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The malaria parasite mitochondrion senses cytosolic Ca^{2+} fluctuations

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Abstract

By using the fluorescent dye Rhod-2, we have investigated the ability of *Plasmodium* mitochondria to participate in cellular Ca^{2+} homeostasis. To this end, isolated parasites were simultaneously loaded with the mitochondrial Ca^{2+} probe Rhod-2 and the cytosolic Ca^{2+} dye Fluo-3 and their fluorescent intensities were monitored in the same cells by confocal microscopy. We here demonstrate that Ca^{2+} increases, as elicited by treatment of parasites with sarco-endoplasmic reticulum Ca^{2+} ATPase inhibitors or the hormone melatonin, induce rapid and reversible increases of the Ca^{2+} concentration in the mitochondria of both human and murine parasites. Pre-treatment of parasites with the mitochondrial uncoupler, FCCP, suppresses mitochondrial Ca^{2+} accumulation. Our data demonstrate that mitochondria of malaria parasites are able to reversibly accumulate part of the Ca^{2+} released in the cytoplasm by pharmacological and physiological agents and thus suggest that this organelle participate in the maintenance of Ca^{2+} homeostasis of *Plasmodia*.

Keywords: *Plasmodium*; Malaria parasites; Calcium signalling; Mitochondria

Intracellular calcium signaling is responsible for activation of different cellular functions [1]. As prolonged rise in cytosolic Ca^{2+} is lethal, its fluctuations are compensated by mechanisms of calcium storage and extrusion. In mammalian cells, the classical store, IP_3-sensitive, the endoplasmic reticulum, ER, plays an essential role [2], but other organelles such as mitochondria and Golgi [2] also participate in intracellular Ca^{2+} handling.

The search of calcium handling mechanisms in the malaria parasite, *Plasmodium* has been investigated as a potential biological target against one of the most devastating human disease [3–7] affecting millions of individuals, particularly in the developing world. Along with strategies for storing Ca^{2+}, the parasite has adapted to use Ca^{2+} as a signaling messenger [8,9]. This is in agreement with the presence of at least 30 proteins containing the Ca^{2+} binding motif EF hand in the *Plasmodium* genome [10].

The role of organelles in parasite physiology is thought to be one of the fundamental biological questions [10,11]. The mitochondria of *Plasmodia* have been considered by several investigators potential target for new antimalarial drugs. It has been established that the mitochondria of malaria parasites contain the Krebs cycle enzymes, succinate dehydrogenase (SDH) [12] and NADH dehydrogenase [13,14] while the annotation of the *Plasmodium* genome predict other molecular components [15]. In addition, a homologue of the mammalian mitochondrial heat shock protein 60 (Hsp60), which acts as a chaperone for protein folding and plays a key role in mitochondrial protein transport and degradation, has been cloned from *Plasmodium yoelii* [16] and *Plasmodium falciparum*.

By using mitochondria-targeted aequorin, Rizzuto et al. [17] have shown that mitochondria of mammalian

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cells can efficiently take up Ca^{2+}. This Ca^{2+} accumulation appears to be due to the existence of close physical contacts between the ER and the mitochondria. The latter can thus sense microdomains of high Ca^{2+} concentration generated close to the IP_3 receptors [17]. Hajnóczky et al. [18] have demonstrated that Ca^{2+} oscillations in the cytoplasm are transduced into mitochondrial Ca^{2+} oscillations and result in the activation of Ca^{2+}-sensitive mitochondrial dehydrogenases.

Since a Ca^{2+} signaling apparatus similar to that of mammalian cells exists in the intraerythrocytic forms of the malaria parasites, *P. falciparum* and *Plasmodium chabaudi* [7,19–21], in this contribution we have investigated whether mitochondria play some role in the Ca^{2+} handling mechanisms of the parasites. This problem appears particularly relevant in *Plasmodia* given on the one hand the key role played in these parasites by oxidative metabolism [22,23] and on the other on the relevance of Ca^{2+} signals in the regulation of parasite cell cycle. On this latter point, in fact we have recently proposed a novel mechanism that allows the intracellular protozoan parasite to subvert the host endocrine regulation of its diurnal rhythm for its own coordinated regulation of development and proliferation. The hypothesis is based on the finding that melatonin synchronizes intraerythrocytic malaria parasites in a Ca^{2+}-dependent manner [8].

