The digestive food vacuole of the malaria parasite is a dynamic intracellular Ca\textsuperscript{2+} store

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Key Words: \textit{Plasmodium falciparum}, thapsigargin, SERCA, Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger, heme, signal transduction, chloroquine

Running Title: The malaria food vacuole is a Ca\textsuperscript{2+} storage organelle
SUMMARY

The acidic food vacuole of *Plasmodium falciparum* has been the subject of intense scientific investigation in the 40 years since its role in the digestion of host haemoglobin was first suggested. This proposed role has important implications for the complex host-parasite inter-relationship and also for the mode of action of several of the most effective antimalarial drugs. In addition, adaptive changes in the physiology of this organelle are implicated in drug resistance. Here we show that in addition to these functions, the digestive food vacuole of the malaria parasite is a dynamic internal store for free Ca$^{2+}$; a role hitherto unsuspected. With the aid of live-cell laser scanning confocal imaging, spatio-temporal studies reveal that maintenance of elevated free Ca$^{2+}$ in the digestive food vacuole (relative to cytosolic levels), is achieved by a thapsigargin (and cyclopiazonic acid)-sensitive Ca$^{2+}$-pump in co-operation with a H$^+$-dependent Ca$^{2+}$-transporter. Redistribution of free cytosolic and vacuolar Ca$^{2+}$ during parasite growth also suggests that vacuolar Ca$^{2+}$ plays an essential role in parasite morphogenesis. These data imply that the digestive food vacuole of the malaria parasite is functionally akin to the vacuole of plants (tonoplast) and the small electron dense granules of some parasites (acidocalciosomes) whereby H$^+$-coupled Ca$^{2+}$ transport is involved in ion transport, Ca$^{2+}$ homeostasis and signal transduction. These findings have significant implications for parasite development, antimalarial drug action and mechanisms of drug resistance.
INTRODUCTION

Malaria remains one of the largest global health (& economic) problems, resulting in more than a million deaths, mostly in young African children (1). With this in mind, it is astonishing how little is known about the basic physiology of the intra-erythrocytic malaria parasite and its organelles. The food vacuole is a major digestive organelle of the malaria parasite and a proven chemotherapeutic target. This organelle has a role in degradation of host-derived haemoglobin, is the site of action of important classes of antimalarials and harbours transporters associated with drug resistance (2-4). Hydrolysis of haemoglobin in the malaria parasite digestive food vacuole occurs by the integrated action of aspartic, cysteine and metalloproteases (5), resulting in the production of haemozoin (malaria pigment), a biocrystal of the toxic precursor ferriprotoporphyrin IX (FPIX (6)). The internal pH of the digestive food vacuole is lower than the parasite cytosol, although the precise pH value is under debate (7). In collaboration with Prof. Kiaran Kirk’s laboratory, we have recently described the function of two H⁺-pumping mechanisms in the digestive food vacuole, one is a bafilomycin-sensitive V-type H⁺-ATPase, the other is a NaF-sensitive H⁺-pyrophosphatase showing a partial dependence on K⁺ (8). The combination of a H⁺-ATPase and a H⁺-pyrophosphatase acting in the digestive vacuole is analogous to the situation in acidic tonoplasts of plant cells (9) and of the acidocalciosome of some protozoa (10). It should be noted that although there may be similarities with acidocalcisomes (which are small electron dense granules/organelles which store Ca²⁺ as a phosphate precipitate) and the malarial parasite food vacuole, they are morphologically (and functionally, with regards to haemoglobin digestion) very distinct. Based on the known H⁺ coupled Ca²⁺ transport in these non malarial organelles we hypothesised that an analogous coupled pathway would be present in the malaria parasite digestive food vacuole thereby contributing to cellular Ca²⁺ trafficking and storage.

By using live-cell confocal laser scanning microscopy and a judicious choice of Ca²⁺ probes, we have uncovered a novel function for this unique organelle. We show that the food vacuole of the malaria parasite is involved in dynamic Ca²⁺ storage. Evidence is
presented showing that Ca\textsuperscript{2+} storage by the digestive food vacuole is linked directly to parasite cell signalling and asexual development. This novel discovery raises many new questions about the functions, physiology and regulation of this critical malarial parasite organelle. As the target for the actions of a number of drugs, the requirement for tightly regulated Ca\textsuperscript{2+} movement and storage within this organelle may represent a new chemotherapeutic target.

