JCB Article

Calcium signaling in a low calcium environment: how the intracellular malaria parasite solves the problem

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Malaria parasites, *Plasmodia*, spend most of their asexual life cycle within red blood cells, where they proliferate and mature. The erythrocyte cytoplasm has very low $[Ca^{2+}]$ (<100 nM), which is very different from the extracellular environment encountered by most eukaryotic cells. The absence of extracellular Ca^{2+} is usually incompatible with normal cell functions and survival. In the present work, we have tested the possibility that *Plasmodia* overcome the limitation posed by the erythrocyte intracellular environment through the maintenance of a high $[Ca^{2+}]$ within the parasitophorous vacuole (PV), the compartment formed during invasion and within which the parasites grow and divide. Thus, *Plasmodia* were allowed to invade erythrocytes in the presence of Ca^{2+} indicator dyes. This allowed selective loading of the Ca^{2+} probes within the PV. The $[Ca^{2+}]$ within this compartment was found to be ~40 μM, i.e., high enough to be compatible with a normal loading of the *Plasmodia* intracellular Ca^{2+} stores, a prerequisite for the use of a Ca^{2+}-based signaling mechanism. We also show that reduction of extracellular $[Ca^{2+}]$ results in a slow depletion of the $[Ca^{2+}]$ within the PV. A transient drop of $[Ca^{2+}]$ in the PV for a period as short as 2 h affects the maturation process of the parasites within the erythrocytes, with a major reduction 48 h later in the percentage of schizonts, the form that re-invades the red blood cells.

Introduction

Malaria is one of the major causes of morbidity and mortality in the third world. *Plasmodia*, the causal agent of malaria, are unicellular parasites that in humans spend most of their life within intact cells. After initial infection from a mosquito bite, the *Plasmodia* first invade and differentiate in hepatocytes, and this is followed by repetitive cycles of proliferation and re-invasion in RBCs (Howard, 1982; Sherman, 1985). Within the latter cells, *Plasmodia* multiply and mature inside a membrane-bound vacuole that fully surrounds the parasites, known as the parasitophorous vacuole (PV).* Mature merozoite-stage parasites are released by RBC lysis and go on to invade other RBCs. The parasitophorous vacuole membrane (PVM) is formed around the parasite at the time of RBC invasion, most likely from invagination of the RBC plasma membrane with additional components contributed by secretion from intracellular organelles of the parasite, known as rhoptries (Bannister and Mitchell, 1989; Ward et al., 1993; Dluzewski et al., 1995). Indirect evidence suggests that the PVM is a molecular sieve highly permeable to solutes up to a mol wt of 2,000 (Desai et al., 1993; Schwab et al., 1994; Desai and Rosenberg, 1997; Kirk, 2001). Accordingly, the ionic composition of the PV is expected to be very similar, if not identical, to the cytoplasm of intact cells. Thus, although the intracellular environment provided by the PVM represents a protected ecological niche where *Plasmodia* can safely escape the immunological reaction of the host, the ionic milieu of the PV, very different from the extracellular medium experienced by most other eukaryotic cells, poses important survival problems. In common with other mammalian cells, the erythrocyte cytoplasm maintains high $[K^+]$ (~140 mM) and very low $[Ca^{2+}]$ (~100 nM). High $[K^+]$ may result in a drastic reduction of the parasite membrane potential, and consequently, in the inhibition of a series of membrane potential–controlled nutrient uptake mechanisms. With respect to $[Ca^{2+}]$, the problem of the low cytoplasmic concentration may be even more dramatic. In

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*Abbreviations used in this paper: PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; THG, thapsigargin.

Key words: parasitophorous vacuole; signal transduction; *Plasmodium*; calcium indicators; melatonin

The Journal of Cell Biology, Volume 161, Number 1, April 14, 2003 103–110
http://www.jcb.org/cgi/doi/10.1083/jcb.200212130

fact, extracellular [Ca^{2+}] is relatively constant (a few mmol/l) in the body fluids of most multicellular organisms (Pozzan et al., 1994). On the contrary, unicellular eukaryotes may experience dramatic changes in extracellular [Ca^{2+}]. However, Ca^{2+} in natural aqueous environments is unlikely to drop below a few tens of a micromolar. Given its ubiquitous role not only as a second messenger, but also as a permissive factor in many biological reactions, how can *Plasmodia* survive, multiply, and differentiate in the low [Ca^{2+}] of the RBC cytoplasm? Indeed, we have previously demonstrated that calcium signaling is likely to be an important mechanism in the stimulation of parasite growth and development by host melatonin (Hotta et al., 2000; Garcia et al., 2001). When other cells are artificially exposed to such low [Ca^{2+}] concentrations, the cation continues to be pumped out from the cytoplasm, and the cytosolic concentration drops to levels that first lead to depletion of [Ca^{2+}] from organelles, and then eventually to cell death.

Here, we propose that the solution to the above question, in the case of *Plasmodia*, resides in the nature and sidedness of the PVM. Specifically, if the PVM is derived at least in part (Dluzewski et al., 1995) from the RBC membrane, its sidedness is predicted to be inside-out (i.e., the former extracellular surface of the RBC plasma membrane facing the lumen of the PV). Thus, if the erythrocyte plasma membrane Ca^{2+} ATPase is still present in the PVM, it should pump Ca^{2+} into the PV, generating a high [Ca^{2+}] microenvironment in the space between the PVM and the parasite plasma membrane. If this is the case, the *Plasmodium* during its intraerythrocytic life is not exposed to the low [Ca^{2+}] of the cytosol, but rather to a [Ca^{2+}] not very different from that experienced by any other eukaryotic cell. Last but not least, this model implies that the PVM should be less permeable to ions and other small molecules than predicted from some in vitro data (Desai et al., 1993).

