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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

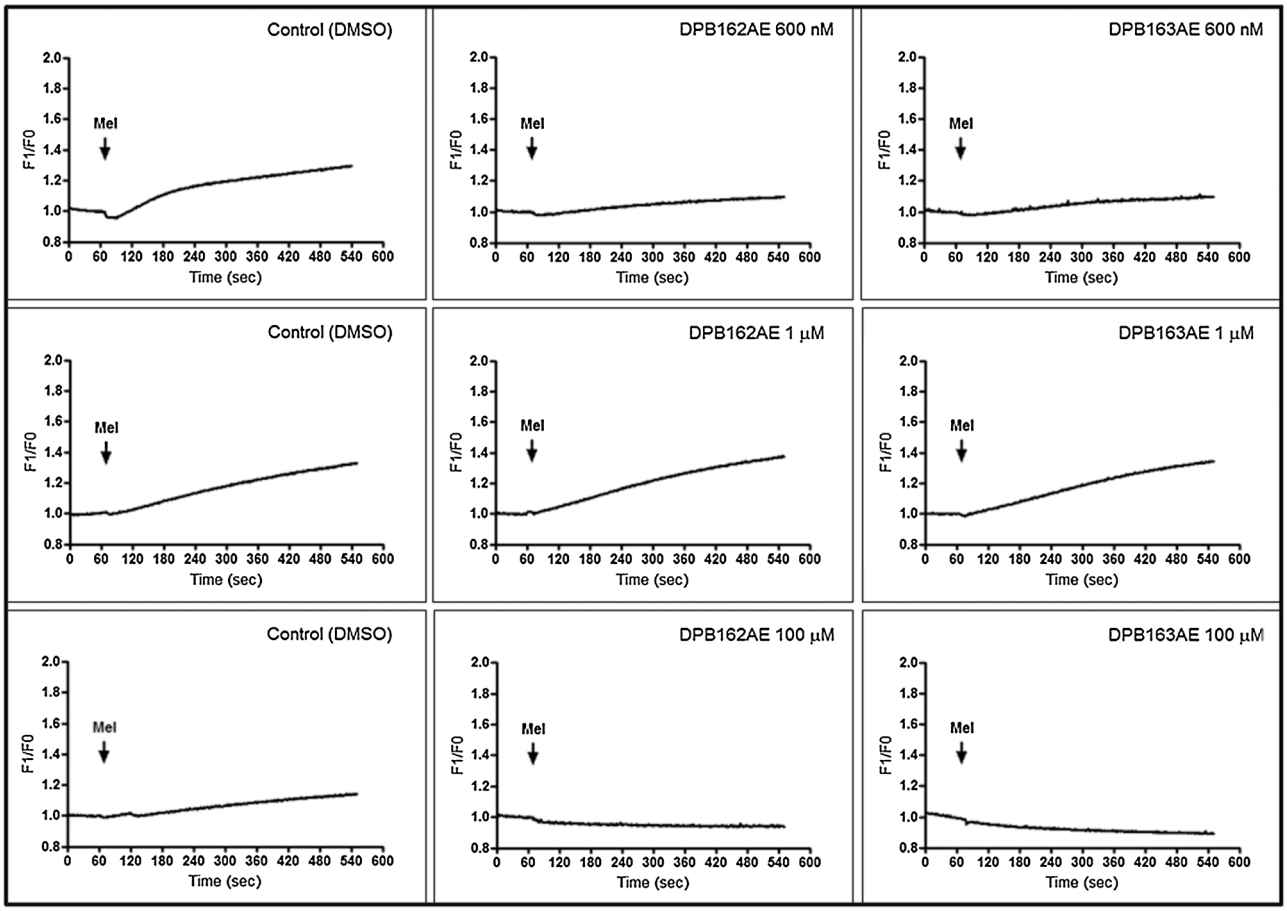

Fig. 1. Effect of 2-APB derivatives on melatonin- induced $[Ca^{2+}]_{cyt}$ rise in *Plasmodium falciparum*. Isolated sorbitol-synchronized *P. falciparum* (3D7 strain) parasites at the trophozoite stage (26–30 h) were loaded with Fluo-4/AM and treated with the indicated compounds (or DMSO solvent for the respective controls) in a buffer containing 2 mM $CaCl_2$. A total of 10^7/mL cells were incubated in the stirred 1 mL cuvette of a spectrofluorometer. The time-course of $[Ca^{2+}]_{cyt}$ changes in response to 100 nM melatonin (Mel) was monitored using the Fluo-4/AM fluorescence signal normalized to basal (F1/F0), in which 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 540-500. A drastic reduction in the $[Ca^{2+}]_{cyt}$ rise was observed after addition of melatonin in parasites previously pre-incubated with the compounds at 100 μM, while no effect was observed with lower concentrations (see Table 1 for mean data and statistics).