By using the Ca^{2+} indicator Rhod-2 that targets rather selectively in the mitochondrial matrix we here demonstrate that *Plasmodium* mitochondria can undergo significant increases in matrix Ca^{2+} when challenged with stimuli that increase cytosolic [Ca^{2+}].

Materials and methods

Parasites

Plasmodium falciparum (Palo alto) parasites were maintained in continuous in vitro culture in adult red blood cells [24] and synchronization was achieved by sorbitol treatment [25]. Parasitemias were determined from Giemsa-stained thin films.

Plasmodium chabaudi parasites were maintained synchronously in female mice (Balb/C strain) by weekly transfer infection. Leukocytes and platelets were removed from whole blood by filtration through a powdered cellulose column (Whatman CF11). The infected erythrocytes were then washed three times by centrifugation at 1500*g* for 5 min in PBS.

Fluorescence digital imaging

Parasites. Infected red cells $10^7(ml^{-1})$ were lysed with $10\,\mu g \cdot ml^{-1}$ saponin (in PBS). RBC membrane were removed by centrifugation, the parasites were washed twice in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 50 mM Hepes, and 1 mM $CaCl_2$, pH 7.2) and resuspended in the same buffer [8].

Rhod-2 fluorescence. For measurement of $[Ca^{2+}]_m$, the cells were loaded with 5 μM Rhod-2/AM in the presence of 40 μM probenecid for 50 min at 37 °C to allow the hydrolysis of the acetoxymethyl esters (AM) and washed twice in buffer A. Rhod-2 fluorescence was excited at 543 nm and emitted fluorescence was collected through a 560 nm pass barrier filter. Dynamic measurements were performed in a confocal laser scanning microscope (model LSM 510 Carl Zeiss).

Simultaneous recording of Rhod-2 and Fluo-3 fluorescence. The parasites were loaded with 5 μM Rhod-2/AM and 5 μM Fluo-3/AM for 50 min at 37 °C. The dyes were excited at 543 and 488 nm and emission was passed through filters—band pass 505–550 nm (Fluo-3 AM) and 560 nm pass barrier filter (Rhod-2 AM).

Organelle localization. For localization of mitochondria in parasite cytosol, we loaded the parasites with 5 μM Mitofluor Green or Rhod-2-AM. Cells were illuminated with an argon laser at 488 nm (Mitofluor Green) and 543 nm (Rhod-2). Emission fluorescence was collected with filters: 505–550 nm (Mitofluor Green), $\lambda \geqslant 560$ nm (Rhod-2-AM).

For Ca^{2+} imaging the labeled parasites were deposited on a coverslip, coated with L-polylysine by immersion in a solution of 1 mg/ml and left to stand at room temperature for approximately 10 min before use. The coverslip was then introduced into a stainless steel chamber containing 100 μl of buffer A, which was placed on the stage of the microscope (Zeiss, Axiovert 100 M). Thapsigargin and other reagents were added directly to the chamber containing the loaded cells.

Confocal imaging

Dynamic imaging was performed with the LSM 510 laser scanning microscope (Carl Zeiss), using the LSM 510 software, version 2.5. The Axiovert 100 M microscope is equipped with a 63× water immersion objective. The samples were illuminated at 488 and 543 nm and emission wavelength collected by a bandpass filter at 505–550 nm (Fluo-3) and pass filter $\lambda \geqslant 560$ nm (Rhod-2). At the beginning and end of each experiment, the cells were observed in transmission to ensure integrity.