Results

Loading the PV with Ca^{2+} indicators

Malaria parasites were allowed to invade RBCs in vitro in a medium containing the fluorescent calcium indicator Fluo-3 in its cell-impermeant, free acid form. This approach allows the Ca^{2+} indicator dye to be trapped in the PV during invasion. If the PVM is permeable to high mol wt solutes, the dye should be at about the same concentration in both the PV and the RBC cytoplasm. On the contrary, if the PVM is impermeable to Fluo-3, the dye should remain in the PV and thus allow selective measurement of [Ca^{2+}] within that microenvironment. Fig. 1 A shows a confocal image of a human RBC invaded by *Plasmodium falciparum* in the presence of Fluo-3. The fluorescence of Fluo-3 forms a ring around the parasite, although no staining is observed in either the RBC cytosol or in the parasite itself. Fig. 1 B shows the phase-contrast image of the same *Plasmodium*-infected RBC, and Fig. 1 C shows the fluorescence intensity profile along the white line overlaying the parasite and the RBC (inset). The fluorescence intensity profile shows two large peaks on opposite sides of the parasite (corresponding to the PV), and a fluorescence level indistinguishable from that of background in the RBC cytoplasm. The mean values of fluorescence intensity obtained in two independent experiments (and 10 different cells) performed with either *P. falciparum*

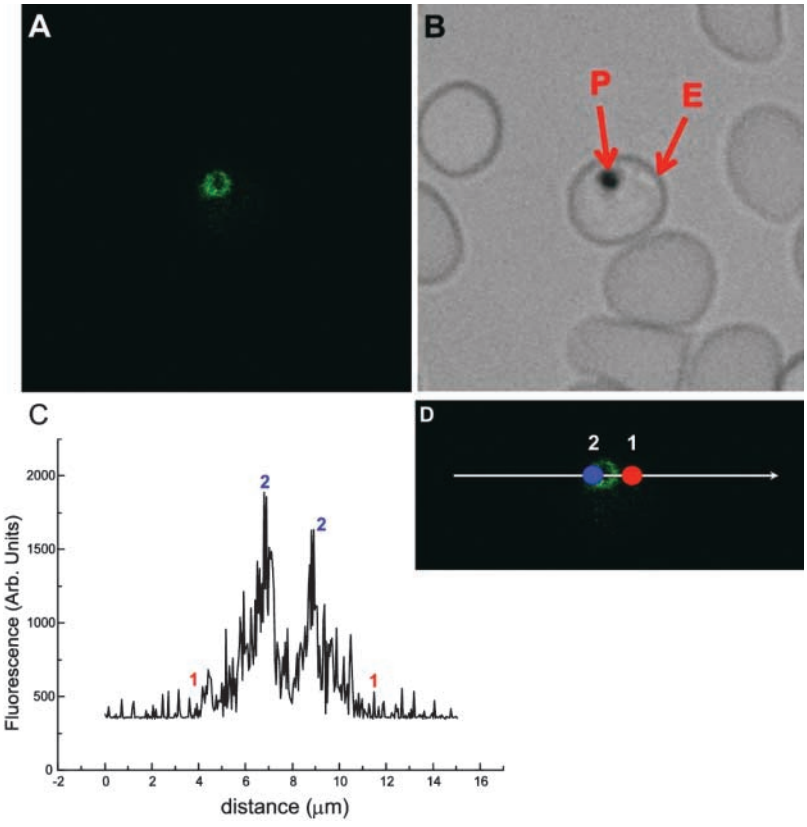

Figure 1. **Trapping of Fluo-3 (free acid) in the PV of infected RBCs.** (A) Confocal fluorescence image of a human RBC invaded by *P. falciparum* in the presence of 10 μM Fluo-3 (acid) in the medium. (B) Phase-contrast image of the same cell. P refers to the parasite, and E to the invaded RBC. (C) Intensity histogram of the fluorescence image shown in A. The fluorescence intensity (expressed as arbitrary units) was measured along the axis shown in D. The numbers 1 and 2 refer to the position along the axis.

Table I. ***P. chabaudi* and *P. falciparum* were allowed to invade RBCs in the presence of 10 μM Fluo-3 acid**

Parasite	Erythrocyte cytosol	Parasitophorous vacuole
Plasmodium chabaudi	<5 A.U.	1,614 ± 342 A.U.
Plasmodium falciparum	<5 A.U.	1,870 ± 388 A.U.

After invasion, the cells were washed and analyzed by confocal microscopy. For each invaded cell, fluorescence was measured in two regions, corresponding to (1) the cytoplasm of the RBC; and (2) the PV. The values of fluorescence were corrected for the background measured outside the RBC and expressed in arbitrary units (A.U.). Results are means ± SEM from 10 cells for each *Plasmodium* species of three independent experiments.

or *Plasmodium chabaudi* are shown in Table 1. Control experiments where RBCs were incubated for the same period of time with Fluo-3 (but without parasites) resulted in no trapping of the dye, nor was any Fluo-3 sequestered if the indicator was added after invasion (unpublished data). Similarly, isolated parasites incubated with Fluo-3 (acid) did not accumulate any dye (unpublished data). When RBCs infected by the parasites in the presence of Fluo-3 were allowed to stay at RT for 30 min–1 h, the staining pattern did not change significantly.