**EXPERIMENTAL PROCEDURES**

*Parasite Culture.* *Plasmodium falciparum* TM6 strain, was kindly provided by Dr. P. Tan-Ariya (Mahidol University, Bangkok, Thailand) and maintained in continuous culture. Cultures contained a 2 % suspension of O\textsuperscript{+} erythrocytes in RPMI 1640 (R8758) medium supplemented with 10 % pooled human AB serum, 25 mM HEPES (pH 7.4) and 20 µM Gentamicin Sulphate (11).

*Effect of ferriprotoporphyrin IX (FPIX) on the fluorescence quantum yield of Ca\textsuperscript{2+} indicators.* Fluorescence emission spectra for pentapotassium salts (1 µM) of Fura-2 (excitation 340 nm) and Fluo-4 (excitation 488 nm) in 39 µM free Ca\textsuperscript{2+} buffer (10 mM CaEGTA in 100 mM KCl, 30 mM MOPS pH 7.2) with 0, 10, 20 & 50 µM ferriprotoporphyrin IX was performed in a Shimadzu RF-5001PC spectrofluorophotometer.

*Real Time Ca\textsuperscript{2+} Confocal Laser Scanning Microscopy.* For experimentation, suspensions (1 %) of infected erythrocytes in HEPES buffered RPMI medium (no serum) were loaded with Fluo-4 AM (3 µM, Molecular Probes BV, The Netherlands) for 20 min at 37 °C. For imaging, malaria parasite infected erythrocytes were immobilised using poly-lysine coated cover slips in a Bioptechs FCS2 perfusion chamber and maintained at 37 °C in growth medium (no serum). Addition of inhibitors were made to the perfusate and the Ca\textsuperscript{2+}-dependent fluorescence responses were monitored in real time. Fluorescence signals from Fluo-4 were collected on a Zeiss LSM510 confocal microscope through a Plan-Apochromat 63× 1.4 NA objective using `tracking mode'. Excitation of Fluo-4 was performed using an Argon ion laser at 488 nm. Emitted light was reflected through a 505–550-nm band pass filter from a 540-nm dichroic mirror.
Photobleaching (the irreversible damage of Fluo-4AM producing a less fluorescent species) was assessed by continuous exposure (5 min) of loaded cells to laser illumination. For each experiment, the laser illumination setting which gave a minimal reduction in cytosolic and food vacuole fluorescence was used (this varied according to dye loading and CLSM settings). Data capture and extraction was carried out with LSM510 version 3 software (Zeiss, Germany). Cytosolic and vacuolar [Ca\(^{2+}\)] was calibrated in situ by addition of known amounts of [Ca\(^{2+}\)]\(_{\text{free}}\) in the presence of nigericin (0.8 µM) and the Ca\(^{2+}\) ionophore A23187 (1 µM). Using Calcium Calibration Buffer Kit #1 solutions (Molecular Probes), the [Ca\(^{2+}\)]\(_{\text{free}}\) was calculated from the K\(_d\) of EGTA for Ca\(^{2+}\) (107.9 nM at 37°C (12)) using the equation [Ca\(^{2+}\)]\(_{\text{free}}\)=K\(_d\)\(_{\text{EGTA}}\) x [CaEGTA/K\(_2\)EGTA].

**RESULTS**

**Effect of ferriprotoporphyrin IX (FPIX) on the fluorescence quantum yield of Ca\(^{2+}\) indicators.** Haeme (FPIX) is a major component of the digestive food vacuole of *P. falciparum*. As the fluorescence quantum yield of some food vacuole targeted probes can be dramatically reduced by haeme (FPIX), we decided to test the effect of monomeric haeme on a number of Ca\(^{2+}\)-specific UV- and visible light-excitable fluorescent probes. Titration of FPIX to solutions of Fura-2 or Fluo-4 resulted in a progressive reduction of fluorescence signal (Fig 1). However, this reduction was more pronounced in the case of the UV-excitable probe (e.g. Fura-2) than for visible light-excitable probes (e.g. Fluo-4 & Fluo-3(not shown)); whereby Fluo-4 retained ~60% of its fluorescent emission signal at FPIX levels which eliminated the Fura-2 signal. Fluo-4 was therefore used as the probe of choice to measure cytosolic and food vacuole Ca\(^{2+}\) fluctuations.

