The genetic Ca\textsuperscript{2+} sensor GCaMP3 reveals multiple Ca\textsuperscript{2+} stores differentially coupled to Ca\textsuperscript{2+} entry in the human malaria parasite \textit{Plasmodium falciparum}

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Abstract
Cytosolic Ca\textsuperscript{2+} regulates multiple steps in the host cell invasion, growth, proliferation and egress of blood-stage \textit{Plasmodium falciparum}, yet our understanding of Ca\textsuperscript{2+} signaling in this endemic malaria parasite is incomplete. By using a newly generated transgenic line of \textit{P. falciparum} (PfGCaMP3) that expresses constitutively the genetically-encoded Ca\textsuperscript{2+} indicator GCaMP3, we have investigated the dynamics of Ca\textsuperscript{2+} release and influx elicited by inhibitors of the SERCA pumps, cyclopiazonic acid (CPA) and Thapsigargin (Thg).

Here we show that in isolated trophozoite phase parasites: i) both CPA and Thg release Ca\textsuperscript{2+} from intracellular stores in \textit{P. falciparum} parasites; ii) Thg is able to induce Ca\textsuperscript{2+} release from an intracellular compartment insensitive to CPA; iii) only Thg is able to activate Ca\textsuperscript{2+} influx from extracellular media, through a mechanism resembling Store-Operated Ca\textsuperscript{2+} Entry (SOCE), typical of mammalian cells; iv) the Thg-sensitive Ca\textsuperscript{2+} pool is unaffected by collapsing the mitochondria membrane potential with the uncoupler carbonyl cyanide m-chlorophenyl hydrazione, or the release of acidic Ca\textsuperscript{2+} stores with nigericin. These data suggest the presence of two Ca\textsuperscript{2+} pools in \textit{P. falciparum} with differential sensitivity to the SERCA pump inhibitors, and only the release of the Thg-sensitive Ca\textsuperscript{2+} store induces Ca\textsuperscript{2+} influx. Activation of the SOCE-like Ca\textsuperscript{2+} influx may be relevant for controlling processes such as parasite invasion, egress and development mediated by kinases, phosphatases and proteases that rely on Ca\textsuperscript{2+} levels for their activation.

Introduction
Malaria infection by \textit{Plasmodium} parasites is responsible for a high rate of morbidity and mortality in humans, with over 200 million people infected and around half million deaths annually. \textit{P. falciparum} infection results in the most lethal form of malaria in humans and resistance to front line drugs is a growing issue (1). Fluctuations in the intracellular Ca\textsuperscript{2+} concentration plays a crucial role in controlling vital processes within mammalian cells such as fertilization, differentiation, proliferation and cell death (2). Similarly, key phases of the \textit{Plasmodium} life cycle have been shown to depend on changes in Ca\textsuperscript{2+} concentration.
including intra-erythrocytic parasite proliferation invasion and egress from the host cell, protein secretion, and cell cycle regulation (3-12). Significantly, these Ca\(^{2+}\) signaling pathways can be coupled to extracellular ligands, including melatonin and ATP (4,5,10,11). Advancing our understanding of the Ca\(^{2+}\) signaling pathways utilized in \textit{P. falciparum} to trigger these essential processes has the potential to identify novel therapeutic targets.

Ca\(^{2+}\) ions inside the cell can bind to specific proteins or be sequestered within organelles. The endoplasmic/sarcoplasmic reticulum (ER/SR) is the major intracellular Ca\(^{2+}\) stores in mammalian cells and in most, though not all, eukaryotic cells (13). Ca\(^{2+}\) accumulation into the ER/SR depends on an ATP driven Ca\(^{2+}\) pump named sarcoplasmic reticulum Ca\(^{2+}\) ATPase, SERCA. In mammalian cells several isoforms of SERCA are expressed in different cells and ER-SR sub-compartments, while Ca\(^{2+}\) accumulation in the Golgi and other post Golgi, lumenally acidic organelles (secretory granules, lysosomes, post Golgi vesicles) depends on the expression of different Ca\(^{2+}\) pumps, the so called Secretory Pathway Ca\(^{2+}\) ATPases, SPCAs, encoded by two genes, ATP2C1 and ATP2C2, and several spliced variants (14). Thg and CPA are known to be highly selective inhibitors of all mammalian SERCAs, while both drugs are totally ineffective on mammalian SPCAs (15). Only one, poorly characterized inhibitor, has been described for SPCA1, namely Bis(2-hydroxy-3-tert-butyl-5-methyl-phenyl)-methane (bis-phenol) (16,17). Other mechanism of Ca\(^{2+}\) accumulation in acidic compartments have been suggested to exist in mammalian cells, e.g. a H\(^{+}\)/Ca\(^{2+}\) exchange mechanism, but based largely on indirect evidence (18,19).

Evidence exists supporting the existence in most eukaryotes (and in some prokaryotes) of acidocalcisomes, intracellular compartments with an acidic lumen and containing large amounts of Ca\(^{2+}\) (and polyphosphates, in addition to Mg\(^{2+}\), Zn\(^{2+}\)). The histological nature of acidocalcisomes, however, is far from clear and this term has been used to define different intracellular compartments, such as lysosomes, vacuoles etc (20). As to the mechanism of Ca\(^{2+}\) uptake, in unicellular organisms and particularly in trypanosomes, the most convincing evidence is that acidocalcisome are endowed with a plasma membrane type of Ca\(^{2+}\) ATPase (21).

The importance and relevant organelle(s) underlying Ca\(^{2+}\) stores in \textit{Plasmodium falciparum} is still debated. While most investigators agree on the importance of the ER as an inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive compartment, different results have been obtained concerning the acidic Ca\(^{2+}\) compartment (22). For example Biagini et al. have suggested that in \textit{P. falciparum} the digestive vacuole, in which hemoglobin is metabolized and degraded, represents the major dynamic acidic Ca\(^{2+}\) compartment of the cell (23). According to these authors, both Thg and CPA cause a drastic loss of Ca\(^{2+}\) from this organelle. On the contrary, an analysis with different fluorescent Ca\(^{2+}\) indicators by Rohrbach et al. suggests that minor changes in the vacuole Ca\(^{2+}\) concentration occur upon addition of the two SERCA inhibitors (24). A major interpretation problem with all these studies is that the fluorescent Ca\(^{2+}\) indicators used are trapped not only in the cytosol or in specific organelles but also in cellular compartments, i.e., cytosol, nucleus, ER and digestive vacuole, which differ in terms of protein composition and luminal pH.

