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Blocking IP_3 signal transduction pathways inhibits melatonin-induced Ca^{2+} signals and impairs *P. falciparum* development and proliferation in erythrocytes

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ABSTRACT

Inositol 1,4,5 trisphosphate (IP_3) signaling plays a crucial role in a wide range of eukaryotic processes. In *Plasmodium falciparum*, IP_3 elicits Ca^{2+} release from intracellular Ca^{2+} stores, even though no IP_3 receptor homolog has been identified to date. The human host hormone melatonin plays a key role in entraining the *P. falciparum* life cycle in the intraerythrocytic stages, apparently through an IP_3-dependent Ca^{2+} signal. The melatonin-induced cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) increase and malaria cell cycle can be blocked by the IP_3 receptor blocker 2-aminoethyl diphenylborinate (2-APB). However, 2-APB also inhibits store-operated Ca^{2+} entry (SOCE). Therefore, we have used two novel 2-APB derivatives, DPB162-AE and DPB163-AE, which are 100-fold more potent than 2-APB in blocking SOCE in mammalian cells, and appear to act by interfering with clustering of STIM proteins. In the present work we report that DPB162-AE and DPB163-AE block the $[Ca^{2+}]_{cyt}$ rise in response to melatonin in *P. falciparum*, but only at high concentrations. These compounds also block SOCE in the parasite at similarly high concentrations suggesting that *P. falciparum* SOCE is not activated in the same way as in mammalian cells. We further find that DPB162-AE and DPB163-AE affect the development of the intraerythrocytic parasites and invasion of new red blood cells. Our efforts to episomally express proteins that compete with native IP_3 receptor like IP_3-sponge and an IP_3 sensor such as IRIS proved to be lethal to *P. falciparum* during intraerythrocytic cycle. The present findings point to an important role of IP_3-induced Ca^{2+} release in intraerythrocytic stage of *P. falciparum.*

1. Introduction

Malaria is the most lethal parasitic disease in the world, being responsible for approximately 660,000 deaths and 200 million cases annually worldwide, with the greatest mortality affecting children under 5 years of age [1]. Malaria parasites follow a complex life cycle and sequentially infect vertebrate and invertebrate hosts [2,3]. In order to survive within the Red Blood Cells (RBCs) *Plasmodium* employs a diverse set of strategies such as disrupting the cytoskeleton network and remodeling the host cell membranes [2,4].

A number of genes involved in signaling have been identified in the *Plasmodium* genome database [5], and these likely play a role in mechanisms by which the parasite senses the environment during its development. These include events such as parasite synchronous development within RBCs [6], male gamete formation [7], hepatocyte invasion [8,9], and RBC invasion [10], all of which require that the parasite senses the extracellular milieu to trigger distinct intracellular processes. Therefore, dissecting the *Plasmodium* signaling pathways that engender the control of its cell cycle is fundamental for the development of new strategies to combat this disease [11–13].

Ca^{2+} signaling is responsible for triggering a plethora of cellular processes in mammalian cells including excitation, contraction, fertilization, cell growth and secretion [14–16]. In apicomplexan parasites, such as *Plasmodium, Toxoplasma and Cryptosporidium,* Ca^{2+} signaling

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controls various vital functions such as protein secretion, motility, cell invasion and differentiation [17–19].

Melatonin follows a circadian rhythm of production in the pineal gland of vertebrates [20]. Previous results from our group showed that melatonin is able to synchronize *P. falciparum*, and this is associated with a rise in the parasite cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) that is initially derived from intracellular stores and involves activation of phospholipase C [6,21,22]. Melatonin also increases parasitemia in the synchronous infection of the rodent malaria parasite *P. chabaudi*, where surgical ablation of the pineal gland or blockade of melatonin receptors with luzindole leads to desynchronization of the parasite life cycle [6]. Melatonin signal transduction pathways in *P. falciparum* seem to be in part due to inositol 1,4,5-trisphosphate (IP_3) production, which is a well-known second messenger, responsible for mobilizing Ca^{2+} from intracellular organelles [23,24]. Due to its influence on *Plasmodium* biology drugs with anti-melatonergic activity could be effective in combatting this disease [6].

Sequencing of *Plasmodium falciparum* genome revealed a lack of an IP_3 receptor homologous to that in mammalian cells in this parasite [25]. Nevertheless, results published by Alves et al. (2011) showed that *P. falciparum* is able to respond to uncaging of IP_3 with an increase in intracellular calcium concentration, and that this IP_3-sensitive Ca^{2+} store is the same as that mobilized by melatonin [26]. Moreover, these authors showed that melatonin increases inositol polyphosphate production in *P. falciparum*. There is also cross-talk between signaling pathways in malaria parasites, with Ca^{2+} inducing a rise of cAMP and activation of protein kinase A [27]. In a related study performed by Furuyama et al. [28], it was shown that melatonin increased cAMP concentrations in the parasite in ring and late trophozoite stages, and these effects were abolished by luzindole treatment. Moreover, luzindole blocks spontaneous $[Ca^{2+}]_{cyt}$ oscillations in the parasite ring stage. These findings suggest that *P. falciparum* development relies on a cAMP signaling pathway situated downstream of the melatonin receptor and the IP_3–Ca^{2+} signaling pathway.

