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 [Ca2+] (<100 nM), which is very different from the extracellular environment encountered by most eukaryotic cells. The absence of extracellular Ca2+ 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 [Ca2+] 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 Ca2+ indicator dyes. This allowed selective loading of the Ca2+ probes within the PV. The [Ca2+] within this compartment was found to be ~40 μM, i.e., high enough to be compatible with a normal loading of the Plasmodia intracellular Ca2+ stores, a prerequisite for the use of a Ca2+-based signaling mechanism. We also show that reduction of extracellular [Ca2+] results in a slow depletion of the [Ca2+] within the PV. A transient drop of [Ca2+] 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; Schab 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 [Ca2+] (~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 [Ca2+], the problem of the low cytoplasmic concentration may be even more dramatic. In
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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{P. falciparum}
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.

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 presence of Fluo-3 and ionomycin. The different compartments respond differently to the same agent.
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\(^{2+}\)] (Gryniewicz 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 \(\mu\)M Mag-Fura-2 acid. After invasion, the cells were resuspended in a spectrophotometer 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 (Gryniewicz 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 K_{d} of 53 \(\mu\)M for the Mag-Fura-2-Ca\(^{2+}\) complex (Hofer et al., 1998). Using this approach, Ca\(^{2+}\) in the PV was found to be \(41 \pm 1\ \mu\)M (\(n = 5\)).

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,
The parasite is exposed not to the $[\text{Ca}^{2+}]$ of the PV, but to that of the extracellular medium. Addition of THG under these conditions resulted in no $[\text{Ca}^{2+}]$ increase, revealing that exposure of the parasite to a low $[\text{Ca}^{2+}]$ medium for 90 min results in almost complete emptying of its intracellular $\text{Ca}^{2+}$ stores (Fig. 5 C). On the contrary, if the cells were permeabilized with digitonin (see previous paragraph), but incubated in a medium containing 100 $\mu$M 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 $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$ (Fig. 5 B). Essentially, the same cytosolic $[\text{Ca}^{2+}]$ response to melatonin was observed after permeabilization of the erythrocyte and PVM with digitonin if medium $[\text{Ca}^{2+}]$ was maintained at 100 $\mu$M (Fig. 5 F), whereas there was no melatonin response when medium $[\text{Ca}^{2+}]$ was buffered at 100 nM (Fig. 5 D).

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

The final question is whether the relatively high level of $\text{Ca}^{2+}$ in the PV 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 $[\text{Ca}^{2+}]$ 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 $[\text{Ca}^{2+}]$-containing medium containing Fluo-3 (free acid, to monitor the $[\text{Ca}^{2+}]$ within the PV), and then they were incubated in $\text{Ca}^{2+}$-free medium. As shown in Fig. 6 A, the Fluo-3 signal representing PV $[\text{Ca}^{2+}]$ 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,

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**Figure 5. Effects of extracellular $\text{Ca}^{2+}$ on the $\text{Ca}^{2+}$ 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 $\mu$M EGTA was included (i.e., about 100 nM); in E and F, the medium was supplemented with 100 $\mu$M CaCl$_2$. For the experiments in C–F, the cells were loaded with Fluo-3 before treating with 10 $\mu$M digitonin. The detergent was washed away after 5 min. The arrows indicate the additions of 10 $\mu$M THG and 100 $\mu$M of melatonin (MLT). The kinetics of the fluorescence of three typical single cells are presented in each panel.

**Figure 6. Effects of extracellular $\text{Ca}^{2+}$ on the $[\text{Ca}^{2+}]$ in the PV and on parasite development.** P. falciparum was allowed to invade RBC in $\text{Ca}^{2+}$ medium and Fluo-3 acid followed by 30 min incubation either in medium containing 1 mM CaCl$_2$ or in $[\text{Ca}^{2+}]$-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 $[\text{Ca}^{2+}]$ medium for 90 min in complete absence of its intracellular $\text{Ca}^{2+}$ stores (Fig. 5 C). On the contrary, if the cells were permeabilized with digitonin (see previous paragraph), but incubated in a medium containing 100 $\mu$M 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).

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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 observed 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 Ins\(P_3\) (Hotta et al., 2000). Ins\(P_3\), 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 outer 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).
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 FIP-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 infected 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 Mg\(_2\)SO\(_4\), 5.5 n-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 \(\mu\)M 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 (Lambros and Vanderberg, 1979). The infected RBCs were washed twice with RPMI 1640 medium (GIBCO BRL) supplemented with 10% cell 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 \(\mu\)M). 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 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% cell serum (*P. chabaudi*) or 10% human serum (*P. falciparum*), and finally resuspended in the medium described above.

**Ca\(^{2+}\) measurements**

Single-cell confocal microscopy (model LSM-510; Carl Zeiss Microlmaging, 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 excitation and emission wavelengths for Mag-Fura-2 were 345/380 nm and 510 nm, respectively. A Kd of 53 \(\mu\)M for the Mag-Fura-2 Ca\(^{2+}\) complex was assumed (Höfer et al., 1998).

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).

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