BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 155–160 (1998)
ARTICLE NO. RC988338

Inositol 1,4,5-Trisphosphate Induced Ca^{2+} Release from Chloroquine-Sensitive and -Insensitive Intracellular Stores in the Intraerythrocytic Stage of the Malaria Parasite *P. chabaudi*

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Received February 5, 1998

Isolated *P. chabaudi* parasites were permeabilized with digitonin and the function of intracellular Ca^{2+} stores was studied using the Ca^{2+} indicators arsenazo III or Fluo 3-acid in the medium. Addition of the second messenger $InsP_3$ (5 μM) to permeabilized parasites leads to Ca^{2+} release into the medium, with the mean extent of release being 40 nmol $Ca^{2+}/10^8$ cells. This Ca^{2+} release was completely abolished in the presence of heparin, an $InsP_3$ receptor antagonist. The amount of Ca^{2+} released was approximately 50% reduced when $InsP_3$ was added subsequent to the discharge of the endoplasmic reticulum (ER) Ca^{2+} pool with the SERCA (sarcoplasmic ER Ca^{2+} ATPase) inhibitors thapsigargin and tBHQ (2,5-di(ter-butyl)-1,4 benzohydroquinone). The thapsigargin- and tBHQ-sensitive pool account for 20 nmol of $Ca^{2+}/10^8$ cells. If $InsP_3$ was added after the discharge of the residual Ca^{2+} by addition of either the K^+/H^+ uncoupler nigericin or the antimalarial drug chloroquine, no further Ca^{2+} release was observed. This is the first report of $InsP_3$-induced Ca^{2+} release in a parasite protozoa. In addition our finding that chloroquine depletes an $InsP_3$-sensitive Ca^{2+} compartment, raises the possibility that the $InsP_3$-dependent Ca^{2+} release from this store might be important for the regulation of growth and differentiation of the parasite.

Key Words: **malaria; *Plasmodium chabaudi*; Ca^{2+} homeostasis; endoplasmic reticulum; thapsigargin; 1,4,5-inositol trisphosphate.**

Understanding the signaling pathways in malaria parasites is an important step toward the elucidation of their complex biology. After the hepatocyte cycle, the asexual stage takes place within red blood cells (RBC) through defined stages known as ring, throphozoite and schizogony up to the time of rupture of the host cell when merozoites are free to invade new RBC. The maturation process of Plasmodium is marked by several physiological and structural features at both pathogen and host cell (1–5). Extensive investigation of signal transduction in malaria parasites at the asexual stage has provided information at the molecular level implicating the possible involvement of Ca^{2+}. Genes with moderate homology to SERCA have been reported in *P. falciparum* (6) and *P. yoelli* (7); as well as the genes encoding: the Ca^{2+} binding protein calmodulin (8) and a Ca^{2+} dependent protein kinase with an E-F hand motif (9). Ca^{2+} homeostasis has also been investigated in Plasmodium-infected RBC at the biochemical level (10–16). Therefore, it is likely that Ca^{2+} mobilization and Ca^{2+} dependent regulation of biological mechanisms underlying the maturation process could occur in these parasites.

Ca^{2+} mobilization from intracellular pools is a ubiquitous mechanism of eukaryotic cells leading to multiple processes such as differentiation, cell division, secretion and cytoskeleton rearrangements (17). Phosphoinositide hydrolysis plays an important role in signal transduction pathways by generating the intracellular second messengers $InsP_3$ and dyacylglicerol (DAG). $InsP_3$, in turn, modulates the opening of a Ca^{2+} channel in the endoplasmic reticulum, thus releasing Ca^{2+} into the cytosol, while DAG activates kinase C.