Data analysis

The Fluo-3 and Rhod 2 fluorescence intensities were normalized as (F_1—maximal fluorescence after drug addition/F_0—fluorescence before drug addition). Software-based analysis allowed fluorescence imaging of the whole field or of a selected cell as a function of time. This was accomplished by defining areas of interest on a given image frame and instructing the software to construct a graphical representation of intensity against time.

Experiments were carried out with at least three different cell preparations, and 10–20 cells were monitored in each experiment. Traces represent single cell responses unless indicated otherwise.

Results

The malaria parasites *P. falciparum* and *P. chabaudi* during their intraerythrocytic stages were treated with the fluorescent, mitochondrial specific, dye Mitofluor Green and analyzed by confocal microscopy. Figs. 1A–C show the phase contrast images of infected-cells at the ring, trophozoite and schizont stages, while Figs. 1D–F show the localization of their mitochondria, as revealed by the fluorescence marker. Similar results were also obtained for the rodent malaria parasite, *P. chabaudi* (Figs. 2A–F).

The distribution and intensity of the mitochondrial fluorescent probe is quite distinct in the different phases, as previously observed by Tanabe [26]. Using similar approach, Divo et al. [27] reported the mitochondrial development progresses from a fine thread-like organelle

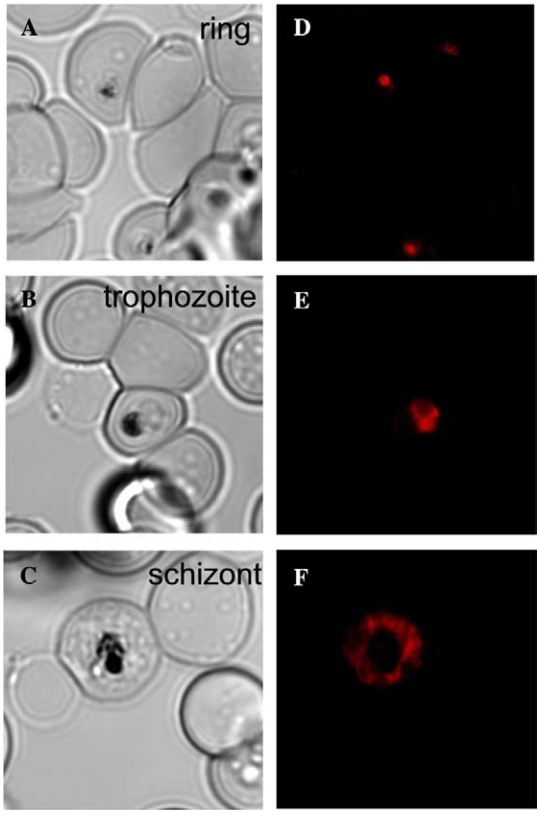

Fig. 1. Mitochondria localization during the intraerythrocytic cycle (ring, trophozoite, and schizont stage) of *P. falciparum* with the mitochondrial probe—Mitofluor green. (A–C) Phase contrast. (D–F) Fluorescence images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

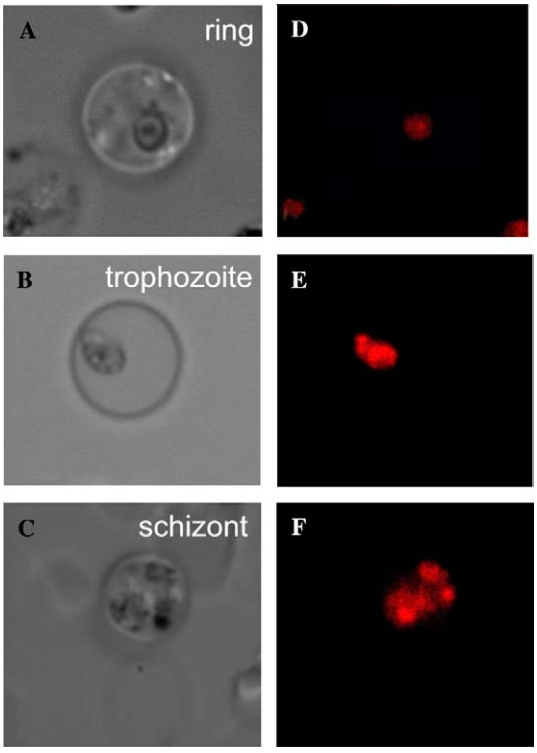

Fig. 2. Mitochondria localization in the intraerythrocytic cycle of *P. chabaudi* with Mitofluor green (ring, trophozoite, and schizont stage). (A–C) Phase contrast. (D–F) Fluorescence images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