In mammalian cells a key tool to investigate the role of SERCA in Ca\(^{2+}\) signaling has been Thg, because of its high specificity in blocking selectively and at low nanomolar concentrations all SERCA isoforms. On the contrary, inhibition of \textit{Plasmodium} SERCA (PfSERCA or PfATP6) by Thg is still debated. Data published by our group and others have shown that \textit{Plasmodium} has a Thg-sensitive Ca\(^{2+}\) pump inhibited by the drug, albeit at higher concentrations than typically observed in mammalian cells (25-27). Other groups, on the contrary, have found no effect of Thg, including on saponin-isolated \textit{Plasmodium} parasites (28). In a recent study published by Pandey et al. the authors employed a transgenic line of \textit{P. falciparum} expressing the Ca\(^{2+}\) sensor Yellow Cameleon (YC)-Nano, and in this work Thg and the antimalarial dihydroartemisinin also did not appear to affect cytosolic Ca\(^{2+}\) whereas CPA was effective (29). Eckstein-Ludwig et al. have employed heterologous expression of PfATP6 in \textit{Xenopus} oocytes, and in their studies 0.8 \(\mu\)M Thg inhibited PfATP6 similarly to 1 \(\mu\)M CPA (26). However, employing the purified protein \textit{in vitro}, Arnou et al. showed that the IC\(_{50}\) of Thg for PfATP6 ATPase
activity was about 100 µM, much higher than for mammalian SERCA. By contrast, the IC50 for CPA acting on PfATP6 was actually about 10-fold lower than for rabbit skeletal muscle SERCA1a (30). Measurements of Ca2+ dynamics are usually performed by loading the cells with chemical fluorescent indicator dyes such as Fluo4 and Fura2. However, as shown by several groups (23,24) (and mentioned above) these dyes are trapped not only in the cytosol, but also in other cellular compartments, complicating the interpretation of the results and possibly explaining, at least in part, the discrepancy between the different published studies. To overcome this problem, we decided to utilize parasites expressing Genetically Encoded Calcium Indicators (GECIs) whose intracellular localization is highly selective in different cells, including protist parasites (29,31,32).

The GCaMP family, a group of GECIs derived from circularly-permutated green fluorescent protein with an engineered calmodulin inserted, were developed by Looger and coworkers (33). The GCaMP Ca2+ indicators offer improved photostability, increased brightness and dynamic range, and a range of affinities for Ca2+, providing greatly enhanced signal to noise compared with FRET-based GECIs (33). The affinity of GCaMP3 for Ca2+ is 660 ±19 nM, and it appears to be nontoxic even when constitutively expressed in living animals (34). We generated a P. falciparum (3D7) stably expressing GCaMP3 as a cytosol-located GECI, referred to as PfGCaMP3 (32).

Results

PfGCaMP3 reveal that both CPA and Thg release Ca2+ from internal stores.

In the past years a large body of work by us and others has employed chemical Ca2+ indicators trapped within the parasites using the intracellularly cleaved acetoxyethyl esters to characterize Ca2+ signaling pathways in malaria parasites (5,6,35,36). These dyes, however, are not exclusively located in the cytosol of Plasmodium spp., but are also trapped within organelles, the relative distribution and localization depending on the experimental protocols and the strain utilized. More recently we reported the generation of a new class of transgenic P. falciparum constitutively expressing the GECI GCaMP3 (PfGCaMP3) that could be employed to monitor selectively the dynamics of [Ca2+] only in the cytosol of the parasites (32). For all experiments described here, PfGCaMP3 cultures were synchronized by sorbitol treatment 12-16 h prior to experiment, then PfGCaMP3 parasites were isolated from the red blood cells with saponin. Figure 1A shows the bright field, basal GCaMP3 fluorescence and merged images of trophozoite parasites. Cultures were confirmed to be in trophozoite phase using Giemsa smears (Fig. 1A) prior to isolation.

The ability of CPA and Thg to mobilize Ca2+ from intracellular stores has been studied in vitro by a number of groups, with varying results (26,30): while the doses of CPA necessary for maximal Ca2+ release are similar to those used in mammalian cells, in P. falciparum those of Thg are at least one order of magnitude higher than those employed in mammalian systems (25,27-29). Kinetic measurements of CPA- and Thg-mediated Ca2+ rises in isolated PfGCaMP3 parasites immobilized on poly-L-lysine were carried out using a plate reader with microfluidic injection capabilities. This assay has advantages for throughput and small sample volume. Figure 1B shows the dose-dependent effect of CPA to increase [Ca2+]c (increased PfGCaMP3 fluorescence). The dose-response to CPA revealed an IC50 of 1.5 µM (Fig. 1C), consistent with previous studies. We have
shown previously in isolated parasites that the maximal effect of Thg is obtained at about 25 µM (25). However, we were not able to accurately determine the IC50 of Thg in the plate reader assay, due to the need to inject aqueous solutions at concentrations that exceed the solubility of Thg. However, at a final concentration of 1 µM, the rise in Ca2+ induced by Thg was equivalent to that caused by 10 nM CPA (data not shown), confirming that Thg is substantially less potent compared to mammalian cells, as discussed above (and see below).

**CPA and Thg show differential effects on [Ca2+]c in *P. falciparum.**

To overcome the limitations of the plate reader assay, we utilized population measurements of isolated parasite suspensions in a fluorometer where Thg could be added directly from a concentrated DMSO stock solution (37). In this preparation the parasites are constantly stirred in the cuvette to prevent them from settling during the experiment and this also allows rapid mixing of applied drugs, enabling us to test higher concentrations of Thg. In these experiments, in the presence of 2 mM CaCl2 in the medium, CPA (30 µM) (Fig. 2A) and Thg (10 µM) (Fig. 2B) elicited a robust [Ca2+]c increase in isolated PfGCaMP3 populations, with no change in [Ca2+]c observed with vehicle control (Fig. 2C). However, the response to each drug was distinct; CPA caused a rapid [Ca2+]c rise followed by a slowly declining phase, whereas Thg elicited an initial very rapid fluorescence increase, followed by a second slower rise and finally by a sustained, larger (compared to that caused by CPA) Ca2+ response that reached a plateau after approximately 200 seconds. Notably, the first rapid and small fluorescence rise caused by Thg is an artefact as it is also observed when Thg is added to a medium without cells (Fig. S1), so the quantitative data presented below were corrected for this artifact. Figure 2D shows a summary of the peak Ca2+ data obtained in this series of experiments, all carried out in the presence of extracellular Ca2+.