By measuring intracellular Ca^{2+} levels with the fluorescent probe Fura-2 we recently reported that cytosolic Ca^{2+} homeostasis is maintained at a level similar to mammalian cells in the rodent malaria parasite *P. chabaudi* as well as in the human malaria parasite *P. falciparum* (18). We also showed that Ca^{2+} can be stored in ER and acidic pools in these parasites (19, 20). Determining the role and interaction of these different Ca^{2+} pools in the regulation of intracellular Ca^{2+} is crucial to understand the signal transduction pathways in these parasites. In this respect, we have now investigated the ability of the second messenger $InsP_3$

0006-291X/98 $25.00

to mobilize Ca^{2+} in permeabilized *P. chabaudi* parasites. Specifically, in the present study we show that $InsP_3$ mobilizes Ca^{2+} in the intraerythrocytic stage of the malaria parasite *P. chabaudi*. Our results demonstrate that the $InsP_3$-releasable Ca^{2+} is accumulated within permeabilized parasites in two distinguishable pools, i.e., a Ca^{2+} pool that is released by the SERCA pump inhibitors thapsigargin and tBHQ and a nigericin-and chloroquine-sensitive pool.

MATERIALS AND METHODS

The experiments described here were carried out using synchronous parasitemia in *Plasmodium chabaudi* maintained in female mice (Balb C strain) by weekly transfer infection. Leukocytes and platelets were removed from whole blood by filtration through a powdered cellulose column (Whatman CF11) (21). The infected erythrocytes were then washed three times by centrifugation at 1500 g for 5 min in PBS.

To isolate the parasites, infected red cells were lysed with 10 □g ml^{-1} saponin. After pelleting to remove red cell membrane material, the parasites were washed twice in PBS by centrifugation at room temperature at 2000 × g for 5 min and resuspended at 10^8 parasites/ml in PBS.

Ca^{2+} flux measurements with arsenazo III were carried out spectrophotometrically as described by Scarpa (22). The parasites were resuspended in buffer A containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes, 1 mM MgCl2, 1 mM ATP, 2.5 mM potassium phosphate, 3.5 μM $CaCl_2$, pH 7.2, in the presence of 40 μM arsenazo III. A total volume of 1 ml parasites (10^8 cells) were permeabilized by the addition of 5 μM digitonin. The mean values shown are the average of 4 experiments. The spectrophotometric measurements with arsenazo III were made using a SLM AMINCO DW 2000 dual wavelength spectrophotometer at 675-685 nm wavelenght pair. The experiments were carried out at 37°C.

Ca^{2+} flux measurements with Fluo-3, free acid, were performed using a Hitachi spectrofluorimeter model F4500 (Tokyo, Japan) with excitation at 505 nm and emission 530 nm. For these experiments we use permeabilized *P. chabaudi* parasites in buffer A containing 1 μM fluo-3 instead of arsenazo III. The experiments were performed at 37°C.

RESULTS

$InsP_3$-Sensitive Ca^{2+} Pools

In order to characterize the nature of the Ca^{2+} storage compartments of *P. chabaudi* parasites, cells isolated from infected red blood cells were resuspended in a Ca^{2+} medium and their plasma membrane permeabilized with the cholesterol preferring detergent digitonin. Fig. 1 A shows Ca^{2+} uptake and release by permeabilized *P. chabaudi* parasites at the trophozoite stage measured with the Ca^{2+} indicator arsenazo III. Active Ca^{2+} uptake by the parasites starts after permeabilization of the cells with digitonin. The uptake of Ca^{2+} can not be attributed to mitochondria, given that the experiments presented in Fig 1 were carried out in the presence of the mitochondrial poisons antimycyn A and oligomycin, thus assuring the inhibition of H^+ ATPase and the electron flow through the respiratory chain, respectively. Addition of 5 μM $InsP_3$ elicited Ca^{2+} release into the medium of the order of 40 nmol Ca^{2+}/10^8 cells. This result demonstrate that also in malaria parasite there are intracellular membrane enclosed compartments capable of accumulating and releasing Ca^{2+} in response to the second messenger $InsP_3$.

In order to characterize the organelle involved in the $InsP_3$-induced Ca^{2+} release in these parasites, we tested the ability of this second messenger to elicit Ca^{2+} release after specifically emptying the ER Ca^{2+} pool. Fig 1 B shows the effect of $InsP_3$ addition after the ER pool was depleted by adding 5 μM thapsigargin, a Ca^{2+} ATPase inhibitor (23). The amount of Ca^{2+} released by thapsigargin was 20 nmoles of $Ca^{2+}/10^8$ cells, a value consistent with the one that we recently reported in *P. chabaudi* parasites (19). Subsequently, the addition of $InsP_3$ elicited the release of 20 nmol of $Ca^{2+}/10^8$ cells into the medium.

