Antimalarial drugs disrupt ion homeostasis in malarial parasites

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Plasmodium chabaudi *malaria parasite organelles are major elements for ion homeostasis and cellular signaling and also target for antimalarial drugs. By using confocal imaging of intraerythrocytic parasites we demonstrated that the dye acridine orange (AO) is accumulated into* P. chabaudi *subcellular compartments. The AO could be released from the parasite organelles by collapsing the pH gradient with the K^+/H^+ ionophore nigericin (20 μM), or by inhibiting the H^+-pump with bafilomycin (4 μM). Similarly, in isolated parasites loaded with calcium indicator Fluo 3-AM, bafilomycin caused calcium mobilization of the acidic calcium pool that could also be release with nigericin. Interestingly after complete release of the acidic compartments, addition of thapsigargin at 10 μM was still effective in releasing parasite intracellular calcium stores in parasites at trophozoite stage. The addition of antimalarial drugs chloroquine and artemisinin resulted in AO release from acidic compartments and also affected maintenance of calcium in ER store by using different drug concentrations.*

Key words: malaria - *Plasmodium chabaudi* - calcium - chloroquine - artemisinin - acidic compartment

During the course of its intraerythocytic life, *Plasmodium* causes well documented biochemical and morphological changes in both pathogen and host cell (Howard 1982, Coppel 1986, Garcia et al. 1997, Kirk 2001). Acidic compartments are organelles present in *Plasmodium* and several trypanosomatids (Docampo & Moreno 2001). For *Plasmodium*, it has been suggested that these organelles are the food vacuole in which hemoglobin is digested and antimalarial drugs such as chloroquine are accumulated (Yayon et al. 1984). Chloroquine is though to act by preventing the monomeric hemin from forming the inert and insoluble polymer hemozoin which is derived from digestion of hemoglobin in the acidic vacuole (Slater & Ceram 1992). Defining the sites of chloroquine action in *Plasmodium*, as well as determining how it is related to proton activity in the acidic compartment is of major importance in the physiology of these parasites.It has been reported that the antimalarial artemisinin act on *PfATP6* (Ca^{2+} ATPase) in *P. falciparum*, thus affecting *Plasmodium* Ca^{2+} homeostasis (Uhlemann et al. 2005).

Regulation of internal pH is important to parasite survival as this sets the environment for intracellular enzymes to function. It has been reported that *P. falciparum* and *P. chabaudi* extrude H^+ to the extracellular medium by a Na^+ independent mechanism (Saliba &

Kirk 1999). Other mechanisms for pH regulation described in malaria parasites may involve the $V-H^+-ATPase$ and the $V-H^+-PPase$ in acidocalcisomes (Marchesini et al. 2000, McIntosh & Vaidya 2002, Saliba et al. 2003, Biagini et al. 2003). We have reported the existence of Ca^{2+} pools in the acidic compartment of rodent as well as human malaria parasites as a possible mechanism for the regulation of calcium concentration in these parasites (Garcia et al. 1998, Passos & Garcia 1998). Calcium homeostasis in malaria parasites has been extensively studied by several authors (Tanabe et al. 1982, Krungkrai & Yuthavong 1983, Scheibel et al. 1987, Wasserman et al. 1990, Adovelande et al. 1993, Garcia et al. 1997, 1998, Gazarini et al. 2003, Gazarini & Garcia 2004, Beraldo et al, 2005, Budu et al. 2007). Interestingly, in *P. chabaudi*, the second messenger IP_3, mobilizes Ca^{2+} not only from the classical ER-like pool but also from an acidic compartment, implying that these parasites utilize a calcium-mediated cell signaling mechanism similar to higher organisms (Passos & Garcia 1998). Nevertheless, we still have no clear picture of signaling events and the role of the acidic compartment in malaria parasite signaling, or its significance in the therapeutic actions of antimalarial drugs such as chloroquine (Yayon et al. 1984, Dzekunov et al. 2000). Ca^{2+} and pH changes are crucial for parasite enzyme activity such as proteases (Na et al. 2004, Farias et al. 2005).