To determine whether Ca2+ influx across the plasma membrane contributes to the Thg response we repeated the experiments in the absence of extracellular Ca2+ (Fig. 2E-G). Under these conditions the transient CPA response was similar to that in the presence of extracellular Ca2+, whereas the sustained phase of the Thg response was abolished. Thus, it appears that there is a significantly larger Thg-induced [Ca2+]c rise only in the presence of Ca2+ in the medium (Fig. 2H).

In mammalian cells, Ca2+ release from the ER is often followed by Ca2+ entry across the plasma membrane into the cytosol, by a process described as SOCE. The usual protocol to reveal SOCE in mammalian cells is to treat the cells with Thg or CPA in Ca2+-free medium to empty the intracellular Ca2+ stores, followed by the re-addition of Ca2+ to the medium. When SOCE is activated, Ca2+ re-addition results in a large and sustained increase in [Ca2+]c. Ca2+ add-back in the experiments of Figures 2E-G resulted in an increase in [Ca2+]c that was much larger after Thg treatment than with CPA or the DMSO control. These data are summarized in Figure 2I and suggest that in *P. falciparum* some form of SOCE appears to be selectively activated by Thg but not by CPA.

**Thg but not CPA targets an intracellular Ca2+ pool that is linked to SOCE.**

Based on in vitro evidence (30) the pharmacological target of both CPA and Thg is assumed to be PfATPase6. However, as these two drugs appear to have differential effects on SOCE in *P. falciparum*, we decided to investigate whether other Plasmodium Ca2+ pumps, in common or distinct intracellular Ca2+ pools, could be targeted by the drugs. To determine whether CPA and Thg release Ca2+ from the same pool, we investigated whether CPA and Thg interfere with the Ca2+ response of each other by adding the two SERCA inhibitors in sequence (Fig. 3). Addition of Thg abolished the Ca2+ rise elicited by subsequent CPA addition, both in the presence and absence of extracellular Ca2+ (Fig. 3A-B). By contrast, pretreatment with CPA only partially reduced the Ca2+ response to subsequent Thg addition (Fig. 3C-D). As expected, no [Ca2+]c rise was observed after addition of the DMSO solvent control (Fig. 3E-F). These data are summarized in Fig. 3G-H. Significantly, when Ca2+ was added back to cells incubated in the absence of extracellular Ca2+ (Fig. 3B,D,F), Ca2+ influx was activated whether Thg was added before or after CPA, and these data are
also summarized in Figure 3H. One interpretation of these findings is that there are two Ca\(^{2+}\) pools, one sensitive to both CPA and Thg, and the other sensitive to only Thg. However, as noted in the Discussion, it is also possible that CPA and Thg differentially target distinct Ca\(^{2+}\) pumps in a single organelle.

**Single cell measurements suggest that Thg targets an additional Ca\(^{2+}\) pool.**

Population measurements of CPA- and Thg-induced GCaMP3 signal changes in a large number of cells provide robust and reproducible data; however, we wanted to confirm that the effects we report above are occurring in the same cell, and not in two distinct cell populations. To this aim trophozoite stage PfGCaMP3 parasites were saponin-isolated as above and then immobilized onto borosilicate glass coverslips with CellTak. GCaMP3 images (excitation 488 nm, emission 510 nm) were collected at 1 Hz on a wide-field epifluorescent microscope at 37°C. Since individual PfGCaMP3 cells differ in expression levels and basal signals are low, the single cell imaging traces were normalized to the peak signal obtained with 10 µM ionomycin in the presence of 2 mM extracellular Ca\(^{2+}\) (Fig. 4).

Consistent with the population data collected in the fluorimeter shown in Figure 2, CPA elicits a robust, transient [Ca\(^{2+}\)]\(_c\) response (Fig. 4B), whereas no [Ca\(^{2+}\)]\(_c\) rise was observed in the solvent control (Fig. 4A). However, in this type of single cell imaging experiments, we observed that Thg elicits a markedly different [Ca\(^{2+}\)]\(_c\) response pattern characterized by a delay of several minutes followed by fast, oscillatory behavior of variable frequency (Fig. 4C). By contrast, Ca\(^{3+}\) oscillations were never observed with CPA. As to the time-to-response discrepancy between CPA and Thg effects in single cell imaging and population measurements, we believe that this again reflects the lower solubility of Thg, and the time taken for Thg diffusion into the cells. In this setup Thg could not be added through a perfusion system because the drug binds strongly to the plastic tubes and never reaches an effective concentration in the cells. These problems do not occur in the cuvette of the fluorometer thanks to the constant rapid mixing in these experiments. Individual Ca\(^{2+}\) oscillations are not observed in the population experiments with the fluorimeter as they are not synchronous; thus, as expected, the mean trace data of all cells in the imaging field yields a similar monophasic sustained Ca\(^{2+}\) increase, albeit much slower than in the stirred cuvette system (Fig. S2). The time lag between Thg addition and onset of response may account for some discrepancies in the literature where Thg is reported not to act in *P. falciparum* (29).

We also performed single cell imaging experiments in Ca\(^{2+}\)-free buffer supplemented with 100 µM EGTA that confirmed the population data showing that only Thg, but not CPA, induces SOCE in *P. falciparum*. CPA and Thg gave similar response patterns under Ca\(^{2+}\)-free conditions (Fig 4D-F); however, only Thg was able to trigger Ca\(^{2+}\) entry into the cell following the addition of 2 mM CaCl\(_2\) into the buffer. Both the amplitude (Fig. 4G) and rate of [Ca\(^{2+}\)]\(_c\) increase (Fig. 4H) on Ca\(^{2+}\) addition following Thg treatment were significantly greater than with CPA or vehicle control. The amplitude was 2-fold larger following Thg compared to CPA, and the rate of rise of [Ca\(^{2+}\)]\(_c\) was 6-fold faster. The response to Ca\(^{2+}\) add-back following Thg treatment was not significantly different from that observed in the vehicle control, but both rates were relatively rapid. This reflects a constitutive Ca\(^{2+}\) entry component in these cells, which is demonstrated by the similar amplitude decrease of [Ca\(^{2+}\)]\(_c\) when extracellular Ca\(^{2+}\) is removed (Fig. S3).

The interactions between CPA and Thg were further investigated at the single cell level. For these experiments sequential additions of both drugs were performed in the presence of 2 mM extracellular Ca\(^{2+}\). When CPA was added first, Thg was still able to elicit a [Ca\(^{2+}\)]\(_c\) response, which was typically oscillatory in nature (Fig. 5A). However, when Thg was added first, the addition of CPA failed to further increase [Ca\(^{2+}\)]\(_c\) (Fig. 5B). We found that on average 80% of cells responded to both drugs when CPA was added first, whereas only 20% of cells responded to both drugs when Thg was added first (Fig. 4C). In the latter experiments the residual response to CPA could be because the slow onset Thg response had yet to fully release the Ca\(^{2+}\) store.