compounds DPB162-AE and DPB163-AE for 48 h. The effect of each compound on parasite development and invasion was assessed after 24 and 48 h of incubation. The parasites were fixed with 2% paraformaldehyde in PBS overnight at room temperature and permeabilized with 0.1% Triton X-100 (Merck) and 20 μg/mL RNAse (Invitrogen) for 15 min at 37 °C. Subsequently, parasites were stained with 5 nM YOYO-1 (Molecular Probes) as previously described [44,45]. Parasitemia was determined in dot plots [side scatter (SSC) versus fluorescence] of 100,000 cells using a FACS Calibur flow cytometer with CELLQUEST software (Becton & Dickinson). YOYO-1 was excited at 488 nm with an Argon laser and fluorescence emission was collected between 520 and 530 nm. The effect of the compounds on parasite development was assessed in panoptic-stained smears after 24 and 48 h incubation. The assays were performed in triplicates in 3 independent experiments.

2.5. Generation of IP_3-Sponge-GFP-pARL and IRIS-pARL plasmid

The mouse type 1 IP_3R ligand binding region (224aa-604aa) with a single point mutation of R441Q, named as IP_3-Sponge (Uchiyama et al., 2002) was amplified with the oligos sense 5′ GGTACCATGAAATGGA GTGATAAC 3′ and antisense 5′ CCTAGGTTTCCGGTTGTTGTGGAG 3′ which removes the stop codon and insert the restriction enzyme sites of KpnI and AvrII. The 1143 bp PCR product was digested with KpnI/AvrII and inserted into pARL plasmid (courtesy of Prof. Castern Wrenger) that contains GFP under the control of CRT promoter to obtain the IP_3-Sponge-GFP-pARL episomal plasmid. A fluorescent IP_3 sensor based on the mouse type 1 IP_3R named IRIS [40] was amplified with oligos sense 5′ GGTACCATGGTGAGCAAGGGCGAG 3′ and antisense 5′ CTCGAGTT ACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGC 3′. The 2500 bp PCR product was digested with KpnI/XhoI restriction enzyme and insert on pARL plasmid without GFP to obtain IRIS-pARL episomal plasmid.

2.6. Plasmodium falciparum transfection

RBC infected with ring stage *P. falciparum* (Dd2 strain) at 5% parasitemia was transfected either with 80 μg of GFP-pARL, IP_3-Sponge-GFP-pARL or IRIS-pARL using a Gene Pulser II electroporation device (Bio-Rad) set up at 0.31 KV, 950 μF and maximum capacitance as previously recommended [46,47]. 24 h after electroporation, the cultures were under 2,5 nM of WR99210 drug pressure for 3–4 weeks until parasitemia reach 1%.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism (Graphpad Software), using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post test. P values < 0.05 were considered significant.

3. Results

3.1. Effect of 2-APB derivatives on calcium signaling of P. falciparum

It is well established that melatonin modulates the *Plasmodium* life cycle through a calcium dependent mechanism, and this appears to play a key role in synchronizing and entraining RBC lysis to the host circadian rhythm [6,21,22,26]. Our previous studies demonstrated that 2-APB is able to block the melatonin-induced Ca^{2+} rise in *P. falciparum* [37]. We set out to investigate the mechanism of 2-APB action on the melatonin-induced Ca^{2+} rise in *P. falciparum* using two derivatives of 2-APB that are more selective than 2-APB for blocking the SOCE mechanism in mammalian cells [31,34]. Unlike 2-APB, which acts at high micromolar concentrations, DPB162-AE and DPB163-AE inhibit SOCE at submicromolar concentrations, whereas they have a low affinity similar to 2-APB for inhibition of IP_3-induced intracellular Ca^{2+} release. Therefore, we examined the effects of these compounds on the melatonin-induced Ca^{2+} response in the human malaria parasite *P. falciparum*.

Isolated sorbitol-synchronized *P. falciparum* parasites at the trophozoite stage (26–30 h) were loaded with Fluo-4/AM and incubated in buffer with 2 mM $CaCl_2$ in the stirred cuvette of a spectrofluorometer. The cells were preincubated for 15 min with the compounds DPB162-AE and DPB163-AE at different concentrations, and then the $[Ca^{2+}]_{cyt}$ response was assessed after melatonin addition. Since the number of conditions that could be tested was limited by the yield of isolated parasites, we selected test conditions of 600 nM, 1 μM and 100 μM of the compounds to cover the range where they have been shown to inhibit SOCE and IP_3-induced Ca^{2+} release in mammalian cells [31]. As shown in Fig. 1, only the highest concentration of DPB162-AE and DPB163-AE blocked the $[Ca^{2+}]_{cyt}$ increase elicited by 100 nM melatonin. These data are summarized in Table 1. Based on the findings in mammalian cells with these compounds, the data of Fig. 1 and Table 1 are most consistent with the participation of an IP_3 receptor in the inhibition of the $[Ca^{2+}]_{cyt}$ rise by 2-APB and derivatives.