*The digestive food vacuole of *P. falciparum* contains elevated free Ca\(^{2+}\).* With the use of Confocal Laser Scanning Microscopy (CLSM) and the cell permeant AM ester of Fluo-4 to probe for Ca\(^{2+}\) in trophozoites of infected red blood cells, we observed a strong fluorescence signal deriving from the digestive food vacuole (e.g. Fig. 3e). Some Ca\(^{2+}\) indicators have a tendency to compartmentalise and differences in apparent Ca\(^{2+}\) affinities in different compartments/organelles within a cell can sometimes lead to the
formation of artifactual Ca\textsuperscript{2+} gradients (13). To overcome these possible artefacts, free Ca\textsuperscript{2+} was independently calibrated \textit{in situ} in both the parasite cytosolic and food vacuole compartments (Fig. 2). The differing slopes (and fluorescence quantum yields in response to Ca\textsuperscript{2+}) of the parasite cytosol and food vacuole calibration curves reveal that there was a difference in the respective apparent Ca\textsuperscript{2+} binding affinities, probably resulting from differences in the Ca\textsuperscript{2+}-buffering capacity of the different intracellular milieus and possibly a degree of dye compartmentalisation. However, taking into account the differences in Fluo-4 Ca\textsuperscript{2+} affinities (by using the respective \textit{in situ} calibration curves), the digestive food vacuole was nevertheless measured as having elevated free Ca\textsuperscript{2+} relative to the cytosol, with levels ranging between 250-300 nM \((n=27)\), some 5-6 times higher than the cytosol (measured at approximately 50 nM, \(n=27\)).

\textit{Intracellular distribution of free Ca\textsuperscript{2+} during the intraerythrocytic development of the malaria parasite.} The distribution of free Ca\textsuperscript{2+} in the malaria parasite was observed to change during asexual development. As described, early trophozoites were observed as having a Ca\textsuperscript{2+} gradient between the digestive food vacuole and cytoplasmic compartments (Fig. 3e). However, as parasites underwent schizogony, free Ca\textsuperscript{2+} was observed to leave the digestive food vacuole and move to the inter-membrane space surrounding each segment (Fig. 3f & 3g). On rupture and release of merozoites from the erythrocytic host there was a sustained elevation in free Ca\textsuperscript{2+}, however the resolution of the CLSM did not allow us to determine the specific intracellular distribution of the signal (Fig. 3h). This increase was lost shortly after reinvasion of new red blood cells (i.e. the early ring stage) and this may implicate an important role for elevated merozoite free Ca\textsuperscript{2+} in the invasion process.