**Other Ca\(^{2+}\) stores**
A variety of evidence suggests that in *P. falciparum* the ER represents a major Ca\(^{2+}\) storage compartment endowed with a unique IP\(_3\)-sensitive Ca\(^{2+}\) release channel (5,11,36,38). Additional Ca\(^{2+}\) stores have been identified in *P. falciparum* that may contribute to cytosolic Ca\(^{2+}\) dynamics, including an acidic Ca\(^{2+}\) pool and the mitochondrion (22). The most abundant acidic compartment in *Plasmodium* is the digestive vacuole in which hemoglobin is metabolized (39). It has been proposed that the digestive vacuole can act as a dynamic Ca\(^{2+}\) storage organelle, playing a major role in maintaining *P. falciparum* Ca\(^{2+}\) homeostasis and redistributing Ca\(^{2+}\) during parasite growth and development (23). However, other groups have argued that the free Ca\(^{2+}\) in the vacuole is too low to significantly contribute to Ca\(^{2+}\) release in response to stimulation (24). A classical approach to investigate the presence and role of acidic compartments is that of collapsing their luminal pH.

To investigate whether an acidic compartment is the additional Thg-sensitive Ca\(^{2+}\) pool we used the K\(^+/H^+\) ionophore nigericin (Nig), which results in the release of Ca\(^{2+}\) from acidic compartments, including the digestive vacuole, by abolishing the pH gradient. Single cell imaging experiments with isolated PfGCaMP3 parasites were performed in Ca\(^{2+}\)-free medium to ensure that [Ca\(^{2+}\)]\(_c\) responses were due to intracellular stores and not Ca\(^{2+}\) influx across the plasma membrane. Addition of 250 nM Nig resulted in a large and rapid [Ca\(^{2+}\)]\(_c\) increase detected by GCaMP3 (Fig. 6A; green trace). The [Ca\(^{2+}\)]\(_c\) increase was preceded by a transient decrease in GCaMP3 fluorescence, which likely reflects acidification of the cytosol. This acidification would tend to decrease the Ca\(^{2+}\)-sensitivity of GCaMP3, so the relatively large [Ca\(^{2+}\)]\(_c\) increase with Nig indicates that there is a large acidic pool, most likely the digestive vacuole. As noted above, the isolated parasites have a constitutive Ca\(^{2+}\) influx pathway (CCI), which may preload the intracellular Ca\(^{2+}\) stores since they are prepared in 2 mM extracellular Ca\(^{2+}\). Therefore, in order to be sure the GCaMP3 signal was not too close to saturation to observe differences in Ca\(^{2+}\) release to CPA or Thg, the PfGCaMP3 parasites were co-loaded with the lower affinity ratiometric Ca\(^{2+}\) indicator Fura-FF (5 \(\mu\)M Fura-FF/AM for 1 h) prior to imaging both Ca\(^{2+}\) indicators simultaneously, as described in the methods section.

The blue traces of Figure 6A-C show the Fura-FF fluorescence ratio measured with 340 nm and 380 nm excitation recorded at the same time as the green traces of GCaMP3 fluorescence excited at 488 nm; all detected with 510 nm emission. The lower affinity Fura-FF signals show slower kinetics and lower amplitude relative to the maximum [Ca\(^{2+}\)]\(_c\) after ionomycin. Nevertheless, both indicators reported the [Ca\(^{2+}\)]\(_c\) transient elicited by CPA (Fig. 6B) and the delayed [Ca\(^{2+}\)]\(_c\) oscillations following Thg addition (Fig. 6C), as well as the large amplitude Nig-induced [Ca\(^{2+}\)]\(_c\) elevations. Since the Fura-FF signals were clearly not saturated, these were used to quantitate the effect of CPA and Thg on the Ca\(^{2+}\) response to Nig. Neither agent reduced the amplitude of the Nig-induced [Ca\(^{2+}\)]\(_c\) increase as compared to the vehicle control (Fig. 6D), suggesting that they do not act on the main acidic pool in *P. falciparum*. It was more difficult to quantitate the relatively small responses to CPA and Thg added after Nig, but both agents elicited [Ca\(^{2+}\)]\(_c\) increases in the presence of Nig (Fig. 6E). It has been shown that the *P. falciparum* mitochondrion can also act as a dynamic intracellular Ca\(^{2+}\) store and participates in Ca\(^{2+}\) homeostasis (40). To investigate whether the mitochondrion is involved in Thg-mediated Ca\(^{2+}\) release, we utilized the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP). We first confirmed the required dose needed to collapse the mitochondrial membrane potential by adding CCCP to isolated parasites loaded with the membrane potential dye tetramethylrhodamine ethyl ester (TMRE). We found that 2.5 \(\mu\)M CCCP (added with 2.5 \(\mu\)g ml\(^{-1}\) oligomycin to prevent reversal of the mitochondrial ATP synthase) uncoupled the mitochondrion and caused the maximum decrease in the TMRE signal within the parasite (Fig. 7A). We then treated isolated PfGCaMP3 parasites with the same dose of CCCP/oligomycin to uncouple the mitochondrion before adding CPA followed by Thg (Fig. 7B). Mitochondrial uncoupling did not cause any Ca\(^{2+}\) release on its own, and did not affect the ability of either CPA or Thg to elicit a [Ca\(^{2+}\)]\(_c\) response. After treatment with CCCP/oligomycin, 76.5 \(\pm\) 1.6 \% of cells responded to Thg, similar to the 85.0 \(\pm\) 9.6 \% of cells responding to Thg in controls (mean \(\pm\)...
S.E.M., n=3). These data indicate that the parasite mitochondrion does not contribute to the additional source of Thg-induced Ca\(^{2+}\) release.

**Discussion**

We and other groups have demonstrated that *Plasmodium* parasites have ER-like internal Ca\(^{2+}\) pools sensitive to Thg and CPA, as well as an acidic Ca\(^{2+}\) pool that can be released with nigericin or chloroquine (5,6,25,26,30,41). In higher eukaryotes, Ca\(^{2+}\) efflux from the ER is mediated by IP\(_3\) or ryanodine receptors. We have described IP\(_3\)-dependent Ca\(^{2+}\) release in asexual stages of *P. falciparum* and *P. chabaudi* parasites (41,42), including in the intraerythrocytic parasite using caged IP\(_3\) (5). The production of IP\(_3\) and DAG has also been reported during *Plasmodium* gametogenesis, a fundamental step in parasite transmission to the mosquito vector (43). These findings support the conclusion that *Plasmodium* relies on Ca\(^{2+}\) signaling for its survival in the vertebrate and invertebrate hosts.