We have previously proposed that *P. falciparum* at the trophozoite stage display store-operated calcium entry (SOCE) when stimulated with 8BrcAMP, which appears to rely on extracellular Ca^{2+} to sustain a $[Ca^{2+}]_{cyt}$ increase [37]. We therefore investigated whether a SOCE mechanism is present in isolated *P. falciparum* parasites using a classical experimental protocol in which the intracellular Ca^{2+} stores are first depleted with thapsigargin treatment in the absence of extracellular Ca^{2+}, followed by addition of extracellular Ca^{2+} to initiate the SOCE phase [48]. Fig. 2B shows that *P. falciparum* loaded with Fluo-4/AM in a medium without Ca^{2+} gave a modest $[Ca^{2+}]_{cyt}$ increase in response to thapsigargin, which is an inhibitor of the SERCA-Ca^{2+} ATPase and acts to release Ca^{2+} from the endoplasmic reticulum. To demonstrate the presence of SOCE, extracellular Ca^{2+} was added to a final concentration of 2 mM following thapsigargin treatment. This led to a large and sustained increase in $[Ca^{2+}]_{cyt}$, which was not observed in the absence of thapsigargin (Fig. 2A vs B). These data provide direct evidence for the existence of a store-operated calcium entry mechanism within human malaria parasites.

We tested the ability of the compounds DPB162-AE and DPB163-AE to interfere with this SOCE response in *P. falciparum*, since these compounds have been reported to be much more selective than 2-APB for SOCE in mammalian cells [31,34]. As for the studies of melatonin-induced $[Ca^{2+}]_{cyt}$ increases described above, isolated sorbitol-synchronized *P. falciparum* parasites at the trophozoite stage (26–30 h) loaded with Fluo-4/AMAM were preincubated for 15 min with the 2-APB derivatives as describe under materials and methods. For the experiments shown in Fig. 3, SOCE was assessed as described for Fig. 2, by first treating the cells with thapsigargin in a medium without Ca^{2+} to release Ca^{2+} from the endoplasmic reticulum, followed by addition of extracellular Ca^{2+} to a final concentration of 2 mM to measure the store-operated Ca^{2+} entry. The amplitude of the $[Ca^{2+}]_{cyt}$ response was measured in each case, and the data normalized to the control amplitude in that experiment because dye loading varied between each preparation of isolated parasites. From the conditions tested (600 nM, 1 and 100 μM), only the highest concentration of DPB162-AE and DPB163-AE was able to impair SOCE in the parasite (Fig. 3). These data are summarized in Table 1, which shows approximately 34.8% and 34.6% inhibition, respectively, at the 100 μM concentration. SOCE in mammalian cells is mediated by STIM and Orai proteins [36]. Interestingly there are no identified homologs of these proteins in the *P. falciparum* genome, suggesting that SOCE in this species could occur through an alternative pathway.

Since previous studies of the effect of 2-APB in *P. falciparum* have shown that 2-APB can interfere with melatonin-induced Ca^{2+} signaling [37], block spontaneous Ca^{2+} oscillations [49] and even reverse chloroquine resistance in the chloroquine-resistant *P. falciparum* K-1 strain [41], we further characterized the effects of this agent on intracellular Ca^{2+} release and SOCE. As in the experiments described above, isolated sorbitol-synchronized *P. falciparum* parasites at the trophozoite stage (26–30 h) loaded with Fluo-4/AMAM were preincubated for 15 min with 2-APB or DMSO (control). SOCE was assessed in Fig. 4 by first treating the cells with melatonin or thapsigargin in a medium without Ca^{2+} to release Ca^{2+} from the intracellular stores, followed by addition of extracellular Ca^{2+} to a final concentration of 2 mM to measure the store-operated Ca^{2+} entry. Consistent with the data for DPB162-AE and DPB163-AE, 2-APB (100 μM) partially inhibited SOCE elicited by thapsigargin (data summarized in Fig. 4G). As previously described [6,22,37] melatonin was able to increase $[Ca^{2+}]_{cyt}$ even in the absence of extracellular Ca^{2+}, and this response was completely blocked by 2-APB (Fig. 4H). However, in contrast to the effect of releasing intracellular Ca^{2+} stores with thapsigargin, the intracellular Ca^{2+} mobilization by melatonin did not result in a stimulation of SOCE in *P. falciparum* (Fig. 4B, H). Since 2-APB blocked the melatonin-induced release of intracellular Ca^{2+} in the absence of extracellular Ca^{2+}, no SOCE would be expected when the extracellular Ca^{2+} readdition protocol was applied after melatonin treatment in the presence of 2-APB, as shown in Fig. 4C, I. Nevertheless, the absence of SOCE in response to melatonin in the absence of 2-APB, together with the results shown above for the 2-APB derivatives, indicates that 2-APB acts on intracellular calcium release and supports our previous data showing that melatonin acts *via* an IP_3-dependent intracellular Ca^{2+} release pathway [6,21,26].