Another SERCA inhibitor, tBHQ was tested at 10 μM final concentration (Fig 1C). The amount of Ca^{2+} released by tBHQ corresponded to 20 nmol of Ca^{2+}/10^8 cells, which is compatible with the amount of Ca^{2+} released by thapsigargin, thus corresponding to the ER $InsP_3$-sensitive pool. Subsequent addition of 5 μM of $InsP_3$ resulted in a Ca^{2+} release equivalent to 20 nmol of $Ca^{2+}/10^8$ cells. These results indicated that in *P. chabaudi,* there is a non mitochondrial, $InsP_3$ sensitive, Ca^{2+} storage compartment that is however insensitive to SERCA inhibitors.

To further test the specificity of the $InsP_3$ response, we added heparin, an $InsP_3$receptor blocker (24) to permeabilized parasites under the same experimental conditions as used in the previous experiment. Fig. 2 shows that $InsP_3$, did not promote Ca^{2+} release from these cells in the presence of heparin, thus confirming that an $InsP_3$ receptor is involved in the Ca^{2+} release observed in our experiments.

An indicator with higher sensitivity to Ca^{2+} as well as less sensitivity to pH changes than arsenazo III, the fluorescent probe, fluo-3, was also used to investigate the origin of the $InsP_3$-induced Ca^{2+} release. It should be stressed that the amount of Ca^{2+} released after inhibition of the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA) was on average about 50% of that triggered by $InsP_3$ (Fig. 1 A and B). This suggests that the parasites possess another $InsP_3$ sensitive Ca^{2+} pool. The possibility that this is identified with the acidic Ca^{2+} store recently described in *P. chabaudi* (20) was investigated in Fig. 3. Fig. 3 A shows indeed that after selectively emptying the acidic Ca^{2+} pool with the K^+/H^+ ionophore nigericin (1.2 μM) and the ER Ca^{2+} pool with thapsigargin (0.8 μM) the addition of $InsP_3$ did not elicit Ca^{2+} release. The data obtained with fluo-3 correspond quantitatively and qualitatively to those obtained with arsenazo III. In fact discharge of the ER pool with thapsigargin released 19.7±3.8 nmol of Ca^{2+}/10^8 cells (n=8), but there was still a substantial residual Ca^{2+} release with subsequent challenge with $InsP_3$