In this context, understanding the mechanisms utilized by *Plasmodium* for the maintenance of its ion homeostasis is crucial. In the present work, we have studied the physiological mechanisms by which H^+ and Ca^{2+} activity are related in the acidic compartments of *P. chabaudi*, and how these are affected by the antimalarials chloroquine and artemisinin. Unraveling the mechanisms by which these organelles contribute to cell function may be important for developing alternative strategies for therapeutic approaches to malaria.

MATERIALS AND METHODS

P. chabaudi parasites - Synchronous parasitemia by *P. chabaudi* (clone AJ) was maintained in female mice (Balb/c) by transfer of infection. Parasitemia was about 50%. After collecting the blood, cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4 and 1 mM $CaCl_2$) and leucocytes and platelets were removed by a powdered cellulose column. Parasitemia was determined from Giemsa-stained thin films.

Parasite isolation - The infected RBCs were then washed twice by centrifugation at 1500 g for 7 min in PBS. Infected RBC (10^7 ml^{-1}) was lysed in PBS with 60 µg.ml^{-1} saponin. RBC membranes were removed by centrifugation (9000 g for 10 min at 4°C), the parasites were washed twice in MOPS buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 50 mM MOPS, and 2 mM $CaCl_2$, pH 7.2) and dispensed in the same buffer at room temperature (Passos & Garcia 1998).

Confocal microscopy - In vitro parasite-infected RBCs (10^6 cells ml^{-1}) were loaded with acridine orange (AO) (5 µM) for 5 min at room temperature and washed once with MOPS buffer as described above. Cells were resuspended in the same buffer and placed in a microscopy chamber. The data acquisition was performed with Zeiss confocal microscope (LSM 510) using the LSM 510 software, version 2.5; excitation 488 nm (Argon laser) and emission collected with a bandpass filter of 505-550 nm (Fluo3-AM). Calcium measurements were performed with isolated parasites incubated with Fluo-3 AM (5 µM) for 50 min at 37°C and placed in microscopy chambers at room temperature. The configurations for image acquisition were the EX 488 nm and Em > 560 nm for AO measurements. Software-based analysis allowed fluorescence imaging in the whole field of view or in a selected cell as a function of time. This was accomplished by defining areas of interest on a given image frame and requesting the software to construct a graphical representation of intensity against time.

Experiments were carried out with at least three different cell preparations. Traces represent typical single cell responses unless indicated otherwise.

Spectrofluorimetric measurement - Isolated parasites (10^7 cells ml^{-1}) were incubated with MOPS buffer in 2 ml cuvette. The measurements were performed in a Shimadzu RF-5301 PC spectrofluorimeter at 37°C and dyes fluorescence (Fluo-3 AM and AO) was measured continuously after the drug addition. Parasites were incubated with calcium indicator Fluo-3 AM (5µM) for 50 min at 37°C in MOPS buffer containing 2 mM $CaCl_2$ and probenecid (40 µM) or AO (5 µM) incubated for 5 min at 37°C in the same buffer. The dyes parameters are excitation 490 nm and emission collected at 530 nm for Fluo-3 AM and AO fluorescence measurement was obtained with Ex-495 nm/Em-590 nm.

Extracellular H$^+$ levels - The effect of chloroquine on H$^+$ mobilization in isolated parasites was recorded using a Cytosensor microphysiometer (Mol Devices, US), a silicon-based biosensor system. This instrument continuously monitors the extracellular pH surrounding cells in culture, and reports cell activation by measuring the increases in extracellular acidification rate occurring in response to agonist stimulation (McConnell et al. 1992). The isolated parasites was resuspended in DMEM (GIBCO) plus 30% agarose to improve parasites adherence onto the polycarbonate membrane of the transwell capsules (3 µm pore size) at a density of 2.5×10^5 cells.