The compound 2-aminoethyl diphenylborinate (2-APB) was first reported to be an antagonist of the IP_3 receptor [29]. Evidence suggests that the pharmacological effects of 2-APB on intracellular Ca^{2+} signaling may be more complex than previously thought. 2-APB inhibits IP_3-induced Ca^{2+} release, despite its variable potency in distinct cell types [30,31]. However, it has also been shown that 2-APB inhibits store-operated Ca^{2+} entry (SOCE) channels in the plasma membrane of many cell types [32–34]. SOCE is activated by depletion of intracellular Ca^{2+} stores and is mediated by clustering of endoplasmic reticulum STIM proteins adjacent to plasma membrane Orai channels that admit Ca^{2+} into the cells [35,36].

We have shown previously that 2-APB blocks the ability of melatonin to synchronize *P. falciparum in vitro*, and also blocks the melatonin-induced $[Ca^{2+}]_{cyt}$ signal [37]. Similarly, a recent study in *P. berghei* sporozoites showed that 2-APB blocked the rise in $[Ca^{2+}]_{cyt}$, adhesion, secretion and motility of activated sporozoites [38]. In both studies 2-APB was used as an inhibitor of the IP_3 receptor, but since Ca^{2+} entry could also occur under the conditions of those experiments, it remains to be determined whether the effects of 2-APB on Ca^{2+} signaling in *Plasmodium* are due to actions on the intracellular Ca^{2+} release channels or SOCE, or both.

Besides the pharmacological use of 2-APB, there are novel approaches to study the IP_3 signaling in Apicomplexan parasites. Those methodologies are based in the expression of a peptide sequence that traps the native IP_3, named IP_3-sponge [39] or expression of IP_3R based IP_3 sensor (IRIS) [40] within the parasite.

In view of the ability of 2-APB to block melatonin-dependent Ca^{2+} signaling and interfere with parasite functions, including synchronization of the symptomatic red blood cell stage of the disease, elucidation of the target of 2-APB action is of therapeutic significance. Indeed, a recent study showed that 2-APB is effective in overcoming chloroquine resistance and reducing parasitemia *in vivo* in a mouse malaria model [41]. In the present study, we have used the 2-APB derivatives DPB162-AE and DPB163-AE to study potential mechanisms of 2-APB action, investigated the effects of these compounds and also the effect of the constitutive expression of IP_3-sponge and IP_3 sensor IRIS on *P. falciparum* parasite development within RBCs.

2. Materials and methods

2.1. Materials

All cell culture reagents were obtained from Cultilab (Brazil). Fluo-4 acetoxymethyl ester (Fluo-4/AM) was from Invitrogen All other reagents were of the highest obtainable grade. DPB162-AE and DPB163-AE [31,34] were kindly supplied by Katsuhiko Mikoshiba.

2.2. P. falciparum: culture, synchronization and isolation of parasites

Plasmodium falciparum (3D7 strain) was maintained in continuous culture according to [42]. The parasites were grown in plastic cell culture flasks (25 cm^2) with RPMI 1640 medium (GibcoBRL) supplemented with 0.5% AlbuMAX I (Gibco) with 5% hematocrit in a 90% N_2; 5% O_2; 5% CO_2 atmosphere at 37 °C. The synchronization of parasites was achieved by the sorbitol method [43]. Parasitemia and synchrony were followed in panoptic-stained smears (Laborclin). The infected erythrocytes were collected and washed twice in PBS, with centrifugation at 2000 rpm for 5 min. Subsequently, the cells were treated with saponin (0.05%) and the lysis of erythrocytes was followed. The parasites were collected by centrifugation at 8.000 rpm for 10 min at 4 °C and washed twice in PBS.

2.3. Spectrofluorometric determinations

Isolated synchronized parasites at the trophozoite stage (28–32 h) were loaded with Fluo4/AM in buffer M (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM D-glucose, 50 mM MOPS and 2 mM $CaCl_2$, pH 7.2) containing 40 μM probenecid (Sigma), an inhibitor of organic anion transport used to avoid the sequestration of Fluo-4/AM/AM in the digestive vacuole of the parasites. Parasites were incubated for 1 h at 37 °C. The cells were washed three times in buffer M to remove the extracellular Fluo4/AM. Experiments were performed either in the presence or absence of extracellular Ca^{2+} (no $CaCl_2$ added plus addition of 500 μM EGTA). Cells were incubated with the compounds DPB162-AE and DPB163-AE for 15 min before addition of the agonist. Cytosolic Ca^{2+} dynamics were monitored using a Shimadzu spectrofluorometer (RF5301PC, Japan) with parasites (10^7 cells ml^{-1}) in a 1 mL stirred cuvette. Excitation of Fluo-4/AM was performed at 488 nm and emission was collected at 525 nm. All assays were performed at 37 °C, in triplicates, with at least three independent experiments. Thapsigargin (5 μM) or Melatonin (100 nM or 1 μM) were added in the buffer, and the $[Ca^{2+}]_{cyt}$ fluctuations were calculated by normalization with the basal fluorescence (F1/F0). F0 corresponds the mean of the fluorescence data points obtained between seconds 50–60, and F1 corresponds the mean of the fluorescence data points obtained between seconds 90–100 for treatment with Thapsigargin, or seconds 540-500 for treatment with Melatonin. SOCE measurements in buffer without Ca^{2+} were done by the re-addition of 2 mM Ca^{2+} in the cuvette, and calculated by F1/F0 equation, in which F0 corresponds the mean of the fluorescence data points obtained between seconds 90–100, and F1 corresponds the mean of the fluorescence data points obtained between seconds 190–200 for Thapsigargin, or between seconds 640–650 for Melatonin.

2.4. Analysis of parasitemia by flow cytometry

A synchronized culture of *P. falciparum* in the ring stage (10–14 h) had the parasitemia adjusted to 2% and was incubated with the