Experiments with intracellularly trappable chemical Ca\(^{2+}\) indicators are plagued by the partial localization of the dyes in different compartments due to compartmentalization into organelles. This problem appears to be particularly relevant in *Plasmodium* spp., where there is a prominent accumulation into the digestive vacuole in the absence of anion transport inhibitors (23). Thus, there is always the possibility that changes in Ca\(^{2+}\) concentration measured with chemical Ca\(^{2+}\) indicators loaded into cells as the acetoxymethyl ester could reflect, at least in part, events not occurring in the cytoplasm. On the contrary, with GECIs, the localization of the expressed Ca\(^{2+}\) indicator protein is highly specific and, in the case of our GCaMP3-expressing *P. falciparum* (PfGCaMP3), exclusively in the cytoplasm. Not only is the GCaMP3 specifically targeted to the cytoplasm, but due to its relatively low affinity for Ca\(^{2+}\) compared to chemical indicators such as Fura2, and its lower diffusion rate, it has a reduced Ca\(^{2+}\) buffering capacity and less effect on the spatial dynamics of Ca\(^{2+}\) within the cell.

Here we show unambiguously, by using the PIGCaMP3 parasites, that in *P. falciparum* both SERCA inhibitors, Thg and CPA are capable of releasing Ca\(^{2+}\) from intracellular stores into the cytoplasm of the parasites. The IC\(_{50}\) of CPA was determined to be 1.5 \(\mu\)M, a concentration that was in a similar range to that usually found in mammalian cells. However, in contrast to mammalian cells where Thg is typically observed to be more potent than CPA, our studies with PfGCaMP3 show that higher concentrations of Thg are required, consistent with other studies of the effects of Thg in *Plasmodium* spp. (25-27,30). The low solubility of Thg makes it difficult to perform dose-response studies in the high concentration range required for maximal effect, but in previous studies with isolated *P. falciparum* parasites (loaded with Fluo-3 and analyzed by confocal microscopy), we observed a maximum Ca\(^{2+}\) release response at 25 \(\mu\)M Thg (25). This falls within the broad concentration range reported for inhibition of the Ca\(^{2+}\)-ATPase activity of purified or heterologously expressed PfATP6 (26,30). Using the GECI yellow cameleon (YC)-Nano in *P. falciparum* parasites, Pandey et al. (29) reported that there was no response to Thg (7.6 \(\mu\)M) in imaging experiments under conditions where they could observe a CPA-induced [Ca\(^{2+}\)]\(_{c}\) increase. However, the low signal to noise obtained using this FRET-based Ca\(^{2+}\) indicator required averaging of data across many cells from multiple experiments to resolve the [Ca\(^{2+}\)]\(_{c}\) increases with CPA and the Ca\(^{2+}\) ionophore A23187 (29). It is likely that this approach would miss the delayed asynchronous [Ca\(^{2+}\)]\(_{c}\) oscillations observed with Thg in our imaging experiments. The slow action of Thg in imaging experiments presumably reflects the diffusion into the imaging chamber, low solubility and the need to accumulate relatively high concentrations of the drug within the parasite. The response to Thg in our cuvette system with rapid stirring is much faster.

The relatively low affinity for Thg-induced Ca\(^{2+}\) release raises the question of whether it acts on the same Ca\(^{2+}\) pump as CPA in *P. falciparum*. To address this question we carried out a series of experiments in which CPA and Thg were added sequentially, in both cell population cuvette experiments and in single cell imaging. These studies showed that pretreatment with Thg eliminated the response to subsequent CPA addition, whereas Thg was still able to cause a [Ca\(^{2+}\)]\(_{c}\) increase after the Ca\(^{2+}\) response to a prior CPA addition was complete. In both cases there
was no washout of the first drug prior to adding the second drug. Thus, it appears that Thg can release the same Ca\(^{2+}\) pool that is released by CPA. However, there is a second target for Thg, with a residual pool of Ca\(^{2+}\) still available for release after treatment with CPA. These data suggest two possibilities (Fig. 8): i) The two drugs target two intracellular Ca\(^{2+}\) pools, one sensitive to both SERCA inhibitors, and one only available for mobilization by Thg. ii) Alternatively, the same Ca\(^{2+}\) pool could express two Ca\(^{2+}\) pumps, one sensitive to both drugs, and one only affected by Thg. The latter case would envision a store that requires the activity of both pumps to be fully loaded with Ca\(^{2+}\), such that inhibition of only one leads to partial emptying, while inhibition of both pumps is required for complete Ca\(^{2+}\) release.

In addition to PfATP6, a P-type ATPase gene, PfATP4, has been described in *P. falciparum* that has characteristics similar to those of eukaryotic P-type ATPases, in particular with conserved amino acids sequences involved in Ca\(^{2+}\) binding (44), and has also been reported to have Ca\(^{2+}\)-ATPase activity that is insensitive to low concentrations of Thg (45). However, PfATP4 is known to function as a plasma membrane sodium extrusion pump, and has not been shown to transport Ca\(^{2+}\) (46,47). Thus, it seems most likely that there is another, as yet unidentified, target of Thg (48-51).

The fact that Thg causes Ca\(^{2+}\) oscillations, whereas CPA does not, points to a two-pool model for the action of Thg, rather than two Ca\(^{2+}\) pumps in the same ER store. For example, Thg might elicit Ca\(^{2+}\)-induced Ca\(^{2+}\) release from a pool that accumulates Ca\(^{2+}\) independent of SERCA activity. In the context of the possibility that Thg could act on a second Ca\(^{2+}\) store, distinct from the ER, we investigated the acidic compartment and the mitochondrion. The primary acidic Ca\(^{2+}\) compartment in *P. falciparum* is the digestive vacuole (23,24) which appears to accumulate a relatively large load of Ca\(^{2+}\) in the isolated parasite preparation. We investigated the role of the acidic compartments in the actions of Thg and CPA using low levels of Nig. These experiments were carried out in the absence of extracellular Ca\(^{2+}\) to avoid any contribution from plasma membrane Ca\(^{2+}\) influx. Neither Thg nor CPA reduced the amplitude of the Ca\(^{2+}\) release response to Nig, even when measured with the low affinity chemical indicator dye FuraFF. Thus, these agents do not appear to deplete Ca\(^{2+}\) from the digestive vacuole, although we cannot rule out an effect of Thg on a minor acidic compartment that might be masked by the large Ca\(^{2+}\) release from the digestive vacuole.