that becomes longer and eventually branched. Recently, *Plasmodium* mitochondria were also target with a GFP construct confirming these initial studies [28].

After imaging *Plasmodium* mitochondria we next investigated the role of this organelle in parasite Ca^{2+} homeostasis. The cells were then loaded with the Ca^{2+} indicator Rhod-2, that is known to be selectively trapped within the mitochondrial matrix of many mammalian cells and thus function as an in situ specific probe of mitochondrial [Ca^{2+}] [29]. The correct mitochondrial localization of Rhod-2 was confirmed by co-staining with another mitochondrial marker, Mitofluor green. By comparing the subcellular distribution of Rhod-2 and Mitofluor green fluorescence in erythrocytes infected with *P. falciparum* (Fig. 3) or *P. chabaudi* (not shown) we can conclude that, also in *Plasmodia*, Rhod-2 accumulates preferentially within mitochondria, though a low signal in the parasite cytosol was also noticed.

Fig. 4 shows an experiment in which Ca^{2+} release from the ER of the parasites was triggered by the addition of the sarco-endoplasmic reticulum ATPase, SERCA, inhibitors thapsigargin (THG) or cyclopiazonic acid (CPA). We have previously demonstrated that

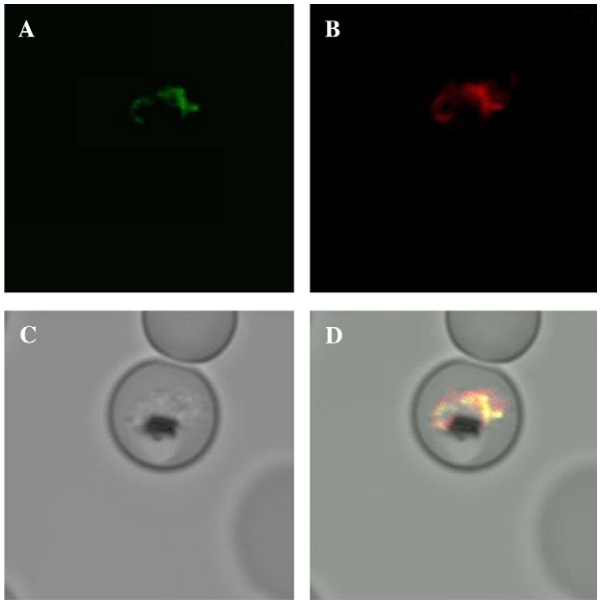

Fig. 3. Colocalization of fluorescence images of mitochondrial probes Rhod-2 AM and Mitofluor green in *P. falciparum*. (A) Mitofluor green fluorescence. (B) Rhod-2 AM fluorescence. (C) Phase contrast. (D) Merged image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

