

Malaria parasites solve the problem of a low calcium environment

Patricia Camacho

Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229

The parasite responsible for malaria, *Plasmodium falciparum*, spends much of its life in the RBC under conditions of low cytosolic Ca^{2+} . This poses an interesting problem for a parasite that depends on a Ca^{2+} signaling system to carry out its vital functions. This long standing puzzle has now been resolved by a clever series of experiments performed by Gazarini et al. (2003). Using advances in fluorescent Ca^{2+} imaging (Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. *J. Biol. Chem.* 260:3440–3450; Hofer, A., and T. Machen. 1994. *Am. J. Physiol.* 267:G442–G451; Hofer, A.M., B. Landolfi, L. Debellis, T. Pozzan, and S. Curci. 1998. *EMBO J.* 17:1986–1995), these authors have elucidated the source of the Ca^{2+} gradient that allows the accumulation of intracellular Ca^{2+} within the parasite.

Malaria is a prevalent disease in tropical and subtropical countries affecting 300–500 million people a year (Hoffman et al., 2002). It is estimated that one to three million deaths occur worldwide, mostly involving children under the age of five. Even in the United States, more than a thousand people are infected annually (Jerrard et al., 2002). Malaria transmission requires a vector, the *Anopheles* mosquito. When a mosquito bites an infected victim, the parasite is taken up into the insect gut where it undergoes a series of developmental transformations until it forms a sporozoite. The sporozoite moves to the mosquito salivary gland where it stays until it is injected into its next host when the mosquito bites (Ghosh et al., 2000). The sporozoites enter the host via small blood vessels and, in a somewhat obscure process, come to reside in the liver (Preiser et al., 2000; Mota and Rodriguez, 2001). Here, the parasite penetrates cells and develops into a spherical, multinucleated form called the schizont. A mature schizont contains 2,000–40,000 uninucleated parasites called merozoites that are released into the bloodstream when the schizont ruptures. The merozoites then enter RBCs where they reproduce until the cell lyses,

releasing the parasite into the bloodstream to infect new RBCs. Multiple rounds of infection are responsible for the cyclical nature of fever attacks that are characteristic of the disease. Although much is known about malaria, including the developmental stages of the parasite in the vector and host, the mode of transmission, and the demographics of affected populations, we have yet to eradicate the disease. Consequently, malaria still seriously undermines the economic development of many developing countries (Sachs and Malaney, 2002).

Plasmodium falciparum is known to require Ca^{2+} for the regulation of its cell cycle and for its long term survival. Eukaryotic cells normally need an extracellular Ca^{2+} concentration that is close to 1 mM in order to maintain the intracellular Ca^{2+} stores necessary for Ca^{2+} signaling. Surprisingly, the *Plasmodium* parasite survives in the cytosolic environment of the RBC, considered to be on the order of 100 nM Ca^{2+} (Alleva and Kirk, 2001). The question then is how does this parasite establish a Ca^{2+} signaling system in such an environment? In this issue, the work of Gazarini et al. (2003) provides the solution to this long standing puzzle.

Invasion of RBCs by malaria parasites occurs via membrane invagination and results in the formation of a parasitophorous vacuole (PV)* that surrounds the parasite and shields it from the host cytosol. The PV membrane (PVM) includes components from both the RBC membrane and the parasite (Preiser et al., 2000). Importantly, the plasma membrane of the RBC contains a Ca^{2+} ATPase responsible for pumping Ca^{2+} across the plasma membrane toward the extracellular space. Gazarini et al. (2003) propose that after membrane invagination this pump now faces the parasite and transports Ca^{2+} from the host cytosol across the PVM and into the PV. This system would allow a Ca^{2+} gradient to form and produce an environment that makes Ca^{2+} signaling within the malaria parasite possible. Therein resides the key piece to solving the Ca^{2+} puzzle.

To test this model, Gazarini et al. (2003) made clever use of fluorescent Ca^{2+} indicators with diverse spectral and diffusional properties. They first demonstrated that specific dyes could be targeted to different compartments such as the PV, the host cell cytoplasm, and the parasite cytoplasm, allowing the selective measurement of Ca^{2+} in these environments. Schizonts were allowed to invade RBCs in the presence of the Ca^{2+} indicator Fluo-3 that is cell impermeant in its

Address correspondence to Patricia Camacho, Dept. of Physiology, Mail code 7756, UTHSCSA, 7703 Floyd Curl Dr., San Antonio, TX 78229-3900. Tel.: (210) 567-6558. Fax: (210) 567-4410. E-mail: camacho@uthscsa.edu

*Abbreviations used in this paper: PV, parasitophorous vacuole; PVM, PV membrane; THG, thapsigargin.

free acid form. If the PVM is also impermeable to the dye, it should be trapped in the PV during parasite invasion; however, if the PVM is permeable to this indicator, it should be found at similar concentrations in the PV and the cell cytoplasm. Indeed, the authors observe a ring of fluorescence around the parasite and no fluorescence in the RBC cytosol or inside the parasite. This result demonstrated that a Ca^{2+} indicator could be trapped in the PV and exclusively report the Ca^{2+} concentration in this microenvironment.