\textit{Spatio-temporal dynamics of free Ca\textsuperscript{2+} in the malaria parasite and digestive food vacuole.} Fluo-4 AM is a 1,2-bis(2-aminophenoxy)ethane-\(N,N,N',N'\)-tetraacetic acid (BAPTA)-based indicator with a large dynamic range \((R_f \approx 50-200, 14)\) making it very suitable for monitoring of both elementary and global Ca\textsuperscript{2+} signals (13). Using this indicator, we were able to determine spatio-temporal Ca\textsuperscript{2+} fluctuations in malaria parasite
infected erythrocytes that were immobilised using poly-lysine coated cover slips in a Bioptechs FCS2 perfusion chamber, in response to a variety of inhibitors and agents. As described, trophozoites were shown to posses elevated Ca\textsuperscript{2+} in the digestive vacuoles (Fig. 3e), addition of thapsigargin, a plant sesquiterpene lactone which interacts irreversibly with sarcoplasmic reticulum(SR)-like Ca\textsuperscript{2+} ATPases (15) to trophozoites caused a rapid depletion of vacuolar Ca\textsuperscript{2+} (Figs. 4a (and 4b, QuickTime movie) and 5a). Addition of cyclopiazonic acid (CPA) another known selective irreversible inhibitor of SR-like Ca\textsuperscript{2+}-ATPases (16) also caused a rapid reduction in vacuolar free Ca\textsuperscript{2+} (Figs. 4c (and 4d, QuickTime movie) and 5b). Unlike the case with thapsigargin however, the reduction of food vacuolar Ca\textsuperscript{2+} on addition of CPA was accompanied by a conspicuous transient increase in cytosolic Ca\textsuperscript{2+}. Manoeuvres that caused a perturbation of the trans-vacuolar membrane pH gradient (the food vacuole is acidic relative to the cytosol) such as addition of the selective V-type ATPase inhibitor bafilomycin A\textsubscript{1} (Fig. 5c) and addition of NH\textsubscript{4}Cl (QuickTime movie Fig 4e and Fig. 5c) to the perfusate, also caused a rapid reduction of vacuolar Ca\textsuperscript{2+}. A transient rise in cytosolic Ca\textsuperscript{2+} was also evident on addition of NH\textsubscript{4}Cl to the perfusate (Fig. 5c). The membrane integrity of the digestive food vacuole was maintained on addition of the irreversible inhibitors thapsigargin, CPA and bafilomycin A\textsubscript{1} (see QuickTime movies Fig. 4b, d and e) and the malaria pigment crystals were observed to continue to move inside the vacuoles. Washing infected erythrocytes with Hepes-buffered RPMI (pH 7.4) or Ringers solution after treatment with NH\textsubscript{4}Cl restored the food vacuole Ca\textsuperscript{2+} gradient of the cells (data not shown).

As the digestive food vacuole of the malaria parasite contains a P-glycoprotein pump (PGH1) which can affect the compartmentalisation of some fluorescence indicators, the effect of verapamil, a known PGH1 modulator (17) was added to the perfusate of the single cell chamber system. Verapamil (10 µM) was not shown to effect any change in the fluorescence signal deriving from either the food vacuole or from the cytoplasm, suggesting no interference from PGH1 in our system.

**DISCUSSION**

It has previously been suggested that the malaria parasite contains an acidic Ca\textsuperscript{2+} storage organelle (18-20), however negative staining of the digestive vacuole by UV excitable
Ca\textsuperscript{2+} fluorescent probes precluded its involvement (20). We have shown recently that FPIX can interact with fluorescent probes such as acridine orange, resulting in a dramatic reduction in fluorescent emission, probably occurring from the absorption by the FPIX Soret peak (21). We therefore investigated whether the fluorescent emission of previously used UV-excitable Ca\textsuperscript{2+}-specific probes such as Fura2 (19, 20) as well as a range of visible light-excitable Ca\textsuperscript{2+}-probes were also affected by FPIX. Titration of FPIX to solutions of Fura-2 or Fluo-4 resulted in a progressive reduction of fluorescence signal (Fig 1). However, this reduction was more pronounced in the case of Fura-2 than for Fluo-4 which retained ~60% of its original fluorescent emission signal at FPIX levels which eliminated the Fura-2 signal. Importantly, the probe remained Ca\textsuperscript{2+} sensitive within this organelle albeit with an altered apparent Ca\textsuperscript{2+} affinity (Fig. 2). FPIX is reported to achieve mM concentrations in the food vacuole (22) and this would fully explain the lack of food vacuole derived Fura 2 fluorescence reported by other investigators.

With the use of Confocal Laser Scanning Microscopy (CLSM) and Fluo-4 AM (which is less susceptible to FPIX-fluorescence quenching and has a good dynamic range), to probe for Ca\textsuperscript{2+} in mature trophozoites of infected red blood cells, we observed a free Ca\textsuperscript{2+} pool in the digestive food vacuole (Fig 3e) generating a Ca\textsuperscript{2+} gradient some 5-6 fold greater than that measured in the cytoplasm (at rest).