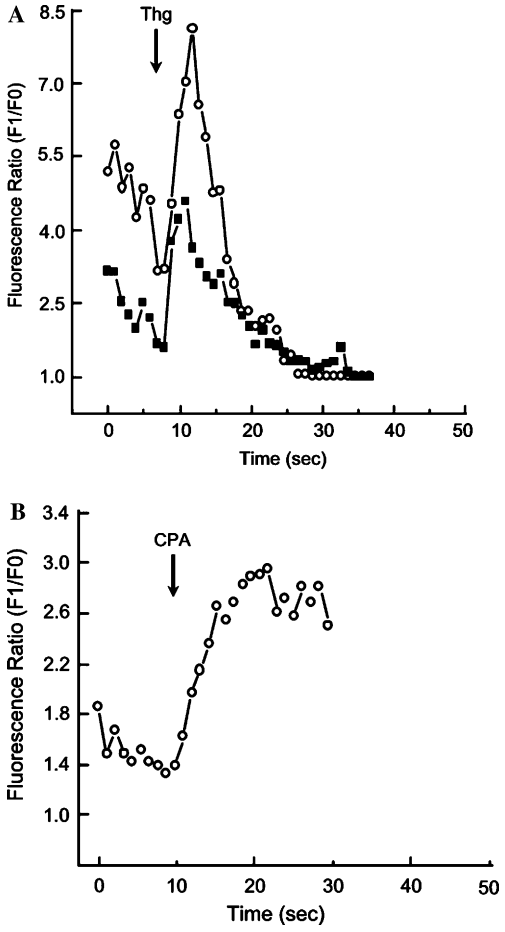

Fig. 4. Correlation of $[Ca^{2+}]_m$ changes with ER Ca^{2+} release by Ca^{2+}ATPases inhibitors in *P. falciparum*. (A) Addition of thapsigargin (10 μM). (B) Addition of cyclopiazonic acid (20 μM). Traces represent ratio of fluorescence intensities from Ca^{2+} probes Rhod-2 AM-mitochondria (open circles) and Fluo-3 AM-cytosol (filled squares).

thapsigargin and CPA induce Ca^{2+} release in both *P. chabaudi* [8,30] and *P. falciparum* [7,9]. Thapsigargin-sensitive Ca^{2+} pools have also been reported by Marchesini et al. [6] in *P. berghei*. Alleva and Kirk [31] have reported a small thapsigargin-sensitive response in *P. falciparum*, when compared to CPA sensitive Ca^{2+} pool. It is worth noting that these latter authors have used a relatively low amount of the SERCA inhibitor (1 μM), while a much larger release is observed at higher doses [7].

In the next experiment, we have used isolated parasites and then simultaneously loaded them with the mitochondrial Ca^{2+} probe Rhod-2 and the cytosolic Ca^{2+} dye Fluo-3. Fig. 4 shows that addition of (10 μM) THG (Fig. 4A) or (20 μM) CPA (Fig. 4B) to *P. falciparum* parasites at the trophozoite stage results in a simultaneous increase of fluorescence in both mitochondria and cytosol. Similar results were obtained in the rodent malaria parasite, *P. chabaudi* at the trophozoite stage (data not shown).

THG and CPA are artificial tools to induce increases in cytosolic Ca^{2+} in *Plasmodia*. We thus turned our attention to stimulus capable of eliciting Ca^{2+} increases in the *Plasmodia* cytosol. In particular we have previously shown that the pineal hormone melatonin is capable of inducing a significant increase in the cytoplasmic $[Ca^{2+}]$ of the parasites [8]. Fig. 5A shows that addition of melatonin (100 μM) causes not only, as expected, a rapid rise of $[Ca^{2+}]$ in the cytosol, but also in the mitochondria (Fig. 5A) of *P. chabaudi*. As a control the response to THG in the same cell batch is also shown (Fig. 5B). However, if the parasites were treated with the mitochondrial uncoupler FCCP (5 μM) addition of melatonin did not induce an increase in Rhod-2 signal, while that of Fluo-3, i.e., in the cytosol, remained unaffected (Fig. 5C).

Similarly, in the presence of FCCP, THG was still able to induce a rise of $[Ca^{2+}]$ in the cytosol (Fig. 5D), but not in the mitochondria. Similar results were obtained with *P. falciparum* (Fig. 6).