Given that Fluo-3 is almost nonfluorescent in a low Ca^{2+} environment, the bright signal surrounding the parasite suggests that the $[Ca^{2+}]$ in the PV is relatively high. A simple test of this conclusion is to treat the cells with the Ca^{2+} ionophore ionomycin. This ionophore is known to transport Ca^{2+} across membranes down its electrochemical gradient.

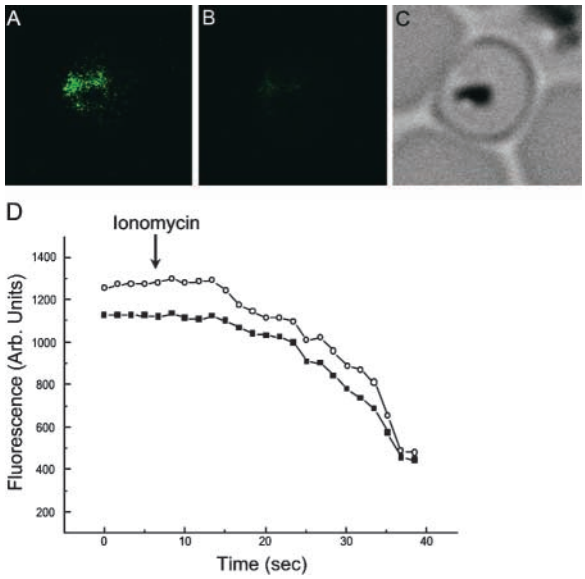

Figure 2. **Effect of ionomycin on the $[Ca^{2+}]$ in the PV.** The PV was loaded with Fluo-3 (acid) by carrying out the invasion by *P. falciparum* in the presence of 10 μM of the dye. (A) Initial fluorescence. (B) 40 s after addition of 15 μM ionomycin. (C) Phase-contrast image of the same invaded RBC. (D) Kinetics of the fluorescence changes of two different cells (open circle and black square). Addition of the solvent alone, DMSO, was without effect. In this and the following experiments, typical results are shown that are representative of at least five similar trials in three or more different batches of cells.

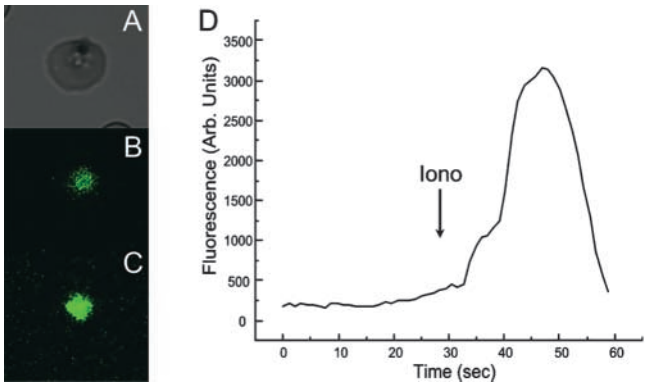

Figure 3. **Effects of ionomycin on the $[Ca^{2+}]$ of the parasite cytoplasm.** *P. falciparum*-infected RBCs were loaded with Fluo-3/AM as described previously (Garcia et al., 1996). Where indicated, 15 μM ionomycin was added. The buffer contained 1 mM $CaCl_2$. Identical results were obtained with *P. chabaudi*. (A) Phase-contrast image of the invaded RBC. (B) Initial Fluo-3 fluorescence. (C) Fluo-3 fluorescence 20 s after addition of 15 μM ionomycin. (D) Typical kinetics of the fluorescence changes.

If the $[Ca^{2+}]$ of the PV is higher than in the RBC or parasite cytoplasm, ionomycin should transport Ca^{2+} from the PV into the RBC and/or parasite and thus cause a decrease of the PV $[Ca^{2+}]$. Fig. 2 shows that addition of ionomycin indeed caused a slow decrease of Fluo-3 fluorescence, indicating that the ionophore was transporting Ca^{2+} out of the vacuolar space. Further evidence that the signal derived from the trapped Fluo-3 (free acid) reflects the PV environment and that the latter behaves differently from the parasite cytoplasm is provided by the experiment presented in Fig. 3. In this experiment, the RBCs infected with parasites were loaded with Fluo-3/AM, which predominantly loads the parasite cytoplasm. Under these conditions, ionomycin caused a large increase in fluorescence (Fig. 3) as expected, given that this ionophore is known to penetrate into cells and release Ca^{2+} from intracellular, membrane bound stores. Results similar to those shown above for *P. falciparum* were obtained in RBCs infected with *P. chabaudi* (unpublished data). The important point is that Fluo-3 responds very differently to ionomycin when trapped within the parasite cytoplasm (as in Fig. 3) or within the PV (as in Fig. 2), indicating that the same dye trapped in the different compartments responds differently to the same agent.

$[Ca^{2+}]$ changes in the cytoplasm affect the $[Ca^{2+}]$ in the PV

The different behavior of the PV with respect to the parasite cytoplasm is even more evident in the experiment presented in Fig. 4 for *P. falciparum*, and identical results were obtained with *P. chabaudi*. In this experiment, invasion was carried out in medium containing the free acid form of another Ca^{2+} indicator, Mag-Fura-2, to monitor $[Ca^{2+}]$ within the PV. After invasion, the infected RBCs were incubated with Fluo-3/AM to load the parasite cytoplasm. The spectral characteristics of Mag-Fura-2 are sufficiently different from those of Fluo-3 that the signals from the two dyes are easily distinguished. Fig. 4 shows the two confocal images of a cell doubly loaded with Mag-Fura-2 (acid) in the