Table 1
Effect of 2-APB derivatives on melatonin-induced $[Ca^{2+}]_{cyt}$ rise and store-operated Ca^{2+} entry *Plasmodium falciparum*. Fluoresce increase (% normalized by the control).

Compound tested Mechanism Analyzed	DPB162-AE (600 nM)	DPB163-AE (600 nM)	DPB162-AE (1 μM)	DPB163-AE (1 μM)	DPB162-AE (100 μM)	DPB163-AE (100 μM)
Melatonin(Fig. 1)	96.1% (± 2.4%)	90.9% (± 4.9%)	107.7% (± 9.1%)	98.4% (± 1.9%)	Not detected	Not detected
SOCE(Fig. 3)	97.1% (± 3.9%)	99.3% (± 5.4%)	99.8% (± 2.6%)	99.7% (± 1.9%)	65.2% (± 5.3%)***	65.4% (± 4.7%)***

Data from a series of experiments similar to those presented in Figs. 1 and 3 were combined by normalizing to the amplitude of control responses to melatonin or Ca^{2+}-addback (SOCE), respectively, in the same preparation of isolated parasites. The results represent the mean ± SEM of three different experiments in triplicate. Control data were obtained in the presence of the DMSO solvent was used to dissolve the drugs. These data were compared by one-way ANOVA followed by Dunnett's multiple comparison post-test.

*** Statistically different from the control values ($p < 0.001$).

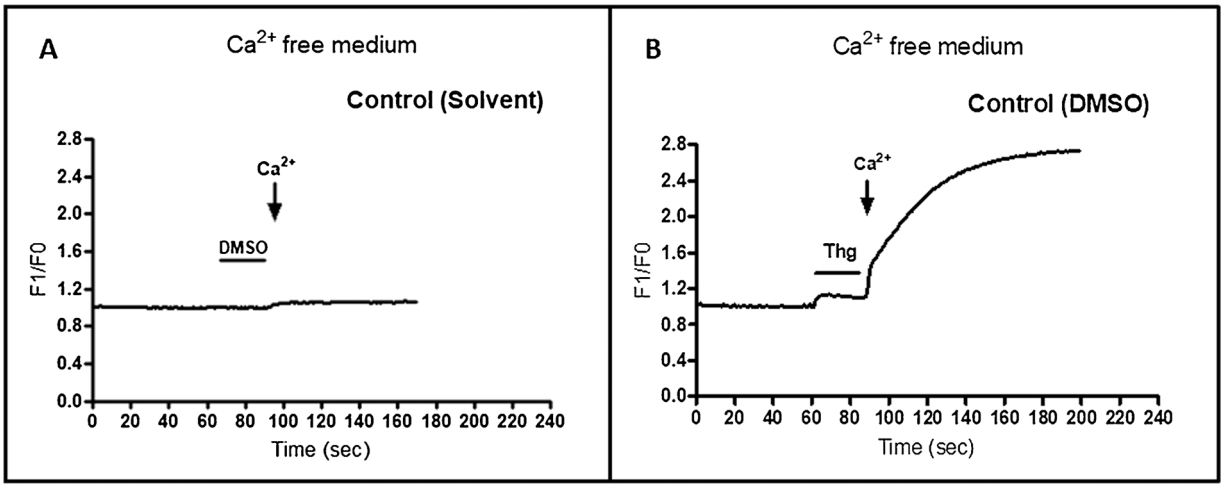

Fig. 2. *Plasmodium falciparum* parasites display store-operated calcium entry. Isolated sorbitol-synchronized *P. falciparum* (3D7 strain) parasites at the trophozoite stage (26–30 h) were loaded with Fluo-4/AM and placed in a medium without Ca^{2+}. A total of 10^7/mL cells were incubated in the stirred 1 mL cuvette of a spectrofluorometer. The cells were first treated with the DMSO (A) as a solvent control, or 10 μM thapsigargin (Thg) (B) to empty the Ca^{2+} content of the endoplasmic reticulum. This was followed by addition of $CaCl_2$ (2 mM) to increase the extracellular $[Ca^{2+}]$ and initiate SOCE. The time-course of $[Ca^{2+}]_{cyt}$ changes was monitored using the Fluo-4/AM fluorescence signal normalized to basal (F1/F0), in which 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. The amplitude of $[Ca^{2+}]_{cyt}$ rise after $CaCl_2$ addition was used as a measure of SOCE. A large $[Ca^{2+}]_{cyt}$ increase can be observed only after Ca^{2+} addition to parasites previously exposed to thapsigargin, indicating the presence of some form of SOCE in *P. falciparum*.