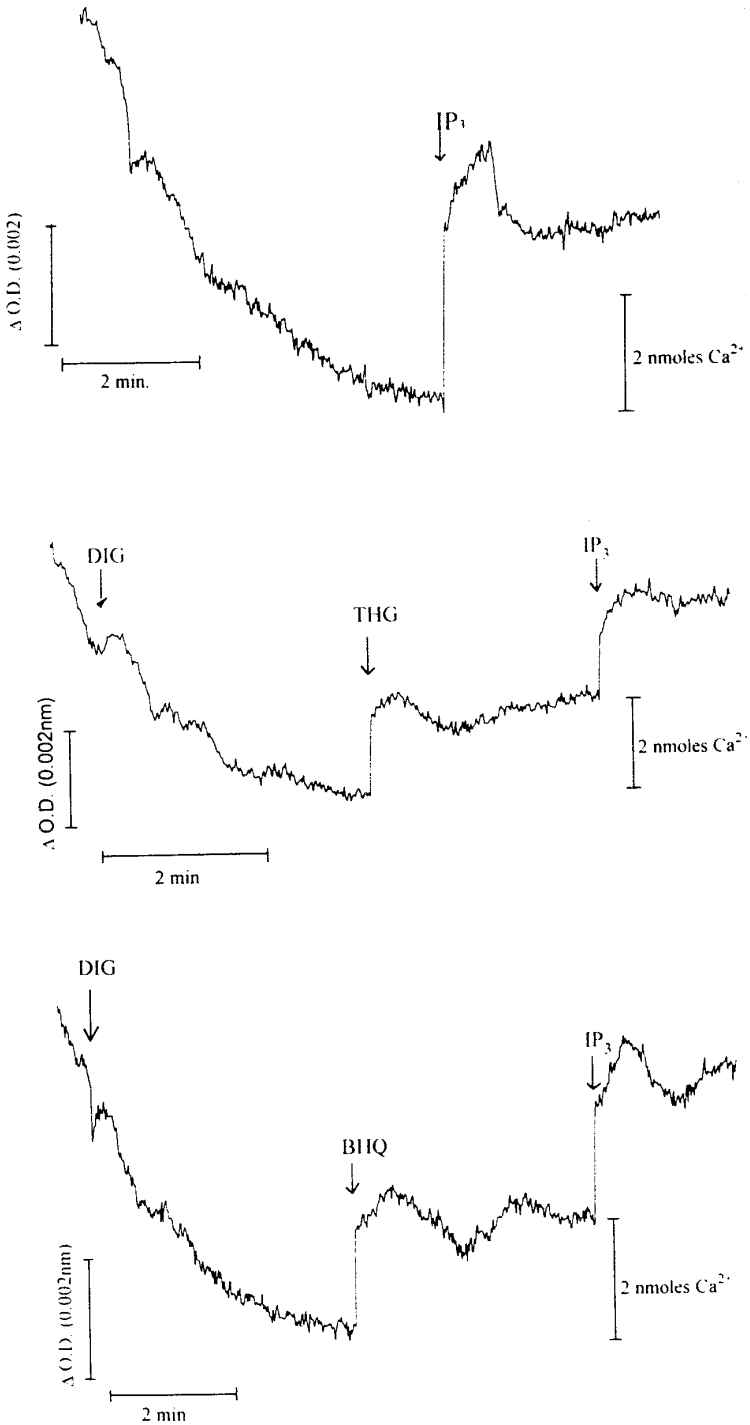

FIG. 1. Ca^{2+} mobilization by $InsP_3$ in *P. chabaudi* permeabilized parasites in the presence of SERCA inhibitors. The reaction medium contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes pH 7.2, 1 mM MgCl2, 1 mM ATP, 2.5 mM potassium phosphate, 3.5 μM $CaCl_2$. and 40 μM arsenazo III in a total volume of 1 ml. Parasites were permeabilized by the addition of 5 μM digitonin. The experiments were carried out in medium containing the mitochondrial poisons antimycin and oligomycin (1 μg/ml). Where indicated, 5 μM $InsP_3$, 5 μM thapsigargin or 10 μM tBHQ were added.

(Fig. 3B). Addition of $InsP_3$ alone released 38.9±3.9 nmol/10^8 cells of Ca^{2+} release (n=5) (Fig. 3C). Addition of nigericin caused a Ca^{2+} release of 16.5±3.7 nmol of $Ca^{2+}/10^8$ cells (n=8) (Fig. 3D). It should be noted that the amount of $InsP_3$ releasable Ca^{2+} matches very closely the sum of that released by the two drugs separately, further confirming that the $InsP_3$ -Ca^{2+} pool is composed of two independent stores, one that utilizes

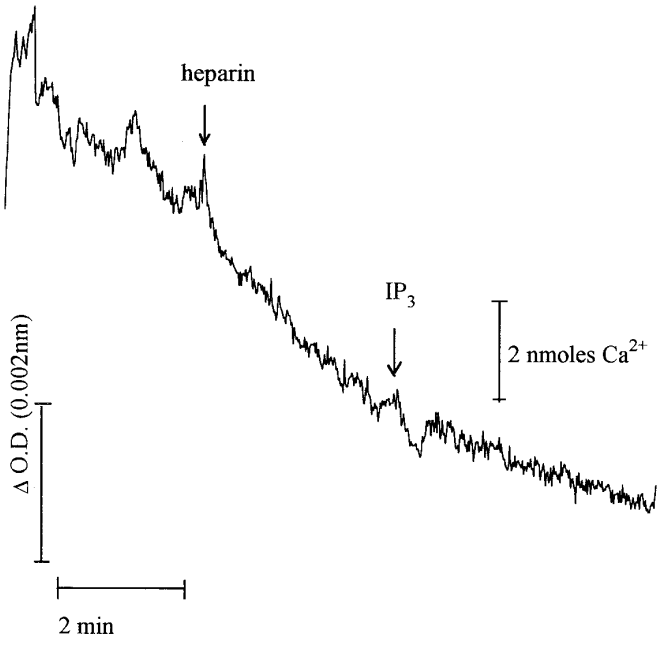

FIG. 2. $InsP_3$ addition on permeabilized *P. chabaudi* parasites in arsenazo III medium containing heparin. The reaction medium and cell permeabilization are described in the legend of Fig 1. The concentration of heparin was 10 μM.