RESULTS

We have investigated the dynamics of solute exchange in the acidic compartments of isolated and intact blood-stage malaria parasite, *P. chabaudi*. To study intracellular pH changes, we loaded infected red blood cells at trophozoite stage (*P. chabaudi*) with AO. Dye mobilization through compartments was measured by selecting areas of interest (cytosol and acid compartment) by confocal imaging (Fig. 1). Addition of H$^+$-ATPase inhibitor bafilomycin (4 mM) to the cells led to alkalinization of acidic compartments as indicated by the partial release of accumulated AO (Fig. 1A), and simultaneously increases the cytosol fluorescence (Fig. 1B). The remaining AO could be released by addition of the K$^+$/H$^+$ ionophore nigericin to the cells (20 µM), which totally released the compartmentalized AO fluorescence (Fig. 1C). Dose-dependent addition of bafilomycin promotes the depletion of AO stores. A remarkable (over 90%) release of AO fluorescence occurs with addition of bafilomycin at 10 µM (Fig. 1D).

The effect of the antimalarial chloroquine on AO accumulation in subcellular compartments (Fig. 2A) in infected erythrocyte (Fig. 2B) shows that chloroquine (80 µM) promotes extrusion of AO from the acidic compartments, presumably as a result of alkalinization. The subsequent addition of H$^+$-ATPase inhibitor bafilomycin (4 µM) caused complete depletion of the residual AO from the acidic compartments (Fig. 2C). Taken together, the data of Figs 1 and 2 suggest that *P. chabaudi* parasites display a bafilomycin-sensitive acidic compartment that can be affected in a dose-dependent manner and is also a target for chloroquine.

By using spectrofluorimeter measurements and isolated parasites (10^7 cells) at trophozoite stage previously incubated with AO (5 µM) for 5 min/37°C we showned that AO could be released in a dose-dependent manner by addition of artemisinin and chloroquine (Fig. 3).

We have previously demonstrated that the H$^+$-pump inhibitor 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) caused release of Ca^{2+} in Fluo-3 loaded *P. falciparum* parasites (Garcia et al. 1998). We therefore investigated the effects of the more selective H$^+$-pump inhibitor, bafilomycin on Ca^{2+} homeostasis. We measured the fluorescence changes in isolated parasites (trophozoite stage) loaded with the calcium probe Fluo-3 AM (5 µM) and placed to microscopy microplates for confocal measurements, in response to effectors of the acidic compartments. Using different concentration of bafilomycin, we observed that bafilomycin-sensitive Ca^{2+} pool is completely discharged at 4 µM (Fig. 4A). However subsequent addition of nigericin (20 µM) after bafilomycin treatment (4 µM) reveals a subsequent Ca^{2+} rise (Fig. 4B). As nigericin was able to release the entire Ca^{2+} pool by itself further addition of bafilomycin had no effect
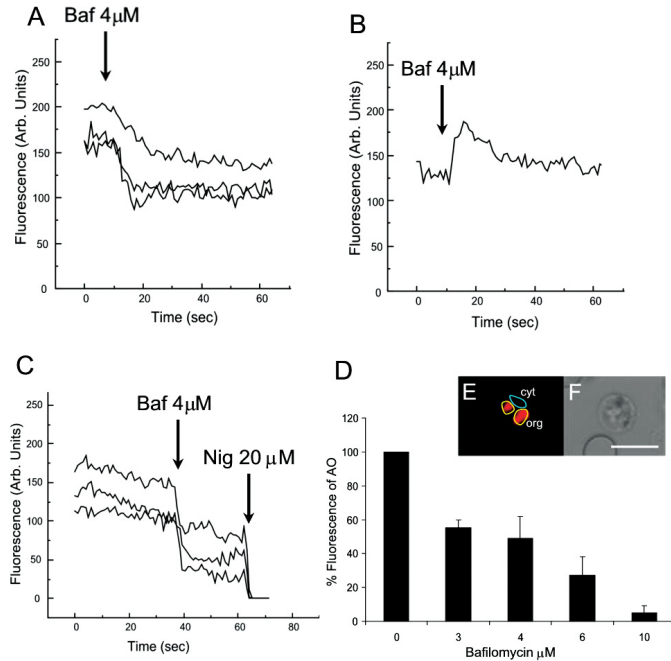
Fig. 1: effect of the H⁺ pump inhibitor bafilomycin on *Plasmodium chabaudi* within the intact RBC. Infected RBC was loaded with AO (5 μM) as described in the Methods Section. A: effect of bafilomycin (Baf) (4 μM) on organelles selected areas stained with AO in single cell; B: cytosolic area with acidification promoted by bafilomycin; C: Bafilomycin and nigericin (Nig) effect on subcellular areas; D: dose-response effect of bafilomycin on AO release from intracellular stores; E: fluorescence imaging with selected areas (cytosol and organelles); F: phase contrast. *(D) bar plot represent % of control fluorescence (arbitrary units) remaining from ten cells in three different trials. Scale bar of 10 μm.