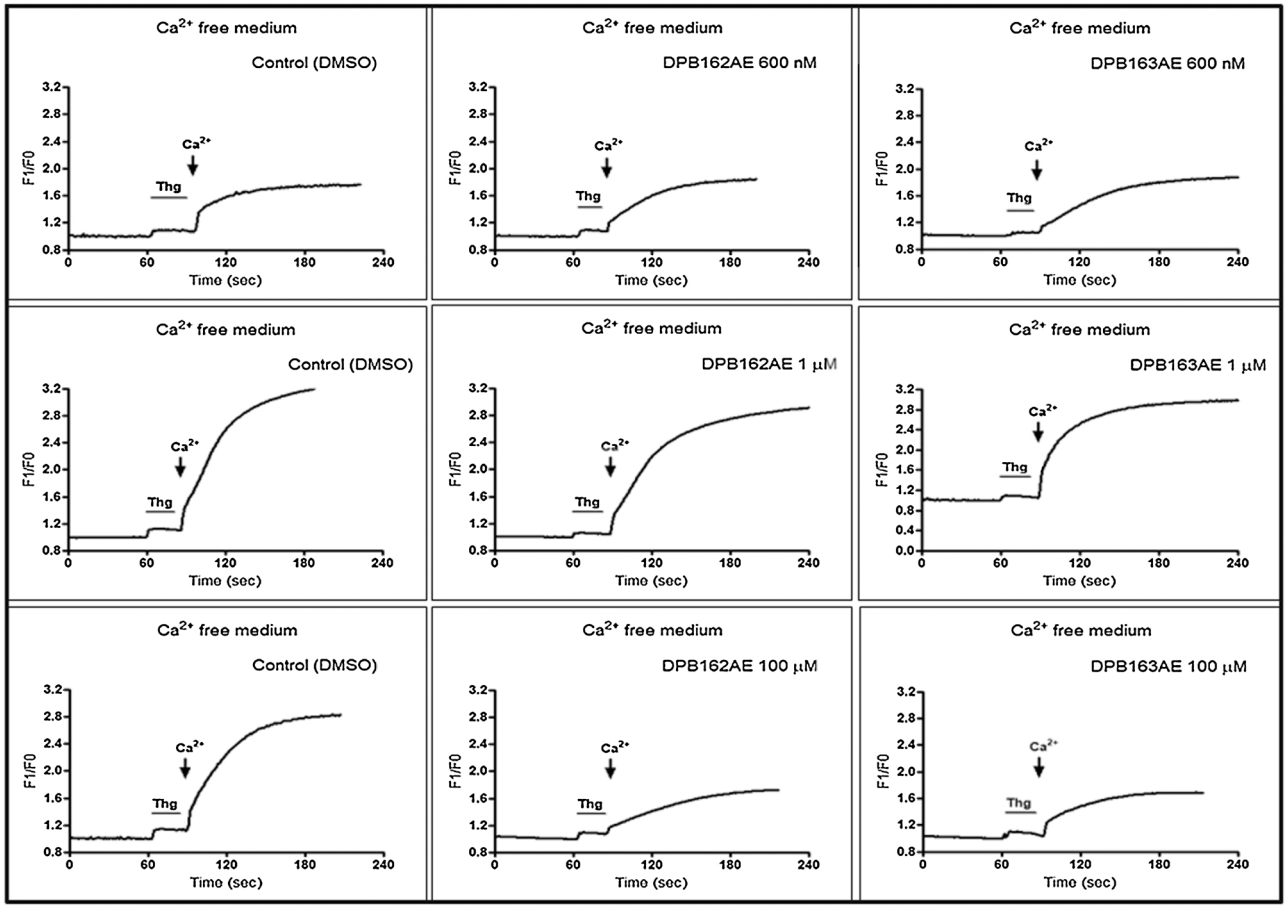

Fig. 3. Effect of 2-APB analogues on store-operated calcium entry in *Plasmodium falciparum.* Isolated sorbitol-synchronized *P. falciparum* (3D7 strain) parasites at the trophozoite stage (26–30 h) were loaded with Fluo-4/AM and pretreated for 15 min with the DMSO solvent (left panels; individual controls for each dose of text compound) or the concentrations of test compounds as indicated on each panel. The parasites were placed in a medium without extracellular Ca^{2+} and 10^7/mL cells were incubated in the stirred 1 mL cuvette of a spectrofluorometer. Thapsigargin (Thg) (10 μM) was used to empty the Ca^{2+} content of the endoplasmic reticulum. $CaCl_2$ (2 mM) was added and the $[Ca^{2+}]_{cyt}$ rise was monitored in a spectrofluorometer as a measure of SOCE (see Table 1 for mean data and statistics), 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.