Discussion

The ability of mitochondria to accumulate Ca^{2+} upon cytosolic Ca^{2+} enhancement has been reported in several systems [32–36]. One of the puzzling questions in the field was the high capacity of mitochondria to accumulate Ca^{2+} above 10 μM when the cytosolic Ca^{2+} rise was about 2 μM [32]. This point was clarified by Rizzuto et al. [17], who showed that the proximity of mitochondria to the ER when IP_3 was generated by agonist addition generates microdomains of sufficiently high Ca^{2+} concentration to account for the rapid and efficient Ca^{2+} uptake by the organelle under these conditions.

Microdomains of high Ca^{2+} could be induced not only by IP_3-generating stimuli but also by Ca^{2+} influx through plasma membrane channels [34]. The rapid uptake of Ca^{2+} by mitochondria stimulates mitochondrial metabolism [35–37], with modulation of Ca^{2+}-sensitive enzymes in the mitochondrial matrix, such as isocitrate, oxoglutarate and pyruvate dehydrogenases [36,37]. The mechanisms of Ca^{2+} fluxes in mitochondria have been widely studied. Ca^{2+} uptake driven by the membrane potential is modulated by the Ca^{2+} uniporter [36,38]. Ca^{2+} extrusion depends on the cell type, and may involve the Na^+ or H^+ counter-transport system [36,39,40], and the non-selective channel (permeability transition pore), which exhibits a dependence on matrix Ca^{2+} [41].

The role of *Plasmodium* mitochondria in Ca^{2+} homeostasis has not been explored as the functioning of this organelle during intraerythrocytic development of parasite is still poorly understood. However, Uyemura et al. [42] reported the existence of Ca^{2+} transport in *P. berghei* mitochondria, indicating the presence of a

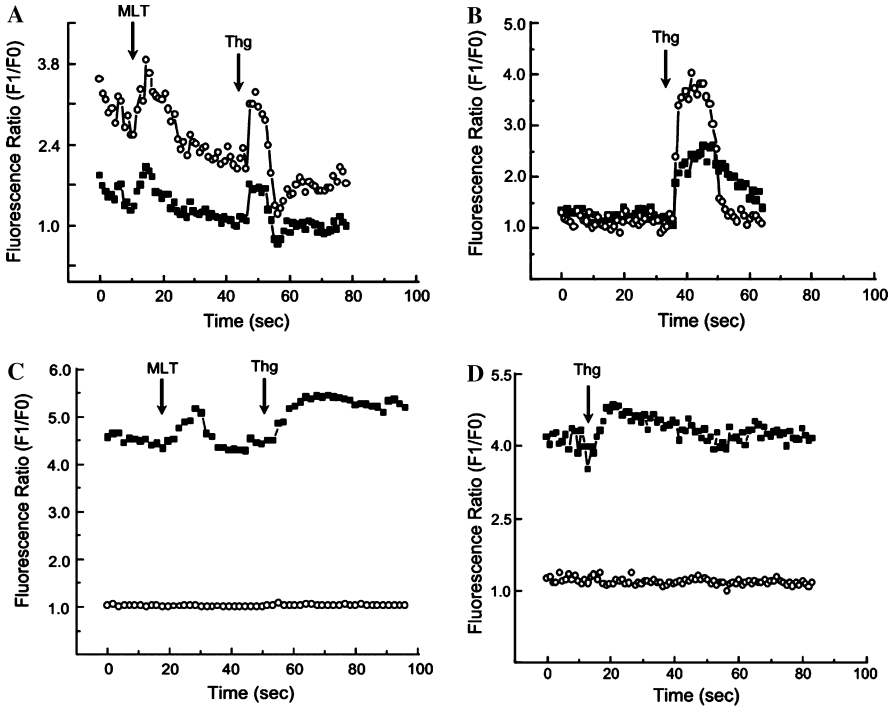

Fig. 5. Relationship between Ca^{2+} indicator fluorescence in the cytosol and mitochondria of *P. chabaudi* parasites. (A) Effect of hormone melatonin (100 μM) and thapsigargin (10 μM) on Ca^{2+} fluorescence. (B) Addition of ER Ca^{2+}ATPase inhibitor-thapsigargin (10 μM). (C) Effect of hormone melatonin (100 μM) and thapsigargin (10 μM) on Ca^{2+} fluorescence in the presence of mitochondrial uncoupler FCCP (5 μM). (D) Addition of thapsigargin (10 μM) in the presence of mitochondrial uncoupler FCCP (5 μM). Traces represent fluorescence intensity ratio of Ca^{2+} probes Rhod-2 AM-mitochondria (open circles) and Fluo-3 AM-cytosol (fill squares).