It was clearly observed that the sequestration of free Ca\textsuperscript{2+} altered during the asexual intra-erythrocytic development of the malaria parasite (Fig 3a-h). For example, as parasites underwent schizogony, free Ca\textsuperscript{2+} was observed to leave the digestive food vacuole and move to the inter-membrane space surrounding each segment (Fig. 3f & 3g) producing ‘wagon wheel’-looking Ca\textsuperscript{2+} distributions. It has been accepted for over 20 years that Ca\textsuperscript{2+} in the culture medium is essential for the complete asexual development of the malaria parasite in red blood cells in culture (23), the results presented here lend further support for a role for Ca\textsuperscript{2+}-dependent processes in the asexual development of the parasite.
Our findings showing Ca$^{2+}$ storage by the digestive food vacuole has allowed us to shed light on a recent controversy relating to the pharmacology of sarco/endoplasmic reticulum Ca$^{2+}$-ATPases (SERCAs) of *P. falciparum*. A recent study demonstrated that the malaria parasite contains an ER-like Ca$^{2+}$ store which is controlled by a thapsigargin-insensitive but cyclopiazonic acid-sensitive Ca$^{2+}$-pump (20), a finding which was at odds to previous studies indicating a thapsigargin-sensitive mechanism (19, 24). It is now apparent that both of these studies adopted methods which were ineffective for the measurement of vacuolar Ca$^{2+}$ and therefore that their fluorescence signals originated from cytosolic Ca$^{2+}$ fluctuations. Using our live cell CLSM system to monitor Fluo-4 loaded infected red blood cells, we observed that upon addition of thapsigargin (1 µM) or cyclopiazonic acid (10 µM) to the perfusate, Ca$^{2+}$ from the digestive food vacuole was rapidly (~60 s) released (Fig. 4a, c, Fig. 5a, b (QuickTime movies Fig. 4b and 4d)). In the case of cyclopiazonic acid (but undetectable for thapsigargin), the vacuolar release of Ca$^{2+}$ was accompanied by a small transient increase in cytosolic Ca$^{2+}$ (Fig. 4c & 5b). These results help explain previous observations of transient elevations of Ca$^{2+}$ on addition of cyclopiazonic acid (20). In addition these results suggest the operation of more than one P-type Ca$^{2+}$ ATPase, one which is sensitive to both thapsigargin and cyclopiazonic acid and one which is only sensitive to cyclopiazonic. Significantly in support of our findings, recent functional characterisation of *P. falciparum* transporters expressed in *Xenopus laevis* oocytes have revealed the presence of both a cyclopiazonic-sensitive Ca$^{2+}$-ATPase (PfATPase4, (25)) and a thapsigargin-sensitive Ca$^{2+}$-ATPase (PfATPase6, Prof. S. Krishna personal communication). At this early stage in the pharmacological analysis of intracellular Ca$^{2+}$ signalling in the malaria parasite, it should be noted however, that thapsigargin and cyclopiazonic acid have previously been shown to have different additional non-selective effects on Ca$^{2+}$ signalling pathway components e.g. capacitative Ca$^{2+}$ entry (26). However, it is unlikely that capacitative Ca$^{2+}$ entry is responsible for the observed increase in cytoplasmic Ca$^{2+}$ on treatment with cyclopiazonic acid, as this effect was observed by Alleva and Kirk (20) to occur in freed *P. falciparum* parasites suspended in Ca$^{2+}$-free solutions.
The generation of a H⁺-gradient across the parasite vacuolar membrane by the action of V-type H⁺ ATPases and H⁺-pumping pyrophosphatases (8) suggests that as with plant tonoplasts and acidocalciosomes of some parasites, the digestive food vacuole of the malaria parasite may also drive Ca²⁺ accumulation by H⁺-coupled transport mechanisms. This hypothesis is supported by our findings that a collapse of the transmembrane H⁺ gradient by addition of NH₄ (Fig. 5c (QuickTime movie 4e)) or by addition of bafilomycin A₁ (a selective V-type H⁺-ATPase inhibitor, Fig. 5c) results in a rapid discharge of vacuolar Ca²⁺. Given the essential role of Ca²⁺ as a secondary messenger molecule and the fact that physiological studies in plants have established a role for vacuolar (tonoplast) H⁺/Ca²⁺ exchange activity in ion transport, Ca²⁺ homeostasis and signal transduction (27), further elucidation of analogous H⁺-dependent mechanisms underlying Ca²⁺ homeostasis in the malaria parasite will be of significant importance.