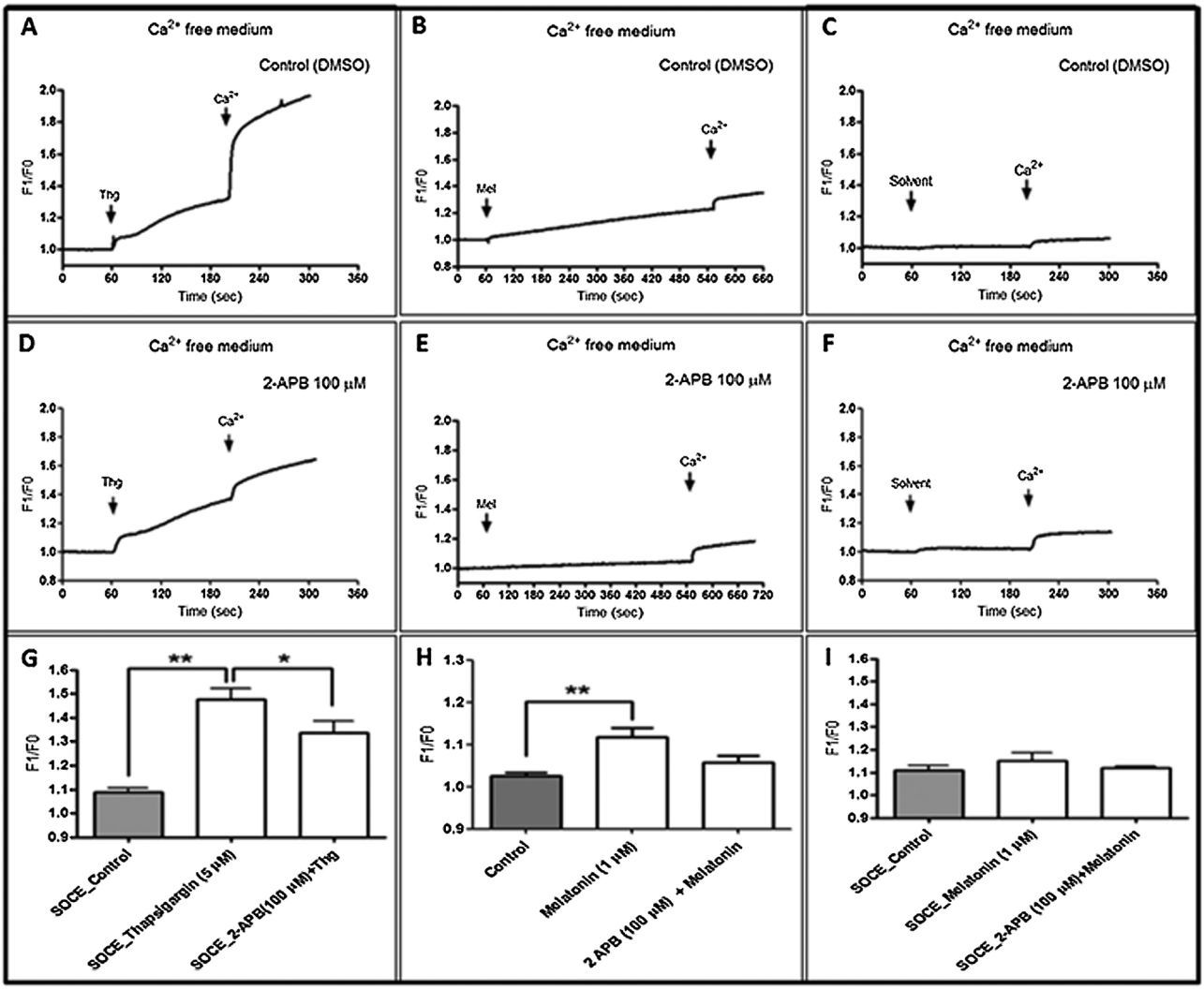

Fig. 4. Effect of 2-APB on melatonin- induced $[Ca^{2+}]_{cyt}$ rise in *Plasmodium falciparum*. Isolated sorbitol-synchronized *P. falciparum* (3D7 strain) parasites at the trophozoite stage (26–30 h) were labeled with Fluo-4/AM and pretreated for 15 min with the solvent or 2-APB (100 µM). The parasites were incubated in medium without extracellular Ca^{2+} and 10^7/mL cells were incubated in the stirred 1 mL cuvette of a spectrofluorometer. Thapsigargin (Thg) (10 µM) (panels A and D) or Melatonin (Mel) (1 µM) (panels B and E) were used to empty the Ca^{2+} content of the endoplasmic reticulum, followed by addition of $CaCl_2$ (2 mM) to initiate SOCE. The time-course of $[Ca^{2+}]_{cyt}$ changes was monitored using the Fluo-4/AM fluorescence signal normalized to basal (F1/F0), in which in which 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 Thapsigargin, or between seconds 540–550 for Melatonin. The amplitude of $[Ca^{2+}]_{cyt}$ rise after $CaCl_2$ addition was used as a measure of SOCE, 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. Panels G, H and I represent the mean ± SEM of six different experiments. Data from panel G were compared by one-way ANOVA followed by Newman-Keuls multiple comparisons post-test. * Statistically different from control values ($p < 0.05$). Data from panels H and I were compared by one-way ANOVA followed by Dunnett's multiple comparisons post-test. * Statistically different from the control values ($p < 0.05$).