PV and Fluo-3 (AM form) in the cytoplasm (Fig. 4, E and D, respectively). The phase image is also presented in C. The different localization of the two dyes appears quite clear; Fluo-3 within the parasite and Mag-Fura-2 around it. Fig. 4 shows the simultaneous dynamic measurements of $[Ca^{2+}]$ in the cytosol (Fig. 4 A, Fluo-3 AM) and PV (Fig. 4 B, Mag-Fura-2 acid) in the same cell. Mag-Fura-2 is a "ratiometric" dye, i.e., Ca^{2+} binding has opposite effects on the fluorescence emitted on excitation at 340 and 380 nm. Thus, an increase in $[Ca^{2+}]$, as revealed by Fluo-3, results in an increase of fluorescence, whereas an increase of the 340/380 nm ratio with Mag-Fura-2 is more directly related to the absolute increase of $[Ca^{2+}]$ (Grynkiewicz et al., 1985). In Fig. 4, the cells were first treated with the inhibitor of the sarco-ER Ca^{2+} ATPase (SERCA) thapsigargin (THG), a drug known to cause the release of Ca^{2+} from internal stores. Addition of THG resulted in elevation of $[Ca^{2+}]$, measured with both Fluo-3 and Mag-Fura-2. However, the subsequent addition of ionomycin had opposite effects on the $[Ca^{2+}]$ monitored by the two indicators; a further increase in $[Ca^{2+}]$ was monitored with cytoplasmic Fluo-3, whereas a large decrease was revealed by Mag-Fura-2 trapped in the PV. The interpretation of this experiment appears straightforward: THG mobilizes Ca^{2+} from *Plasmodium* intracellular stores, thus increasing cytoplasmic $[Ca^{2+}]$; Ca^{2+} is then pumped out into the PV and revealed by Mag-Fura-2. Indeed, a clear delay is observed in the peak of the Mag-Fura-2 signal, as compared with that of Fluo-3. Ionomycin further releases Ca^{2+} from stores and causes an additional small increase in the $[Ca^{2+}]$ of the cytoplasm, but, given that the $[Ca^{2+}]$ of the PV is much higher than in the cytosol of the RBC, the ionophore transports Ca^{2+} out of the PV, leading to a major decrease of the Mag-Fura-2 signal.

Quantitation of the $[Ca^{2+}]$ in the PV is difficult in these single-cell experiments. A calibration of the Mag-Fura-2 signal in the PV was thus carried out in a population of RBC infected by *P. chabaudi*. In the experiment shown in Fig. 4 F, RBCs were infected with *P. chabaudi* in a medium containing 10 μM Mag-Fura-2 acid. After invasion, the cells were resuspended in a spectrofluorimeter cuvette in medium without calcium and were then treated with ionomycin. Addition of ionomycin induced a substantial decrease in the Mag-Fura-2 signal, again indicating that the dye was contained in an environment whose calcium concentration drops on treatment with the ionophore. The cells were then lysed and the values of R_{max} and R_{min} were calculated as described previously (Grynkiewicz et al., 1985). The fluorescent signal of Mag-Fura-2 before lysis was calibrated in terms of $[Ca^{2+}]$ according to the standard ratiometric equation assuming a Kd of 53 μM for the Mag-Fura-2–Ca^{2+} complex (Hofer et al., 1998). Using this approach, Ca^{2+} in the PV was found to be 41 ± 1 μM (n = 5).

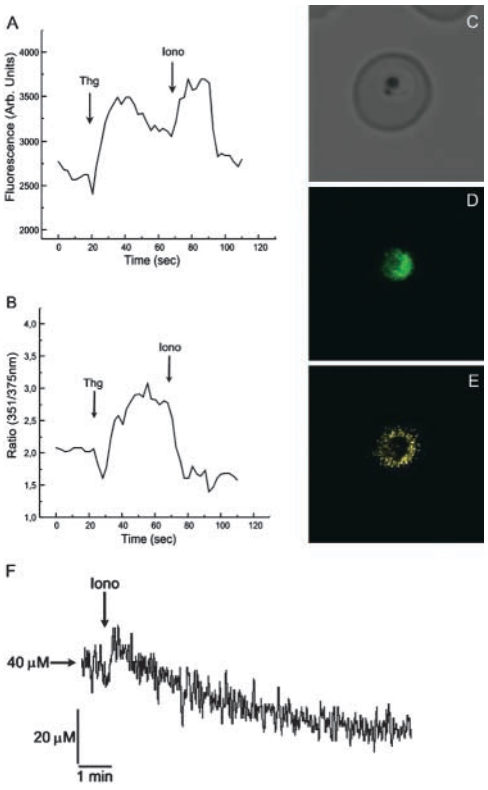

Figure 4. **Simultaneous imaging of the $[Ca^{2+}]$ in the PV and cytosol.** 10 μM Mag-Fura-2 acid was present during invasion with *P. falciparum*, and the cells were then loaded with Fluo-3/AM after invasion. The buffer contained 1 mM $CaCl_2$ and 2 mM Probenecid to avoid release of loaded Fluo-3. (A) Changes in parasite cytosolic $[Ca^{2+}]$ monitored by Fluo-3 fluorescence (arbitrary units) on addition of 10 μM THG and 10 μM ionomycin. (B) Changes of PV $[Ca^{2+}]$ monitored as the ratio of Mag-Fura-2 fluorescence excited at 351 and 375 nm in the same cell. (C) Phase-contrast image of the invaded RBC. (D) Fluo-3 fluorescence signal (in the parasite cytoplasm). (E) Mag-Fura-2 (in the PV) fluorescence. The small reversible drop in fluorescence observed in A and B is an addition artifact (a slight change in focus). In fact, we have also observed it on addition of medium only. (F) Mouse RBCs were infected with *P. chabaudi* in the presence of 10 μM Mag-Fura-2 acid in the standard medium containing $CaCl_2$. After washing the excess dye, the infected cells (10^7 cells) were resuspended in the fluorimeter cuvette, equipped with magnetic stirring, in medium without added $CaCl_2$. The arrow shows the time of addition of 15 μM ionomycin. The Ca^{2+} concentration in the PV was calculated, after lysing the cells, using the F-4500 intracellular cation measurement system software (Hitachi). The $[Ca^{2+}]$ of the PV, measured 30 min after completion of invasion, was found to be 41 ± 1 μM (n = 5).