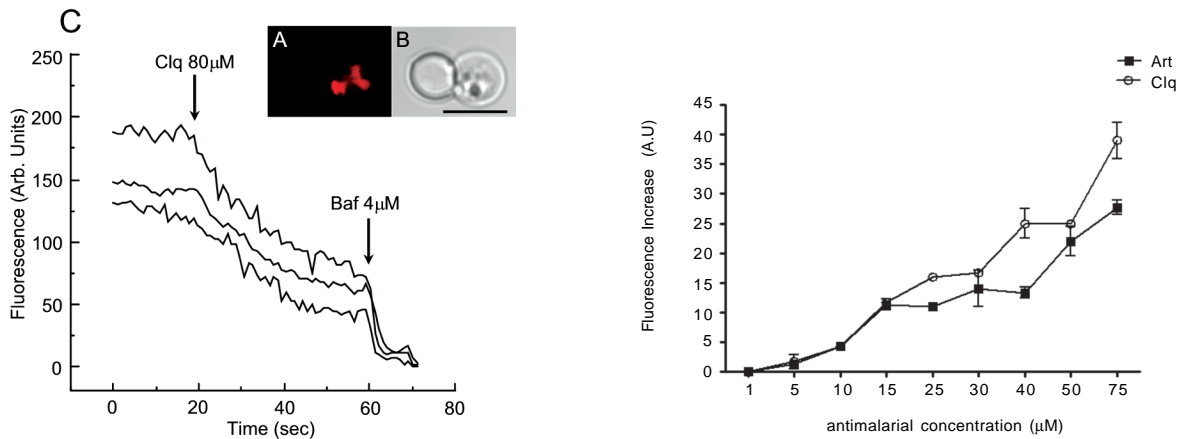

Fig. 2: action of the antimalarial chloroquin and bafilomycin on acidic compartments in *Plasmodium chabaudi*. Infected RBC loaded with 5 μM AO; A: fluorescence image; B: phase contrast; C: changes in AO fluorescence (arbitrary units) in selected subcellular compartments promoted by chloroquine (Clq) (80 μM). Scale bar of 10 μm.