3.2. APB and derivatives impair the development of P. falciparum and interfere with RBC invasion

In order to evaluate the effect of 2-APB, DPB162-AE and DPB163-AE on the *P. falciparum* cell cycle a synchronized culture of parasites at the ring stage was incubated with 100 µM of each of the compounds (since this was the concentration able to interfere with calcium signaling) for 48 h, and the parasitemia was then assessed by flow cytometry. Exposure of parasites to 100 µM of 2-APB, DPB162-AE or DPB163-AE for 24 h caused a reduction in parasitemia of the treated groups (26.5%, 22.3% and 28.8% inhibition, respectively) (Fig. 5A, B). This effect was even more pronounced after 48 h incubation, with 2-APB, DPB162-AE and DPB163-AE yielding 54.1%, 44.5% and 47.7% inhibition, respectively (Fig. 5C, D). This indicates that all three compounds were effective in impairing parasite invasion of RBCs (Fig. 5).

To further verify the effect of 2-APB, DPB162-AE and DPB163-AE on the *P. falciparum* intraerythrocytic cycle, the same parasites submitted to flow cytometry were also used to make thin blood smears and parasite development was observed using a light microscope. The smear analysis of parasites after treatment with the compounds showed that they can interfere with parasite development, thus confirming the reduction in parasitemia (Fig. 5E). 2-APB, DPB162-AE and DPB163-AE caused a delay in parasite development after 24 h, since the treated parasites remained mostly in the early trophozoite stage, whereas control cells had advanced to the schizont stage. After 48 h incubation, the control cultures had gone through a full parasite cell cycle leading to RBC lysis and reinvasion, yielding ring-stage parasites again (Fig. 4E rightmost panels). The 2-APB treated parasite cultures were still delayed when compared with control, and had not advanced beyond the schizont stage. However, the parasite cultures treated with DPB162-AE and DPB163-AE had their life cycle blocked. These results show that exposure to 2-APB and derivatives was able to impair *P. falciparum* development and invasion of RBCs. Taken together these findings demonstrate that signaling pathways involving the elevation of $[Ca^{2+}]_{cyt}$

