through a 3-μm filter and incubated on human foreskin fibroblast–containing coverslips for 1 h. The intracellular buffer was removed by aspiration and replaced with DMEM. Parasites were incubated a further 4 h, during which they were able to invade host cells; then parasites that had failed to invade were removed by washing the host cells three times in DMEM. Infected host cells were cultured for a further 20 h before fixation and counting of the number of parasites in vacuoles. Parasites within at least 100 vacuoles were counted for each condition.

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...ing average was applied to the data shown in Figs. 5, 6, B and C, and 7A. In Figs. 5, 6, B and C, and 7A, the parasites were suspended in physiological saline for the fluorescence measurements. In Fig. 7B, parasites were suspended in a solution that was identical to physiological saline except that it contained 1 mM EGTA (Ca^{2+}-free saline solution) or in Ca^{2+}-free saline solution to which had been added sufficient CaCl₂ to achieve a final free [Ca^{2+}] of 1,100 or 1,000 μM (Ca-EGTA Calculator v1.3).

For [Na⁺]_{cyt} and [Ca^{2+}]_{cyt} experiments, the dye-loaded cells were excited at 340 nm and 380 nm, with fluorescence measured at 515 nm. For pH_{cyt} experiments, the excitation wavelengths were 440 nm and 495 nm, and the emission wavelength was 520 nm. [Ca^{2+}]_{cyt} measurements were calibrated in situ (56) using a K_d of 120 nm for the Fura-2/Ca^{2+} complex. pH_{cyt} measurements were calibrated as described previously (3). Cell number limitations prevented full calibration of Na⁺ measurements; however, the fluorescence ratios corresponding to 130 mM Na⁺ and 20 mM Na⁺ were determined, essentially as described previously (57, 58), by suspending parasites in calibration salines containing either 130 mM or 20 mM Na⁺ and adding the ionophores gramicidin, nigericin, and monensin (each at 4 μM). Segments of the calibration traces are shown before the time courses presented in Figs. 5A and 6B.

HPLC measurements

An HPLC-based method described previously (11) for analysis of intracellular Na⁺ and K⁺ content in Plasmodium parasites was adapted here for use with T. gondii. Parasites were harvested by passage of cultures through a 26-gauge needle. The parasites were then passed through a 3-μm filter to remove host cell debris and centrifuged at 1500 × g for 10 min. The supernatant medium was removed, and the parasites were washed and resuspended in a saline solution and then incubated with cipargamin or DMSO (the conditions tested are detailed in the legend for Fig. S5). The parasites were then sedimented by centrifugation and washed three times (12,000 × g, 1 min) in ice-cold wash buffer (150 mM magnesium acetate (pH 7.1)). The parasites were then resuspended in 100 μl of lysis buffer (40%/60% (v/v) 20 mM ammonium acetate (pH 5)/acetonitrile) and centrifuged (16,000 × g, 5 min) to remove insoluble matter. The supernatant solution was transferred into a vial for HPLC analysis, which was performed as described previously (11).

Statistics

Data were tested for statistical significance using t tests (two-tailed, unpaired) or one-way ANOVA, as stated in the relevant sections. For the fluorescence-based assays of parasite growth (for which data are shown in Fig. 2D), one-way ANOVA was performed on the prenormalized data (fluorescence intensity units, after subtraction of the background), and “experiment” was used as a “blocking factor” to prevent any differences in fluorescence intensity between different experiments from eroding the precision of the test. Post hoc comparisons were then performed using the least significant difference test. For one-way ANOVAs performed without blocking, a post hoc Tukey test was used.

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Characterization of the ATP4 ion pump in *Toxoplasma gondii*

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