Fig. 3: effect of antimalarial drugs chloroquine and artemisinin on acridine orange mobilization from acidic compartment. Isolated parasites loaded with 5 μM AO for 5 min. Different concentrations of drugs (Art-artemisinin and Clq-chloroquine) were added and fluorescence measurement was performed in spectrofluorimeter cuvette. Fluorescence intensities (arbitrary units - AU) represent at least three different cell preparations.
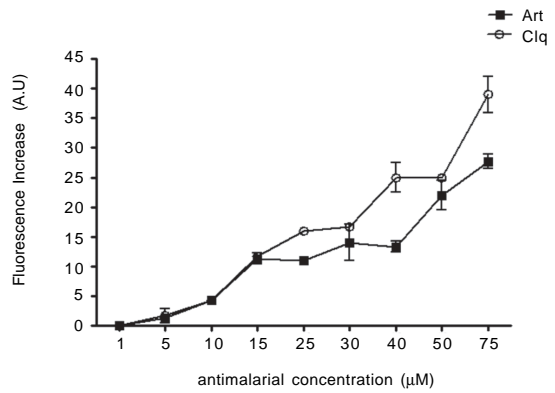
on Ca²⁺ release (Fig. 4C). Interestingly, neither bafilomycin (Fig. 4D) or nigericin (Fig. 4E) was able to deplete ER Ca²⁺ pool, as a subsequent addition of thapsigargin (Thg) promoted a second calcium rise.

To investigate the effect of the antimalarials drugs chloroquine and artemisinin in calcium homeostasis we

carried out experiments with isolated parasites loaded with calcium indicator Fluo-3 AM (5 μM). Fig 5 shows spectrofluorimetric measurements of calcium release by Thg (10 μM) with parasites previously incubated (15 min at 37ºC) with different concentrations of chloroquine and artemisinin (0-80 μM) in a high calcium ex-
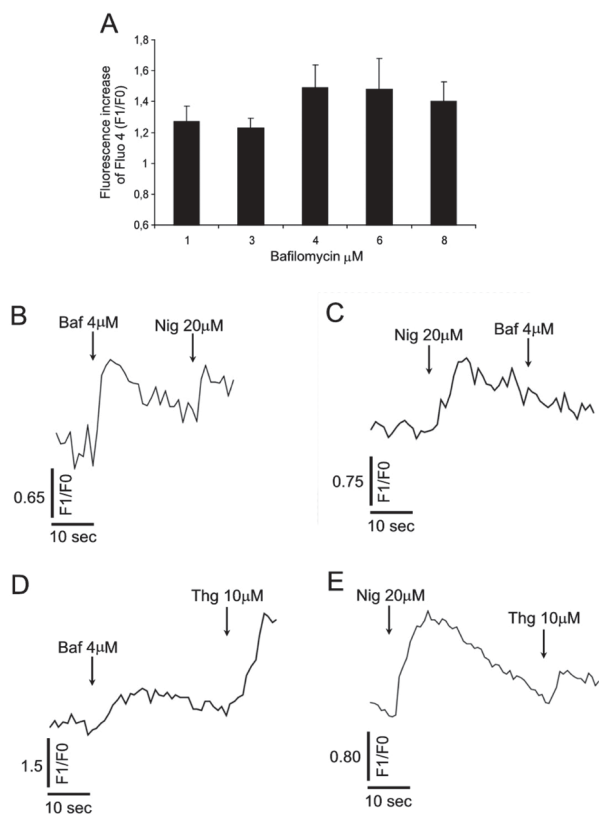
Fig. 4: H$^+$ pump inhibitor-bafilomycin induces Ca^{2+} release in intact *Plasmodium chabaudi* parasites. *P. chabaudi* parasites were loaded with the calcium indicator Fluo-3 AM (5 μM) as described in the Methods Section and cytosolic fluorescence intensities measured in confocal microscopy were normalized as (F1-maximal fluorescence after drug addition/F0-fluorescence before drug addition). A: dose-response effect of bafilomycin (Baf) on cytosolic calcium fluorescence (mean data of fluorescence ratio remaining from ten cells in three different trials); B: addition of bafilomycin (increase of 1.3 ± 0.1; n = 6) and nigericin (Nig) (20 μM); C: addition of nigericin (1.42 ± 0.1; n = 6) and bafilomycin (4 μM); D: addition of bafilomycin (1.5 ± 0.2; n = 6) and thapsigargin (Thg) (10 μM); E: addition of nigericin (1.65 ± 0.3; n = 6) and thapsigargin (10 μM).
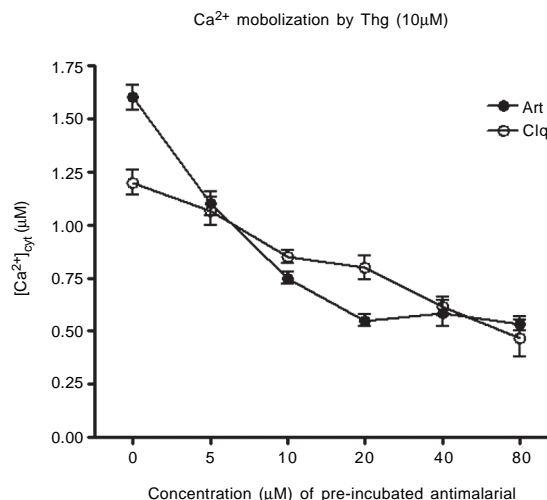