The Ca^{2+} content of the *Plasmodia* intracellular stores depends on the $[Ca^{2+}]$ in the PV

As a final test to prove that parasites within intact RBCs are indeed exposed to a relatively high Ca^{2+} environment, the experiments presented in Fig. 5 were carried out. RBCs infected with *P. chabaudi* were first loaded with Fluo-3/AM, then after 90 min incubation in 1 mM $CaCl_2$, the Ca^{2+} content of the parasite intracellular Ca^{2+} stores was verified by the addition of THG (Fig. 5 A). In a parallel experiment, after loading with Fluo-3/AM, the RBC plasma membrane was permeabilized with digitonin and then, after washing out the digitonin, the cells were incubated in a low $[Ca^{2+}]$ medium (~100 nM) for 90 min (Fig. 5 C). The rationale of the experiment is as follows: digitonin completely permeabilizes the RBC plasma membrane and the PVM. On the other hand, the plasma membrane of the parasites is not affected by the detergent at these concentrations, as revealed by the maintenance of Fluo-3 fluorescent signal within the *Plasmodium*. However, under these conditions,

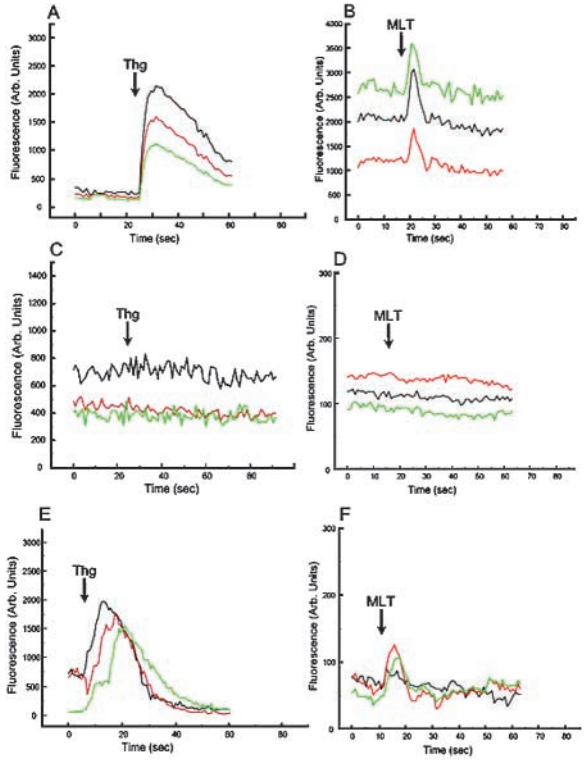

Figure 5. **Effects of extracellular Ca^{2+} on the Ca^{2+} content of the parasite intracellular stores.** *P. chabaudi* were loaded with Fluo-3/AM and analyzed at the confocal level. The loaded cells were incubated for 90 min in media containing different concentrations of $CaCl_2$. (A and B) The medium was supplemented with 1 mM $CaCl_2$; in C and D, no $CaCl_2$ was added and 100 μM EGTA was included ($[Ca^{2+}]$ about 100 nM); in E and F, the medium was supplemented with 100 μM $CaCl_2$. For the experiments in C–F, the cells were loaded with Fluo-3 before treating with 10 μM digitonin. The detergent was washed away after 5 min. The arrows indicate the additions of 10 μM THG and 100 μM of melatonin (MLT). The kinetics of the fluorescence of three typical single cells are presented in each panel.

the parasite is exposed not to the $[Ca^{2+}]$ of the PV, but to that of the extracellular medium. Addition of THG under these conditions resulted in no $[Ca^{2+}]$ increase, revealing that exposure of the parasite to a low $[Ca^{2+}]$ medium for 90 min results in almost complete emptying of its intracellular Ca^{2+} stores (Fig. 5 C). On the contrary, if the cells were permeabilized with digitonin (see previous paragragh), but incubated in a medium containing 100 μM $CaCl_2$ (i.e., a concentration close to that calculated to be in the PV), the intracellular release caused by THG was similar to that observed in controls, i.e., without digitonin permeabilization (compare Fig. 5 E with Fig. 5 A). Results similar to those shown above for *P. chabaudi* were obtained in RBCs infected with *P. falciparum* (unpublished data).

The effects of melatonin on cytosolic $[Ca^{2+}]$ of the intraerythrocytic *Plasmodia* was measured with Fluo-3/AM. In intact erythrocytes incubated in 1 mM physiological $CaCl_2$, melatonin caused a transient increase in parasite cytosolic $[Ca^{2+}]$ (Fig. 5 B). Essentially, the same cytosolic $[Ca^{2+}]$ response to melatonin was observed after permeabilization of the erythrocyte and PVM with digitonin if medium $[Ca^{2+}]$ was maintained at 100 μM (Fig. 5 F), whereas there was no melatonin response when medium $[Ca^{2+}]$ was buffered at 100 nM (Fig. 5 D).