We also examined a potential role of the mitochondrion using the uncoiler CCCP to collapse the mitochondrial membrane potential. In the presence of oligomycin to prevent reversal of the mitochondrial ATP synthase, CCCP did not cause any increase in [Ca\(^{2+}\)]\(_c\) that would reflect Ca\(^{2+}\) loading of the *Plasmodium* mitochondrion in the intact parasite. Moreover, pretreatment with CCCP did not affect the ability of CPA or Thg to elicit Ca\(^{2+}\) responses. Thus, the mitochondrion does not represent an additional Ca\(^{2+}\) store sensitive to Thg.

We have previously shown that Thg activates a form of SOCE in *P. falciparum*, and that this can be blocked by 2-aminoethyl diphenylborinate (2-APB) and 2-APB analogs (11). Significantly, this SOCE pathway is not activated by melatonin (11), which we have shown to elevate [Ca\(^{2+}\)]\(_c\) by mobilizing an IP\(_3\)-linked Ca\(^{2+}\) pool in *Plasmodium* spp., believed to be the ER (4,5). Therefore, in the present study we examined the [Ca\(^{2+}\)]\(_c\) response to the SERCA inhibitors in the presence and absence of extracellular Ca\(^{2+}\). Whereas the [Ca\(^{2+}\)]\(_c\) increase elicited by CPA was always transient, Thg presented a distinct pattern of response in the two conditions, being sustained when extracellular Ca\(^{2+}\) was present and transient in the absence of Ca\(^{2+}\). Moreover, only Thg increased the rate of Ca\(^{2+}\) entry when extracellular Ca\(^{2+}\) was added back after the depletion of intracellular stores. By contrast, CPA pretreatment had no effect on the rate of Ca\(^{2+}\) entry. It might be argued that CPA has a secondary effect to inhibit the SOCE. However, this does not appear to be the case, because Thg was able to activate SOCE whether it was added before or after CPA. The finding that Thg but not CPA can activate SOCE in *P. falciparum* is not necessarily incompatible with a one-pool model (Fig. 8), since it could be that complete emptying of the stores is required to activate SOCE (ie. both the CPA/Thg-sensitive SERCA and the additional Thg-sensitive Ca\(^{2+}\) pump must be inhibited to reach a sufficiently low ER Ca\(^{2+}\) load). Nevertheless, the preponderance of the evidence seems most consistent with a two-pool model, in which CPA and Thg inhibit SERCA in a Ca\(^{2+}\)-containing ER...
compartment, and there is an additional Ca\(^{2+}\)-containing intracellular compartment that is specifically mobilized by Thg. This would best explain the different patterns of [Ca\(^{2+}\)]\(_c\) response observed with Thg and CPA, and the observation that only Thg can activate the SOCE-like Ca\(^{2+}\) influx in \textit{P. falciparum}.

The intraerythrocytic trophozoite phase malaria parasite is sequestered within the parasitophorous vacuole inside the RBC, and this might be expected to preclude significant SOCE because RBC cytosolic Ca\(^{2+}\) is maintained in the nanomolar range. However, the parasitophorous vacuole is formed by invagination of the red cell plasma membrane, and we have previously shown that Ca\(^{2+}\) within the vacuole is at least two orders of magnitude higher than in the parasite or RBC cytosol (35). This elevated Ca\(^{2+}\) level could be maintained by a parasitophorous duct connecting the vacuole to the extra-erythrocytic medium (52), or through Ca\(^{2+}\) transport into the vacuole by plasma membrane-derived RBC Ca\(^{2+}\) pumps. Thus, SOCE at the trophozoite stage can play a physiological role, and may be required to maintain Ca\(^{2+}\) signaling and parasite maturation during this phase of the life cycle (11,35). It is also possible that the \textit{P. falciparum} SOCE participates in Ca\(^{2+}\) signaling during egress of the mature parasite as the RBC membranes break down, and during the invasion of new RBCs by the extracellular merozoites. In mammalian cells SOCE is mediated by STIM and Orai proteins, which have no homologs in the \textit{P. falciparum} genome. Thus, in \textit{Plasmodium}, the SOCE-like process most likely occurs through molecular mechanisms different from those described in mammals, or through functional analogs of STIM and Orai that are molecularly distinct.

**Experimental procedures**

\textit{P. falciparum}: Culture, synchronization and isolation of parasites.

\textit{Plasmodium falciparum} was maintained in continuous culture as previously described (53). A novel, transgenic strain of \textit{P. falciparum} (derived from 3D7) expressing the genetically encoded Ca\(^{2+}\) indicator GCaMP3 (PGCaMP3) was used for all of the experiments in this paper. This clone was developed by Borges-Periera et al (32). Briefly, GCaMP3 (a gift from Loren Looger (34); Addgene plasmid #22692) was cloned and inserted into the \textit{P. falciparum} expression vector pDC, then transfected into trophozoite phase parasites via electroporation. Episomal vector expression is maintained by the continuous presence of the selection agent (WR99210; 5 nM). The parasites were grown in plastic cell culture flasks (175 cm\(^2\)) with RPMI 1640 medium (GibcoBRL) supplemented with 0.5% AlbuMAX I (Gibco) and 5 nM of WR99210 with 5% hematocrit in a 90% N\(_2\); 5% O\(_2\); 5% CO\(_2\) atmosphere at 37°C. Parasites were synchronized approximately 12-18 hours prior to isolation via sorbitol treatment (54) to yield a homogenous population of trophozoite stage parasites, as verified by Giemsa-stained thin blood smears. Synchronized cells were pelleted, resuspended into PBS. Free parasites were isolated from the RBCs by saponin treatment (0.01%), which eliminates the RBC plasma membrane and parasitophorous vacuole, without disrupting the integrity of the parasite plasma membrane (55). The trophozoite stage parasites were collected by centrifugation at 2,000 RPM, and washed twice into a MOPS-based buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO\(_4\), 2 mM CaCl\(_2\), 5.5 mM D-glucose, 50 mM MOPS, pH 7.2), or HEPES-based imaging buffer (25 mM HEPES, 121 mM NaCl, 5 mM NaHCO\(_3\), 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 10 mM glucose, 0.25% BSA, pH 7.4).

**Spectrofluorometric determinations**

Isolated parasites at the trophozoite stage were used to perform experiments either in the presence or absence of extracellular Ca\(^{2+}\) (no CaCl\(_2\) added plus addition of 100 µM EGTA). Cytosolic Ca\(^{2+}\) dynamics after addition of the compounds were monitored using a Shimadzu spectrofluorometer (RF5301PC, Japan) with parasites (10\(^7\) cells ml\(^{-1}\)) in a 1 ml stirred cuvette. Excitation of GCaMP3 fluorescence was performed at 488 nm and emission was measured at 530 nm. All assays were performed at 37°C, in triplicates, with at least three independent experiments.