Fluo-3 fluoresces poorly under low Ca^{2+} conditions; hence, the fact that it emits fluorescence at all in the PV suggests that this space has a relatively high Ca^{2+} concentration. To test if this was the case, Gazarini et al. (2003) treated cells with ionomycin, a Ca^{2+} ionophore that allows the passage of Ca^{2+} ions across membranes. The rationale for the experiment was that if Ca^{2+} was high in the PV space, ionomycin would release Ca^{2+} into the RBC cytoplasm and into the parasite cytoplasm, thereby causing a decrease in Fluo-3 fluorescence in the PV space. This is exactly what the authors observed. In a variation on this experiment, the authors loaded the parasite cytoplasm with Fluo-3/AM, a cell permeant form of the Ca^{2+} indicator, and then treated infected RBCs with ionomycin. Under these conditions, ionomycin caused a large fluorescence increase in the parasite cytoplasm due to the release of Ca^{2+} from internal stores but also due to the movement of Ca^{2+} down the concentration gradient from the PV into the parasite cytoplasm.

In another experiment designed to allow the simultaneous measurement of Ca^{2+} in the parasite cytosol and in the PV, the authors labeled the *Plasmodium* cytoplasm with Fluo-3/AM and used a second indicator, Mag-fura-2, to label the PV. The spectral properties of the two dyes are sufficiently different that one can easily distinguish the signals emanating from either the PV or the parasite cytoplasm. The cells were then exposed to thapsigargin (THG), an irreversible sarcoplasmic reticulum Ca^{2+} ATPase inhibitor, to release Ca^{2+} from the internal stores of the parasite. This resulted in an increase in both the Fluo-3 signal and after a brief lag the Mag-fura-2 signal. Thus, it appears that THG released Ca^{2+} into the parasite cytoplasm and then Ca^{2+} was actively pumped across the plasma membrane to the PV space by Ca^{2+} ATPases of the *Plasmodium* plasma membrane.

Gazarini et al. (2003) continued to demonstrate that the parasite exists in a high Ca^{2+} environment with the following series of experiments. Infected RBCs were preloaded with Fluo-3/AM to label the parasite and then incubated in medium containing various Ca^{2+} concentrations for 90 min. Cells incubated at the physiological Ca^{2+} concentration of 1 mM were then treated with THG to evaluate the state of the parasite's intracellular Ca^{2+} stores. THG treatment was accompanied by a significant increase in Fluo-3 fluorescence, demonstrating that intracellular Ca^{2+} stores are filled under these conditions. This experiment was also performed in the presence of digitonin, which permeabilizes the RBC plasma membrane and the PVM but not the plasma membrane of the parasite. Under these conditions, the concentration of Ca^{2+} to which the parasites are exposed is determined by the extracellular medium of the RBC. The authors showed that THG released Ca^{2+} from the parasitic stores only when the concentration of Ca^{2+} in the extracellular medium was

>100 μM . These experiments were repeated using the physiological agonist melatonin to replace THG. In *Plasmodium* parasites, the hormone melatonin stimulates IP_3 -mediated Ca^{2+} release from both the ER stores and from the acidic Ca^{2+} storage compartment (Garcia, 1999; Hotta et al., 2000). Gazarini et al. (2003) loaded the parasite cytosol with Fluo-3/AM and incubated the RBCs as described above. Application of melatonin caused a transient release of Ca^{2+} from intraparasitic Ca^{2+} stores when intact cells were incubated at physiological Ca^{2+} . If the cells were permeabilized with digitonin, Ca^{2+} was released only when the extracellular Ca^{2+} was buffered above 100 μM . These experiments firmly established the requirement for and presence of a reservoir of Ca^{2+} in the PV space.

Gazarini et al. (2003) go on to calibrate the Ca^{2+} signal in the PV, which obviously becomes one of the more critical experiments reported in the article. These experiments are difficult to do on a single cell level and hence were done using populations of infected RBCs. The cells were placed in a medium containing Mag-fura-2, which was trapped in the PV during parasite invasion. The cells were washed and resuspended in a medium devoid of Ca^{2+} before lysis. Using a spectrophotometer to measure Mag-fura-2 fluorescence, the Ca^{2+} concentration in the PV was estimated following standard procedures assuming a Kd of 53 μM for Mag-fura-2 (Hofer et al., 1998; Hotta et al., 2000). Remarkably, the authors found that the Ca^{2+} concentration in the PV of the parasite was of the order of 40 μM . The implication of this experiment is far reaching. The PV Ca^{2+} concentration is 100–1,000-fold higher than the RBC cytoplasm and, as evidenced from the digitonin experiments, is sufficiently high to fill the parasite intracellular stores and create a functional Ca^{2+} signaling system.

Finally, the authors addressed the very important question of whether the relatively high Ca^{2+} concentration in the PV space was necessary for normal development of the parasite. Interestingly, even a transient drop in the Ca^{2+} concentration in the PV was enough to affect maturation of the parasites and to cause a major reduction in the number of parasites capable of reinvasion (i.e., the schizont). This novel finding underscores the importance of the Ca^{2+} signaling system in the control of parasite development.

In summary, Gazarini et al. (2003) have presented the remarkable finding that the malarial PV provides a sufficiently high Ca^{2+} environment to maintain a functional Ca^{2+} signaling system within the parasite. This allows the parasite to survive, and in fact thrive, in the low Ca^{2+} surroundings of the RBC cytoplasm. In addition, the authors have provided an elegant explanation for how the gradient of Ca^{2+} across the PVM is formed.

They suggest that the RBC plasma membrane Ca^{2+} ATPase is transferred to the PVM during parasite invasion and that it then proceeds to pump cytosolic Ca^{2+} into the PV. This raises the possibility that the RBC Ca^{2+} ATPase could be a potential site of intervention in the control of malaria. Obviously, this would require that the plasma membrane Ca^{2+} ATPase is, or can be, somehow modified in the PVM, allowing it to be specifically targeted. Hopefully, future pharmacological or genetic engineering strategies can be developed to address this point.

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