The Ca^{2+} levels in the PV affects the intraerythrocytic maturation of the parasites

The final question is whether the relatively high level of Ca^{2+} in the PVM is just accidental or whether it is essential for the proper development of the parasites within the RBC. The simplest direct test to answer this question is to artificially decrease the $[Ca^{2+}]$ in the PV and monitor the effect on parasite development. To this end, *P. chabaudi* or *P. falciparum* were allowed to invade RBCs in normal $[Ca^{2+}]$-containing medium containing Fluo-3 (free acid, to monitor the $[Ca^{2+}]$ within the PV), and then they were incubated in Ca^{2+}-free medium. As shown in Fig. 6 A, the Fluo-3 signal representing PV $[Ca^{2+}]$ for *P. falciparum*-infected RBC remained constant for a few minutes and then slowly decreased (similar results were obtained with *P. chabaudi*; not depicted). After 30 min under these conditions, the fluorescence of Fluo-3 was about 50% of the initial value. Continuous incubation for 20 h in EGTA completely prevented the maturation of the parasites,

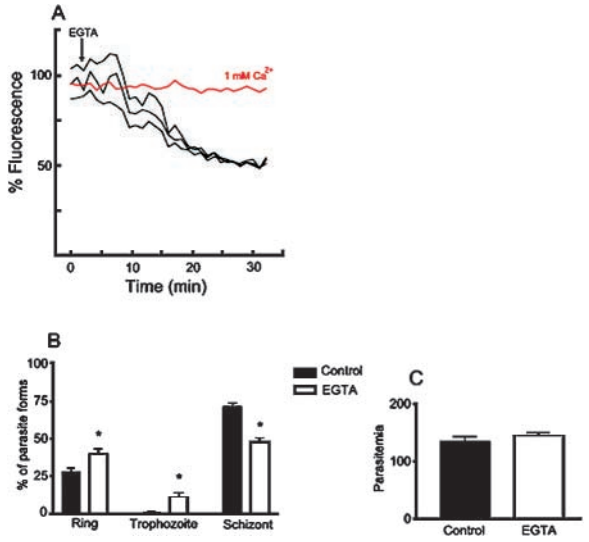

Figure 6. **Effects of extracellular Ca^{2+} on the $[Ca^{2+}]$ in the PV and on parasite development.** *P. falciparum* was allowed to invade RBC in Ca^{2+} medium and Fluo-3 acid followed by 30 min incubation either in medium containing 1 mM $CaCl_2$ or in $[Ca^{2+}]$-free medium supplemented with 10 mM EGTA. Other conditions were as in Fig. 2. (A) Kinetics of the Fluo-3 signal in four typical cells. The black traces refer to three cells incubated initially in Ca^{2+} medium followed by $[Ca^{2+}]$-free medium and EGTA (where indicated). The red trace refers to a single typical control cell continuously incubated in normal calcium-containing medium. The values have been normalized to the fluorescence measured at the beginning of the experiment. For clarity, the three black traces have been offset vertically by 5%. (B) Effect of a 2-h exposure to 10 mM EGTA on the proportions of *P. falciparum* forms measured 48 h after invasion. *P. falciparum* were allowed to invade the RBC in normal medium and were then incubated in medium supplemented with 10 mM EGTA for 2 h, and finally transferred to the normal Ca^{2+}-containing medium. (C) Parasitemia (number of parasites/1,000 cells) in both conditions. Results in B and C are means ± SEM from three independent experiments; data were compared by one-way ANOVA and then by a Newman-Keuls test. Asterisk denotes statistical significance with respect to control values ($P < 0.05$).

but many RBCs appeared damaged under these conditions. A less drastic protocol was thus adopted; the invaded RBCs were incubated for 2 h in an EGTA-containing medium and then were returned to the normal [Ca^{2+}]-containing medium. Fig. 6 (B and C) shows that after 48 h, the number of infected RBCs (parasitemia) is identical in controls and in cells that have been treated for 2 h in EGTA (Fig. 6 C), indicating that the Ca^{2+} removal protocol did not damage the RBC. However, the percentage of immature forms was significantly increased in the Ca^{2+}-depleted condition, and the mature form, the schizonts, was reduced by about 30%. This experiment demonstrates that maintaining a high [Ca^{2+}] in the PV is necessary for a normal maturation of *Plasmodia* within the RBC, and that even a short depletion of [Ca^{2+}] in the PV results in a substantial alteration in the maturation process.

Discussion

Data from the present paper demonstrate that a Ca^{2+} indicator such as Fluo-3, about 700 D, can be trapped within the PV during invasion and does not diffuse into either the RBC cytoplasm or the parasite for at least 1 h. These results are in contrast with the suggestion that the PVM functions as a "molecular sieve" permeable to solutes up to 1.9 kD (Schwab et al., 1994; Desai and Rosenberg, 1997). However, it should be noted that those experiments were performed at later stages of parasite development (15–20 h after invasion). Therefore, we cannot at present exclude that the permeability characteristics of the PVM change during the intraerythrocytic life time of the *Plasmodia*.

It may be argued that the 10-μM doses of THG used here are higher than those used in vertebrate cells to induce Ca^{2+} release from intracellular stores, and thus may reflect a nonspecific effect of the drug on intracellular [Ca^{2+}] (Vercesi et al., 1993). Furthermore, it has been suggested that the SERCAs expressed in *P. falciparum* are insensitive to THG, but sensitive to cyclopiazonic acid (Alleva and Kirk, 2001). We would argue that (1) it is not surprising that the doses of THG necessary to completely inhibit the SERCA in *Plasmodia* are much higher than in mammalian cells, given the large evolutionary distance between these cells (Varotti et al., 2003); and (2) effects similar to those obtained with THG have been obtained with cyclopiazonic acid (another SERCA inhibitor) at the same doses effective in mammalian cells (5–20 μM).