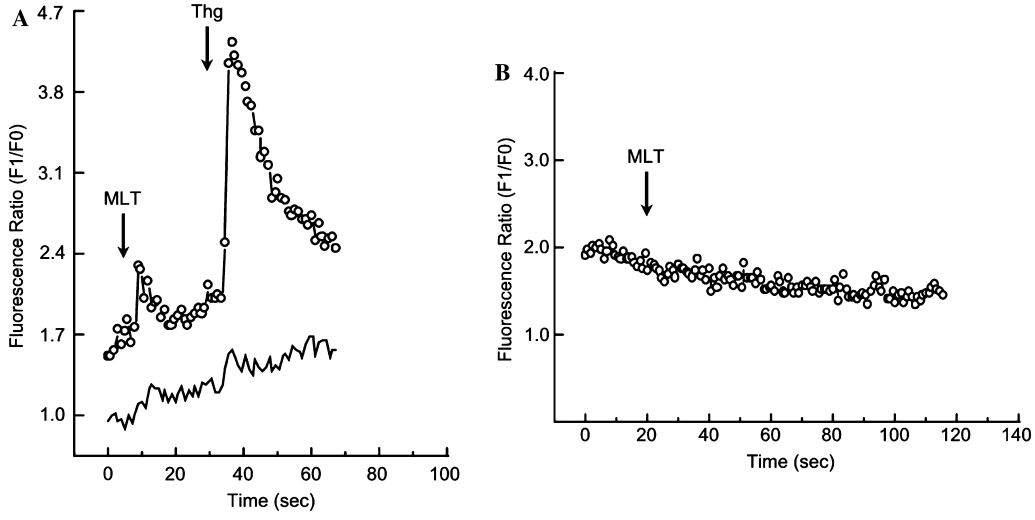

Fig. 6. Relationship between Ca^{2+} indicator fluorescence in the cytosol and mitochondria of *P. falciparum* parasites. (A) Effect of hormone melatonin (100 μM) and thapsigargin (10 μM) on Ca^{2+} indicator fluorescence. (B) Effect of melatonin (100 μM) in the presence of mitochondrial uncoupler FCCP (5 μM) in Rhod-2 AM fluorescence. Traces represent fluorescence intensity ratio of Ca^{2+} probes Rhod-2 AM-mitochondria (open circles) and Fluo-3 AM-cytosol (line).

Ca^{2+} uniporter similar to that present in mammalian mitochondria.

We have used fluorescent probes to measure Ca^{2+} concentration changes in the cytosol and mitochondria in *Plasmodium* . Our work provides for the first time evidence for Ca^{2+} buffering capacity of *P. chabaudi* and *P. falciparum* mitochondria. We have shown a cytosolic [Ca^{2+}] rise from either a discharge of ER Ca^{2+} on addition of SERCA inhibitors (THG and CPA) or stimulation with an agonist. For the latter, we observed that

addition of the host hormone melatonin which promotes synchronization of the intraerythrocytic cycle by Ca^{2+}-signaling mechanism [8] results in a [Ca^2] rise in *Plasmodium* mitochondria (Fig. 5). Ca^{2+} is an important modulator of the activity of mitochondrial enzymes such as dehydrogenases [36]. The *Plasmodium* genome sequence has identified the genes involved in the tricarboxylic acid cycle [15], but Ca^{2+} handling mechanisms in *Plasmodium* are still undefined. The present contribution may shed some light on this question and provides new tools for the analysis of mitochondrial function and the regulation of metabolic pathways in *Plasmodium* species.

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