a SERCA to accumulate Ca^{2+}, the other that requires a pH gradient (acidic inside) to retain (and possibly accumulate) calcium ions in the lumen. The latter result is confirmed by using the antimalarial drug chloroquine which also collapses the pH gradient within acidic compartments. In agreement with our findings that *P. chabaudi* parasites contain an acidic Ca^{2+} pool, chloroquine caused a substantial Ca^{2+} release on its own. Evidence that the chloroquine-induced Ca^{2+} release is derived from the acidic compartment comes from the experiment shown in Fig.3E, which shows that nigericin did not promote further Ca^{2+} release when added after the antimalarial drug. Fig. 3F shows that there was still a residual $InsP_3$-sensitive Ca^{2+} release component after chloroquine treatment, presumably derived from ER. A schematic diagram of the $InsP_3$ Ca^{2+} pools of *P. chabaudi* that takes into account the present findings is presented in Fig. 4.

DISCUSSION

Information concerning the signaling events in Plasmodia is fragmentary and largely incomplete (25). In fact, though we and others have shown previously that tyrosine kinase inhibitors prevent *P. falciparum* invasion and impair its intraerythrocytic development (26, 27), the role and nature of the kinases involved in these phenomena is still obscure. As far as Ca^{2+} signaling is concerned, we have shown that human, as well rodent, malaria parasites accumulate Ca^{2+} in thaspigargin and pH sensitive Ca^{2+} stores (18–20), while Martin et al (28) have demonstrated the production of $InsP_3$ in *P. falciparum* gametocytes during exflagellation. On the basis of the last data, the suggestion has been put forward that Ca^{2+} could be involved in the development of gametocytes and in the transformation into gametes after blood ingestion by the vector mosquito from the vertebrate bloodstream. To our knowledge, however there is no direct indication that $InsP_3$ can indeed mobilize Ca^{2+} in the malaria parasites. In the present report we demonstrate that $InsP_3$ can efficiently release Ca^{2+} in isolated permeabilized *P. chabaudi* parasites at the trophozoite stage. So far, the existence of an $InsP_3$ sensitive Ca^{2+} store has never been demonstrated in any parasite. This finding in *P. chabaudi* is of major significance not only because it demonstrates that the signaling pathway based on this second messenger is operative also in very primitive organisms, but also because it raises the possibility that malaria parasites could utilize $InsP_3$ to mobilize Ca^{2+} during the intraerythrocytic cycle, and thus employ a Ca^{2+} based signaling pathway during division and maturation processes.

In other cell types, the $InsP_3$ and thapsigargin sensitive stores have been identified with the endoplasmic reticulum, but do not represent the only Ca^{2+} storage compartment of eukaryotic cells. In particu-