The intracellular Ca^{2+} stores in malaria parasites appear to play a major role in the signaling pathway initiated by the host hormone melatonin via the production of $InsP_3$ (Hotta et al., 2000). $InsP_3$, in turn, is known to act in *Plasmodia* on both classical ER-like stores and on another Ca^{2+} store, characterized by an acidic lumen, the so-called acidic Ca^{2+} store (Docampo and Moreno, 1999; Garcia, 1999). Therefore, we investigated whether the [Ca^{2+}] in the medium surrounding the *Plasmodia* (PV [Ca^{2+}]) needs to be maintained in the 100-μM range for full response to the physiological agonist, melatonin.

Our experiments demonstrate that, at least as far as Ca^{2+} handling is concerned, the *Plasmodia* are surrounded by a microenvironment whose [Ca^{2+}] is ~40 μM, 100–1,000-fold higher than that in the parasite and RBC cytoplasm (Adovelande et al., 1993; Garcia et al., 1996). This [Ca^{2+}] in the PV is lower than that experienced in the extracellular fluid by cells of multicellular organisms. However, if the RBC plasma membrane is permeabilized and the parasites are exposed to an extracellular medium containing a [Ca^{2+}] in this range, they preserved the Ca^{2+} content of their intracellular stores, a prerequisite for the use by *Plasmodia* of a Ca^{2+}-based signaling mechanism (Wasserman et al., 1982, Passos and Garcia, 1998; Garcia, 1999; Hotta et al., 2000). Several not mutually exclusive hypotheses can be proposed to explain the relatively high [Ca^{2+}] of the PV: (1) extracellular Ca^{2+} remains trapped within the PV during invasion; (2) the RBC plasma membrane Ca^{2+}-ATPase continuously supplies Ca^{2+} to the vacuolar space. Consistent with this latter possibility is the localization on the PVM (Langreth 1977; Caldas and Wasserman, 2001) of a Ca^{2+}-ATPase. The electronmicrographs suggest that the enzyme is located on the inner surface of the PVM membrane, i.e., with the sidedness required to continuously refill Ca^{2+} into the PV; or (3) an alternative explanation would be the diffusion of Ca^{2+} into the PV from the extracellular medium through specialized membrane structures. It has been shown that parasite maturation is accompanied by the development of tubular membrane structures connecting the PV to the extracellular medium. These membranes have been suggested to play a role in the uptake of nutrients and proteins into the developing *Plasmodia* (Pouvelle et al., 1991; Lauer et al., 1997). Although we cannot exclude that these membrane structures could contribute to the maintenance of Ca^{2+} homeostasis at later stages in *Plasmodia* development, it should be stressed that they are not observed during the first hours after infection (Gormley et al., 1992; Garcia et al., 1997).

The sequencing of the *Plasmodium* genome (Gardner et al., 2002) and several recent studies have identified in this parasite a number of signaling molecules related to those of vertebrate cells, including many proteins concerned with Ca^{2+} handling and signaling (Dyer and Day, 2000; Le Roch et al., 2000;

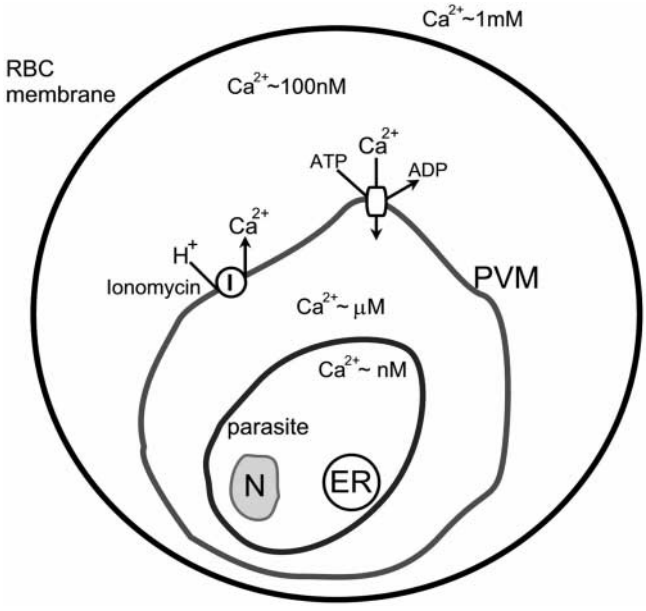

Figure 7. **Schematic model of Ca^{2+} compartmentation in *Plasmodium*-infected RBC.** PVM, parasitophorous vacuolar membrane; ER, endoplasmic reticulum; N, nucleus.

Marchesini et al., 2000). The key question addressed here is how the parasite can use Ca^{2+}-based signaling mechanisms while located within the RBC, where it might be expected to be exposed to a very low [Ca^{2+}]. We have shown unambiguously that the PV provides a sufficiently high [Ca^{2+}] to ensure the maintenance of the parasite Ca^{2+} stores (represented in Fig. 7), and thus the sensitivity to agents, such as melatonin, that use Ca^{2+} as a second messenger to regulate the *Plasmodia* cell cycle (Hotta et al., 2000). In addition, a prolonged decrease of the [Ca^{2+}] of the PV appears to impair the maturation of the parasites, and eventually is incompatible with the survival of the *Plasmodia* within the RBC.