Fig. 5: effect of antimalarials drugs on calcium maintenance on endoplasmic reticulum in *Plasmodium chabaudi*. Isolated parasites (10^7 cells ml^{-1}) were loaded with calcium indicator Fluo-3 AM (5 μM) for 50 min at 37ºC and further incubation with different concentrations of antimalarials chloroquine (Clq) and artemisinin (Art) for 15 min. The resting calcium present in ER was measured in spectrofluorimeter cuvette with addition (10 μM) of Ca^{2+}ATPase inhibitor thapsigargin (Thg) (n = 3).
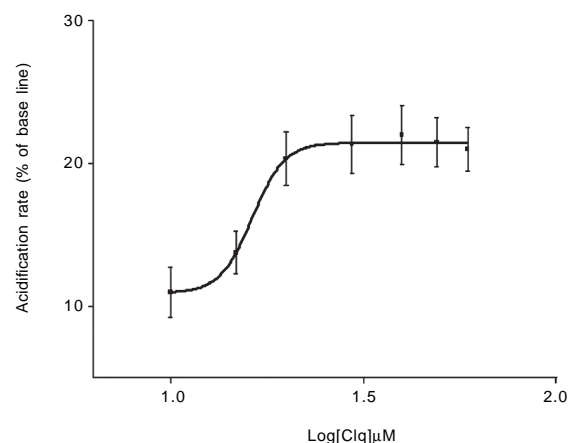

Fig. 6 : concentration-response curve for chloroquine-induced acidification rate in *Plasmodium chabaudi*. Exposure to chloroquine for 7 min with concentrations between 10 μM and 60 μM. Concentration-response curve was calculated as the difference from baseline and the peak. EC$_{50}$ calculated from this curve is 16.29 mM (n = 3).

tracellular medium (2 mM). The results show that previous parasite incubation with chloroquine or artemisinin leads to a dose-dependent reduction of total amount of calcium release by thapsigargin.

When parasites are exposed to chloroquine, the cytosolic pH decrease as a result of extrusion of H$^+$ from acidic compartments and the parasites (*P. falciparum*) try to restore the cytosolic pH level using plasma membrane mechanisms (Saliba & Kirk 1999, Marchesini et al. 2000, Saliba et al. 2003). By using microphysiometry we showed that addition of chloroquine to *P. chabaudi* at the trophozoite stage led to a dose-dependent increase in the extracellular acidification rate (Fig. 6). The mean 50% maximal effective concentration (EC$_{50}$) for three experiments was 16.29 ± 0.4 μM (n = 3) of chloroquine. This data reveal an activation of plasma membrane mechanism of H$^+$ extrusion under chloroquine treatment.

DISCUSSION

The antimalarial drug chloroquine is thought to accumulate in acidic compartments, although its mechanisms of action are controversial (Slater & Cerami 1992, Dorn et al. 1995, Waller et al. 2003). The resistance to chloroquine observed in *P. falciparum* is dependent to mutations in specific transporter (*PfCRT*), by using heterologous expression Reeves et al. (2006) observed a increase of lysosomes acidification in mammalian cells expressing *PfCRT*, providing new information about acidification process and chloroquine resistance.