The Ca\(^{2+}\) dose-response of isolated parasites to CPA was also measured in a FlexStation 3 plate spectrofluorometer (Molecular Devices). For this,
96 well plates (black with clear flat bottom) were treated overnight with 50 µg of poly-L-lysine. Isolated PfGCaMP3 parasites at trophozoite stage were added to each plate well (10^7 per well in 200 µL total volume) and incubated for approximately 30 min allowing parasite adhesion to the plate bottom. After this, the media was replaced to remove the parasites that did not adhere. Cytosolic Ca^{2+} dynamics after addition of CPA was monitored in the 96-well plate spectrofluorometer using the multi-channel fluidic addition capabilities. GCaMP3 was excited at 488 nm and emission was measured at 530 nm.

**Fluorescence Imaging**

Isolated parasites were plated onto borosilicate glass coverslips coated with Cell-Tak via the adsorption method according to manufacturer’s instructions (Sigma) and allowed to settle for 30 min at 37°C to enable cell adherence. Once plated, coverslips were washed twice and mounted in a 37°C temperature controlled cell chamber with 3 ml of imaging buffer on the stage of a wide field epifluorescence inverted microscope (Nikon Eclipse TE300). Imaging was performed using a heated 40X oil immersion objective and an EM-CCD camera (Hamamatsu Photonix ImageEM); GCaMP3 images (excitation 488 nm, emission 510 nm; long band pass filter) were acquired at 1 Hz using the data acquisition software NIS Elements (Nikon).

**Ca^{2+} influx measurements**

Immediately prior to imaging, parasites were switched into a modified imaging buffer without Ca^{2+} supplemented with 100 µM EGTA. Cells were then treated with either CPA (10 µM, Cayman Chem Co), Thg (5 µM, Cayman Chem Co), or vehicle control (DMSO) for 10 min prior to Ca^{2+} add back.

**Mitochondrial membrane potential**

Tetramethylrhodamine, ethyl ester (TMRE) was used to determine the concentration of the mitochondrial uncoupler Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) required to cause complete dissipation of the mitochondrial membrane potential. Images of TMRE fluorescence were obtained using 543 nm excitation and 580 nm emission, and were acquired at 1 Hz. The isolated parasites were loaded with TMRE (5 nM, Sigma) for 13 min, followed by treatment with CCCP (2.5 µM, Sigma) and oligomycin (2.5 µg·ml⁻¹, Sigma).

**Analysis**

Imaging data were acquired and processed using NIS Elements (Nikon) software, and analyzed using a custom in-house program built in MATLAB. Data were normalized to the maximum ionomycin Ca^{2+} response (F/F_{Iono}). Statistical analysis was performed in GraphPad Prism, including one-way ANOVA testing with Bonferroni’s multiple comparison test.

**Data availability:** The authors declare that all data are contained within the article and accompanying supporting information.

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**Author contributions**

L. B. P. performed and designed the experiments in Figs. 1–3, SJT and PJB performed and designed the experiments in Figs. 4-7, ALAS helped to perform the experiments in Fig. 2-3. APT and CRSG are the principal investigators who contributed for the design of the experiments and data discussion as well as managed the project. All the authors contributed to the writing of the paper and interpretation of the data.

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**Conflict of interest**
The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**
Ca\(^{2+}\) (calcium), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), CPA (cyclopiazonic acid), GECIs (Genetically Encoded Calcium Indicators), IP\(_3\) (inositol, 1,4,5 trisphosphate), SERCA (sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase), SOCE (store operated calcium entry), TMRE (tetramethylrhodamine, ethyl ester), Thg (thapsigargin)