Materials and methods

P. chabaudi and *P. falciparum*

P. chabaudi (clone F IP-Pc1) was maintained in female mice (Balb/c) by transfer of infection. *P. falciparum* (Palo alto strain) was maintained in continuous culture (Trager and Jensen, 1976). The gas mixture of the acrylic chamber contained 5% O_2, 7% CO_2, and 88% N_2. The procedure for collecting blood from mice and removing platelets has been described previously (Hotta et al., 2000). By carrying out the experiments at the single cell level, the morphology of the invaded cells was verified and corresponded to that of RBC. The contamination by cells other than RBC in the preparation was <0.1%.

The intraerythrocytic stages of parasites were determined by Giemsa stain smears. All the experiments were carried out with 10^7 cells in a final volume of 2 ml in a buffer containing (mM): 116 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 5.5 D-glucose and 50 Mops, pH 6.8. As indicated in the figure legends, the medium was either supplemented with 1 mM $CaCl_2$ or without added $CaCl_2$ with or without the addition of 100 μM or 10 mM EGTA. The parasitemia was about 25% at schizont stage both in the case of *P. chabaudi* and of *P. falciparum*. The culture was synchronized with sorbitol treatment (Lambros and Vanderberg, 1979). The infected RBCs were washed twice with RPMI 1640 medium (GIBCO BRL) supplemented with 10% calf serum (*P. chabaudi*) or 10% human serum (*P. falciparum*), and finally resuspended in the medium described above.

Loading with the Ca^{2+} indicators

In order to monitor the [Ca^{2+}] in the PV the invasion was carried out at 37°C in the presence of the calcium indicators (acid form) Fluo-3 or Mag-Fura-2 (10 μM). At the end of invasion, cells were stored at 4°C to prevent the possibility of endocytosis of the indicator by the parasite. In order to label the parasite cytoplasm with acetoxymethyl ester forms of the dyes, we used infected cells immediately after invasion by following with Giemsa-staining the stage of the infection. In brief, segmented schizonts were monitored up to the moment of invasion, and early ring stages were used for loading with 10 μM Fluo-3/AM in the presence of 2 mM Probenecid (Sigma-Aldrich). Before analyzing the cells in the confocal microscope or in the spectrofluorimeter, the RBCs were washed twice with PBS and finally resuspended in the standard buffer. The relatively low pH (6.8) of the medium was chosen to reduce the transport of Ca^{2+} by ionomycin from the medium into the RBCs, while its efficacy on intracellular membrane is unaffected (Fasolato and Pozzan, 1989). In a few experiments, loading with Fluo-3/AM was carried out at the segmented schizont stage, i.e., before invasion of new RBCs, with no appreciable difference in the response of the Ca^{2+} indicator to the different treatments. In the experiments where the [Ca^{2+}] of both cytoplasm and PV was measured simultaneously, the RBCs were invaded in the presence of 10 μM Mag-Fura-2, and after invasion, Fluo-3/AM was added to load the parasite cytoplasm.

Ca^{2+} measurements

Single-cell confocal microscope (model LSM-510; Carl Zeiss MicroImaging, Inc.) experiments were carried out at RT. The infected cells were plated on slides previously incubated for 1 h with L-polylysine (Sigma-Aldrich). The dyes were excited sequentially at 488 nm (for Fluo-3), 351 nm, and 375 nm (for Mag-Fura-2), and the emitted fluorescence was collected using band pass filters: 505–530 nm (Fluo-3) and 475–525nm (Mag-Fura-2). Experiments in cell suspension were carried out at 37°C in a spectrofluorimeter (model F-4500; Hitachi) as described previously (Garcia et al., 1996). The emission and excitation wavelengths for Mag-Fura-2 were 345/380 nm and 510 nm, respectively. A Kd of 53 μM for the Mag-Fura-2 Ca^{2+} complex was assumed (Hofer et al., 1998).

The fluorescence traces represent the average signal in the region of interest (ROI). Usually, the ROI covered the whole cell, on the assumption that the dye was distributed only in the PV (Fig. 2) or in the whole cytoplasm (Fig. 3 and Fig. 6). Also, in the case of double labeling with Fluo-3 and Mag-Fura-2 (Fig. 4), the ROI covered the whole cell because the spectra of the two dyes are well separated so that no signal from Fluo-3 was visible when exciting in the UV, and vice versa, no signal from Mag-Fura-2 was detected at 488 nm (i.e., the excitation wavelength of Fluo-3). In a few experiments, we used smaller ROIs centered on the PV or on part of the parasite cytoplasm, but the results were not significantly different from those obtained with the larger ROIs. Each frame (512 × 512 pixels) was acquired with a scan time of 784 ms/image and an interval of 200 ms between consecutive images. Experiments were carried out with both *P. chabaudi* (and mouse RBC) and *P. falciparum* (and human RBC) with no appreciable difference. For simplicity, for each specific experiment, only the results obtained with either *P. falciparum* or *P. chabaudi* are presented. All representative traces are typical of experiments carried out with similar results in at least in three independent trials.

We are grateful to P. Magalhães for critical reading of the manuscript.

We thank Fundação de Amparo à Pesquisa de São Paulo (FAPESP) for funding C.R.S. Garcia (FAPESP 98/00410-2). This work was also partially supported by a United Nations Educational, Scientific, and Cultural Organization grant to T. Pozzan (UVO-Roste 875.635.1).

Submitted: 20 December 2002
Revised: 13 February 2003
Accepted: 24 February 2003

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