Here we add another critical transport function to the malarial parasites repertoire of vacuolar transport mechanisms (Fig. 6). It will be important to determine how the interplay of Ca²⁺ transport and H⁺ transport via either the H⁺-pyrophosphatase or the V-type H⁺-ATPase contributes to organelle function. The rapid redistribution of vacuolar Ca²⁺ at schizogony (asexual multiplication) must implicate communication between the parasite nucleus and the food vacuole. In addition this organelle harbours transporters implicated in drug resistance (notably PfCRT and PGH1 reviewed in 4). The potential for proton coupled Ca²⁺ transport to influence the function of these resistance transporters could have significant implications for malaria chemotherapy in the age of the quinoline resistant parasite.

Acknowledgements
SAW and PGB receive support from the Wellcome Trust.

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Figure Legends

**Fig. 1** Fluorescence emission spectra for pentapotassium salts (1 μM) of Ca^{2+}-saturated Fura-2 (excitation 340 nm) and Fluo-4 (excitation 488 nm) in phosphate buffered saline (pH 7.2) with 0, 10, 20 & 50 μM ferriprotoporphyrin IX (black, red, blue & green lines, respectively).

**Fig. 2** Cytosole (●) and digestive food vacuole (○) *in situ* free Ca^{2+} calibration of Fluo 4-AM-loaded *P. falciparum*-infected erythrocytes. Data points represent mean ± S.E. of ≥ 19 cells.

**Fig. 3** Intracellular distribution of free Ca^{2+} during the intraerythrocytic development of the malaria parasite. Pannels show brightfield/fluorescence images of; a mid-term trophozoite (a & e); parasites in early (b & f) and late (c & g) stages of schizogony; and merozoites after rupture of the erythrocyte host (d & h).

**Fig. 4** Subcellular Ca^{2+} dynamics of the infected red blood cell (RBC), the malaria parasite (parasite), and the digestive food vacuole (FV) in response to (a) thapsigargin (1 μM) and (c) cyclopiazonic acid (10 μM). The images show pseudocolour representation of fluo-4 fluorescence (high Ca^{2+} in red, low Ca^{2+} in blue) obtained using confocal laser scanning microscopy. Still images (bright field and fluorescence, b, d and e) represent links for QuickTime videos of thapsigargin- (1 μM, b), cyclopiazonic acid- (10 μM, d) and NH$_4$Cl- (80 mM, e) treated parasitized red blood cells. Some erythrocytes are shown with double parasite infections (d and e). Addition of inhibitors to the perfusate occurred in frames 4 for thapsigargin (b) and cyclopiazonic acid (d) and frame 3 for NH$_4$Cl (e). The movies do not represent real time.

**Fig. 5** Effect of (a) thapsigargin (1 μM), (b) cyclopiazonic acid (10 μM), (c) NH$_4$Cl (80 mM) and (d) bafilomycin A$_1$ (200 nM) on the concentration of free Ca^{2+} in the malaria parasite cytosol (○) and digestive food vacuole (●). Values show mean ± SE (n ≥ 5).
**Fig. 6** Schematic representation of known and putative transporters/channels on the *P. falciparum* digestive food vacuole membrane. Although morphologically and functionally (e.g. haemoglobin digestion) quite distinct, the digestive food vacuole shares similar properties to the acidocalciosomes of other apicomplexa (as described by Docampo & Moreno, 10).
Fig. 1
Fig. 2
Fig. 4a
Fig. 4c
Fig. 4e
Fig. 5
Fig. 6
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*J. Biol. Chem.* published online May 8, 2003

Access the most updated version of this article at doi: [10.1074/jbc.M304193200](http://10.1074/jbc.M304193200)

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