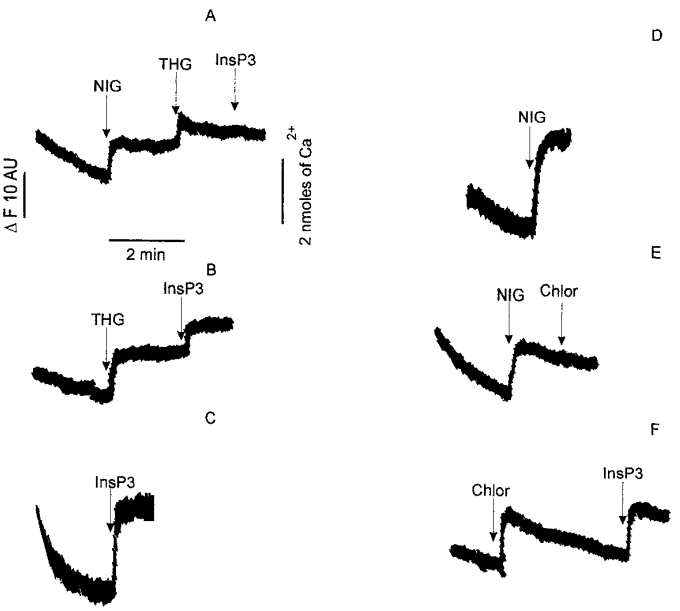

FIG. 3. Ca^{2+} mobilization by $InsP_3$ in *P. chabaudi* permeabilized parasites occurs in two distinguishable pools. The reaction medium and cell permeabilization are the same as those described in the legend of Fig. 1, but for the use of the Ca^{2+} indicator fluo-3 (1 μM). Where indicated 1.2 μM nigericin, 0.8 μM thapsigargin, 5 μM $InsP_3$. and 10 μM chloroquine were added to the parasite suspensions. ΔF10 AU: changes in fluorescence intensity (ΔF) in arbitrary units (AU).

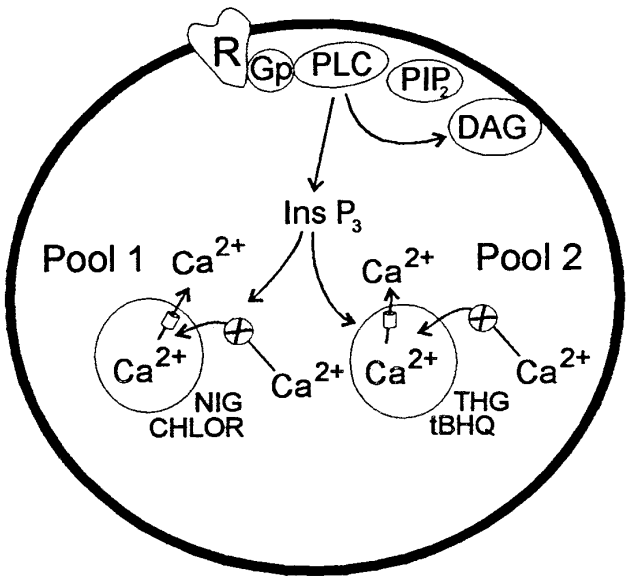

FIG. 4. Schematic illustration of *Plasmodium chabaudi* Ca^{2+} pools. This scheme is based on data of the present report and of previous data. $InsP_3$ induces Ca^{2+} release from an acidic pool (1) and a more typical ER-type pool (2). Pool 1 is thapsigargin-sensitive and pool 2 is nigericin and chloroquine-sensitive.

lar, other organelles, mitochondria and acidic compartments (29–33) have been shown to sequester Ca^{2+} in different cells types. As to the latter, however, much debate exists as to their mechanism of accumulation and $InsP_3$ sensitivity (17, 35, 36). In plant cells, it has been shown that $InsP_3$ is capable of releasing Ca^{2+} from the internal vacuoles (a classical acidic organelle), while in unicellular organisms such as *T. cruzi* and *T. gondi, S. cerevisae* and *D. discoideum*, a major Ca^{2+} release can be induced by neutralization of intracellular acidic pH gradients, though the $InsP_3$ sensitivity of these compartments has not been determined. Here we have shown that the Ca^{2+} containing acidic compartment of *P. chabaudi* (20) can be discharged by $InsP_3$, through a receptor that, at least in terms of heparin sensitivity, is indistinguishable from that expressed in the ER. This finding is of relevance for two reasons: i) it is a direct proof that an acidic compartment is part of the $InsP_3$ sensitive Ca^{2+} pools in a cell other than plants and ii) this compartment is depleted of its Ca^{2+} content upon application of chloroquine, an antimalarial drug. This raises the intriguing question that, at least in part, the therapeutic effect of this drug could depend on impairment of the parasite Ca^{2+} homeostatic mechanisms. Several features of this acidic pool, however remain to be determined, in particular the mechanism coupling the pH gradient to Ca^{2+} accumulation and the nature of the $InsP_3$ receptor expressed in this organelle.

ACKNOWLEDGMENTS

We thank Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and Fundação de Amparo a Pesquisa de São Paulo (Fapesp) for financial support. We are also indebted to Prof Ohara Augusto for the use of SLM AMINCO DW 2000 and to Prof. Robin Irvine for kindly supplying $InsP_3$.

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