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Fig. 1 Measurement of $[\text{Ca}^{2+}]_c$ response of CPA with PfGCaMP3. A synchronized population of trophozoite-stage *P. falciparum* parasites expressing GCaMP3 (PfGCaMP3) were isolated, resuspended into MOPS buffer with 2 mM Ca$^{2+}$ and plated onto a 96 well black plate with clear bottom (10$^7$ cells per well in 200 µl total volume) pre-treated with 50 µg/ml of poly-L-lysine. (A) PfGCaMP3 parasites employed in the experiments. (B) The Ca$^{2+}$ dose-response of isolated parasites to CPA was measured in a FlexStation 3 plate reader. (C) The mean area under curve (AUC) of $[\text{Ca}^{2+}]_c$ in response to CPA was used to calculate the IC50 value. Scale bar = 10 µm
Fig. 2 SOCE is activated by Thg but not by CPA in *P. falciparum* parasites. Isolated PfGCaMP3 parasites at the trophozoite stage were placed in a 1 ml stirred cuvette at a density of 10^7 cells ml^{-1} and maintained at 37°C. Changes in [Ca^{2+}]_c were detected using a Shimadzu spectrofluorometer (RF5301PC, Japan), excitation 488 nm and emission 530 nm. Representative traces of cytosolic Ca^{2+} dynamics after addition of the compounds, CPA 30 µM, Thg 10 µM or DMSO as control are shown in the presence (A-C) and absence (E-G) of extracellular Ca^{2+} (no CaCl2 added plus addition of 100 µM EGTA). In the presence of 2 mM Ca^{2+}, Thg elicits a prolonged [Ca^{2+}]_c response with larger maxima than CPA (D). Removal of extracellular Ca^{2+} did not reduce the response to CPA, whereas the Thg response was smaller in the absence of extracellular Ca^{2+} (H). Peak [Ca^{2+}]_c after add-back of extracellular Ca^{2+} (E-G) was elevated above the control with Thg but not with CPA (I). Data in panels D, H, I are the mean ± S.D. from ≥ 3 independent experiments, with individual data points shown. Statistical significance calculated by one-way ANOVA with Bonferroni’s multiple comparison test: ***p=0.009; ****p<0.001.
Fig. 3 Thg releases Ca\textsuperscript{2+} from more than one compartment in \textit{P. falciparum} parasites. [Ca\textsuperscript{2+}]c was monitored in isolated PfGCaMP3 parasites at the trophozoite stage suspended in the cuvette of a spectrofluorometer, as described in the legend of Fig. 2. The PfGCaMP3 parasites were sequentially exposed to 10 \mu M Thg then 30 \mu M CPA (A,B), CPA then Thg (C,D), or DMSO control (E,F) in the presence (A,C,E) or absence (B,D,F) of extracellular Ca\textsuperscript{2+} (no CaCl\textsubscript{2} added plus addition of 100 \mu M EGTA). The CPA Ca\textsuperscript{2+} response was blocked after Thg addition (A & B), however the Thg Ca\textsuperscript{2+} response was not abolished by CPA (C & D). Panels A-F are representative traces. The mean peak Ca\textsuperscript{2+} response to each drug addition is plotted in the presence (G) or absence (H) of extracellular Ca\textsuperscript{2+}. Data are the mean ± S.D. from ≥ 3 independent experiments with individual data points shown. Statistical significance calculated by one-way ANOVA with Bonferroni’s multiple comparison test: (G) *p=0.017; **p=0.0013; ****p<0.0001 and (H) *p=0.0256; **p=0.0035; ****p<0.0001; φ p=0.0257; Σ p=0.0124.
Fig. 4. The ability of Thg but not CPA to induced SOCE is confirmed at the single cell level. Synchronized trophozoite-stage PfGCaMP3 parasites were isolated, resuspended into MOPS buffer with 2 mM CaCl₂, and plated onto glass coverslips. The cell chamber was mounted on an epifluorescence microscope and GCaMP3 images (excitation 488 nm; emission 510 nm long band pass filter) were acquired at 1 Hz. Representative traces of changes in [Ca²⁺]ᵢ in the presence of extracellular Ca²⁺ in response to (A) DMSO control, (B) 10 µM CPA and (C) 5 µM Thg. The Ca²⁺ ionophore ionomycin (10 µM, Iono) was added at the end of each experiment. To assess SOCE after CPA and Thg addition, the buffer was switched to Ca²⁺-free MOPS buffer with 100 µM EGTA immediately prior to recording. Cells were then treated with DMSO (D) 10 µM CPA (E) or 5 µM Thg (F) for 10 min prior to CaCl₂ addition (2 mM). All responses were normalized to the peak signal with 10 µM ionomycin (F/Fₐₙₖ). The amplitude (G) and rate of [Ca²⁺]ᵢ rise (H) in response to CaCl₂ addition was significantly greater than the vehicle control following treatment with Thg, but not CPA. Data in panels G and H were averaged from at least 15 cells in each experiment, and are the mean ± S.D. from ≥ 3 independent experiments. (G) ** p=0.0053, ## p=0.0082 (H) *p=0.0345, # p=0.0483 one-way ANOVA with Bonferroni’s multiple comparison test.
Fig. 5 Thg blocks the CPA [Ca$^{2+}$]$\text{c}$ response, but CPA does not block the Thg [Ca$^{2+}$]$\text{c}$ response in single \textit{P. falciparum} trophozoites. Synchronized PfGCaMP3 parasites were isolated, resuspended into HEPES buffer with 2 mM CaCl$_2$, and plated on glass coverslips before loading into the incubation chamber of an epifluorescence microscope to image GCaMP3. Cells were treated with sequential additions of 10 µM CPA and 5 µM Thg as indicated (A, B) to determine if the addition of either drug interfered with the ability of the other to mobilize Ca$^{2+}$. Responses were normalized to the peak ionomycin (10 µM, Iono) response ($F/F_{\text{Iono}}$), and the percentage of cells responding to both drugs was measured (C). Data are the mean from ≥ 127 cells ± S.D. from 2 independent experiments.
Fig. 6 The major acidic compartment is not affected by Thg or CPA. Isolated PfGCaMP3 parasites synchronized at the trophozoite stage were loaded with 5 µM Fura-FF/AM for 1 h while plating onto glass coverslips. The dual loaded cells were transferred to the microscope imaging chamber and immediately prior to recording the buffer was switched to Ca^{2+}-free MOPS buffer with 100 µM EGTA. Images were acquired at 1 Hz for both GCaMP3 (excitation 488nm, emission 510nm) and Fura-FF (excitation 340nm & 380nm, emission 510nm). Cells were treated with either the DMSO vehicle control (A), 10 µM CPA (B), or 5 µM Thg (C) for 10-15 min, and then Nig (250 nM) was added for another 10 min prior to adding back 2 mM CaCl\textsubscript{2} followed by 10 µM ionomycin (Iono). GCaMP3 fluorescence and the Fura-FF 340nm/380nm fluorescence ratio were normalized to the peak ionomycin (10 µM, Iono) response. The mean peak height of the Nig Ca^{2+} response was unaffected by CPA or Thg (D). Nigericin does not prevent a subsequent response to CPA and Thg (E). Data are the mean ± S.D. of ≥ 60 cells from 2 independent experiments.
Fig. 7 Mitochondria uncoupling does not affect $[\text{Ca}^{2+}]_{c}$ responses to CPA or Thg. Synchronized PfGCaMP3 parasites were isolated, resuspended into MOPS buffer with 2 mM CaCl$_2$, plated onto glass coverslips, and loaded into the incubation chamber of an epifluorescence microscope. Cells were loaded with 5 nM TMRE and then treated with 2.5 µM CCCP plus 2.5 µg/ml oligomycin, which uncouples the mitochondria (A). TMRE fluorescence was monitored with 543 nm excitation and 580 nm emission. In panel (B), GCaMP3 images were acquired at 1 Hz (excitation 488nm; emission 510nm), and cells were treated sequentially with CCCP plus oligomycin, 10 µM CPA, and 5 µM Thg. GCaMP3 signals were normalized to the peak ionomycin (10 µM, Iono) response ($F/F_{\text{Iono}}$).
Fig. 8 Proposed model for Thg action within *Plasmodium falciparum*. CPA and Thg are both able to release Ca\(^{2+}\) from the ER by inhibiting the *P. falciparum* SERCA (PfATP6). The presence of a Thg-Ca\(^{2+}\) response, even after emptying the ER with CPA, suggests that: i) the two drugs target two intracellular Ca\(^{2+}\) pools, one sensitive to both SERCA inhibitors, one only to Thg; ii) alternatively the same Ca\(^{2+}\) pool could express two SERCA isoforms, one sensitive to both drugs, one only to Thg. If the latter is the case the additional compartment was shown not to be the parasite mitochondrion or acidic pool. This additional compartment is crucial for SOCE in *P. falciparum*, since only Thg was able to activate Ca\(^{2+}\) influx into the parasite cytosol. PM, parasite plasma membrane; CPA, cyclopiazonic acid; Thg, thapsigargin; ER, endoplasmic reticulum; MT; mitochondrion; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; SOCE, store-operated Ca\(^{2+}\) entry; Nig, nigericin; DV, digestive vacuole; CCI, constitutive Ca\(^{2+}\) influx.
The genetic Ca2+ sensor GCaMP3 reveals multiple Ca2+ stores differentially coupled to Ca2+ entry in the human malaria parasite Plasmodium falciparum
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