We have previously shown that the acidic pool also functions as a Ca^{2+} store in permeabilized malaria parasites and that chloroquine causes Ca^{2+} release from this store (Passos & Garcia 1998). In the present study, intact parasites were labeled with the calcium indicator, Fluo-3 AM to measure parasite cytosolic Ca^{2+} mobilization, and we also investigated the acidic pools in intact parasites within the RBC, using confocal microscopy.

The data in the present work indicate the existence of mechanisms for H^+ homeostasis in *P. chabaudi* parasites. The acidic vacuole in malaria parasite is the site of hemoglobin digestion and it is believed of drug action. In the rodent malaria parasite *P. chabaudi* the second messenger IP_3 is known to induced-Ca^{2+} mobilization from this compartment (Passos & Garcia 1998). These findings are of major significance, because they raise the possibility that chloroquine and artemisinin action in malaria parasites may involve alterations in ion homeostasis. This is especially important in view of our work showing that IP_3-dependent Ca^{2+} signaling is involved in the progression of the malarial parasite cell cycle (Hotta et al. 2000, Gazarini et al. 2003, Beraldo et al. 2005).

By using confocal microscopy and analyzing *P. chabaudi* loaded with AO within the intact RBC, we showed a H^+ mobilization, corresponding to a fluorescence decrease when V-type H^+-ATPase was inhibited by bafilomycin (Fig. 1). Simultaneously, parasite cytosol fluorescence was transiently increased as the AO was released, thus indicating that cellular mechanisms were operating to restore cytosolic pH (Fig. 1B). Bafilomycin completely discharge the AO pool at 10 µM (Fig. 1D) while Ca^{2+} release reach the maximal values at addition of 4 µM of H^+ pump inhibitor (Fig. 4A).

The interaction of antimalarial artemisinin in *P. falciparum* was reported to occur through inhibition of *PfATP6*, a SERCA-type ATPase (Uhlemann et al. 2005). Our data shows the ability of both antimalarial in modifying the calcium and proton dynamics in *P. chabaudi* internal stores (Fig. 5).

We also addressed the metabolic response evoked by chloroquine with cytosensor microphysiometer (Fig. 6). These results showed that chloroquine stimulated a H^+ extrusion response with an EC_{50} of 16 µM in *P. chabaudi*, using plasma membrane mechanisms to recover the intracellular pH (Saliba & Kirk 1999).

Our data provide new information of acidic organelle physiology, the high concentration of bafilomycin required for complete reversion fluorescence from AO, suggests a low density of H^+ pumps or susceptibility to the drug. However chloroquine action promoted a more extensively effect on parasite ion homeostasis than the specific H^+ pumps inhibitor bafilomycin. The micromolar concentration of antimalarial drugs required in our assay may represent the cell physiology differences between the *Plasmodium* species and we are search for extension of organelles capacity in ion maintenance.

The fact that Ca^{2+} antagonists block malaria parasite development is well known. In addition, reversal of chloroquine resistance in vitro by several Ca^{2+} antagonists such as verapamil has also been extensively reported (Deloron et al. 1991, Adovelande et al. 1998).

We have demonstrated that host melatonin activates parasite calcium signaling via the second messenger IP_3, which appears to be important in the proliferation and maturation of the intraerythrocytic malaria parasites (Hotta et al. 2000). More recently, we also verify calcium mobilization with AFMK, a product of melatonin degradation in *Plasmodium*-infected erythrocytes (Budu et al. 2006). Finally, although the molecular mechanism of chloroquine and artemisinin action is not fully understood, its affect on calcium and proton activity indicates the importance in elucidate calcium homeostasis and signaling mechanisms on malarial parasites.

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