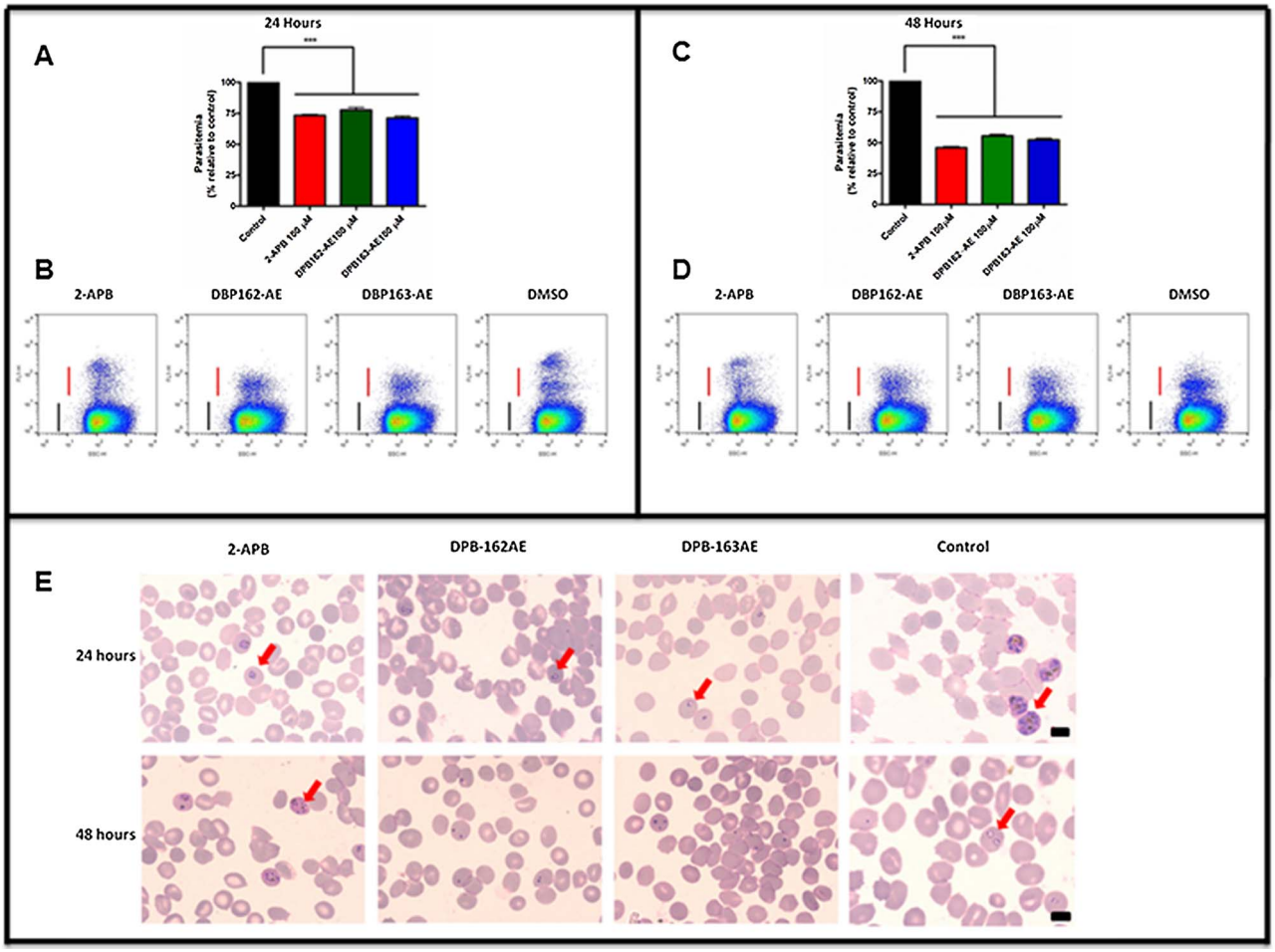

Fig. 5. 2-APB and the DPB derivatives impair *Plasmodium falciparum* development and invasion of RBCs. Synchronized ring-stage (10–14 h) cultures of *P. falciparum* were incubated with the compounds 2-APB, DPB162-AE and DPB163-AE (100 µM of each) for 48 h, allowing time for the parasites to go through a complete life cycle and reinvade other RBCs in the culture. After 24 h (A, B) and 48 h (C, D) samples were collected and the parasitemia was measured by flow cytometry. The histograms show the mean ± SEM of data from three separate experiments in triplicate. The control was carried out in the presence of DMSO, which was used to dissolve the drugs. These data were compared by one-way ANOVA followed by Dunnett's multiple comparison post test. *** Statistically different from the control values ($p < 0.001$). Panel (E) shows the effects of the compounds on parasite development stage. Parasites treated with 2-APB, DPB162-AE and DPB163-AE (100 µM of each) were also used to make thin blood smears, which were stained with giemsa and parasite developmental stages were observed in a light microscope. All the compounds tested were able to delay parasite development after 24 h, whereas DPB162-AE and DPB163-AE also blocked the parasite life cycle after 48 h. Scale bar = 5 µm.

are vital for *P. falciparum* and could be attractive targets for drug development.

3.3. Constitutive expression of IP_3-sponge and IRIS are lethal during erythrocytic cycle of P. falciparum

An episomal plasmid pARL [50] constitutively expressing a mutated mouse IP_3R biding domain known as IP_3-sponge, and mouse IP_3R-based IP_3 sensor named IRIS, have been previously reported to sequestrate the cytosolic IP_3 and compete with native IP_3R in mammalian cells respectively [39,40]. However, when *P. falciparum* are transfected with IP_3-sponge and IRIS no viable parasites are obtain when compare with parasites transfected with same vector containing GFP protein alone (Fig. 6). This data, together with our pharmacological approach of blocking the IP_3R with DPB162-AE and DPB163-AE, highlight the importance of the IP_3 molecule and its receptor on malaria physiology.

4. Discussion

Little is known about the biological significance and molecular machinery of Ca^{2+} signaling systems in Apicomplexan protists, including the malaria parasite. In this study, we have demonstrated that blockade of intracellular $[Ca^{2+}]_{cyt}$ signals by 2-APB and its analogs DPB162-AE, DPB163-AE reduced *P. falciparum* parasitemia or RBCs *in vitro*. Maruyama and collaborators [29] showed that the inhibitory concentration (IC_{50}) of 2-APB for inhibition of intracellular Ca^{2+} release in mammalian cells was approximately 40 µM. Therefore, we believe that the concentration used in this study was sufficient to block Ca^{2+} release in *P. falciparum*. Enomoto and collaborators [49] demonstrated that *P. falciparum* possesses $[Ca^{2+}]_{cyt}$ signal fluctuations during its life cycle and when parasites were treated with 2-APB, those Ca^{2+} fluctuations at the ring and trophozoite stages were blocked. It has also been reported that 50 µM 2-APB can synergize with chloroquine in blocking parasite growth in a chloroquine-resistant P. falciparum strain [41]. The same authors showed *in vivo* antimalarial activity of 2-APB in a chloroquine-resistant mouse malaria model.

SOCE is a critical Ca^{2+} signaling component to sustain $[Ca^{2+}]_{cyt}$ in non-excitable cells [35]. 2-APB has been an important pharmacological tool in the field of SOCE and the underlying ion channel current known as I_{CRAC}, which is mediated by Orai channels in the plasma membrane that become activated by interaction with STIM proteins of the endoplasmic reticulum following Ca^{2+} store depletion. 2-APB has been shown to activate I_{CRAC} at low concentration (~5 µM), but is more commonly used as an inhibitor of SOCE at high concentrations (> 30 µM) [51,52]. Analogs of 2-APB were developed which are structurally related isomers, but have different effects on SOCE.

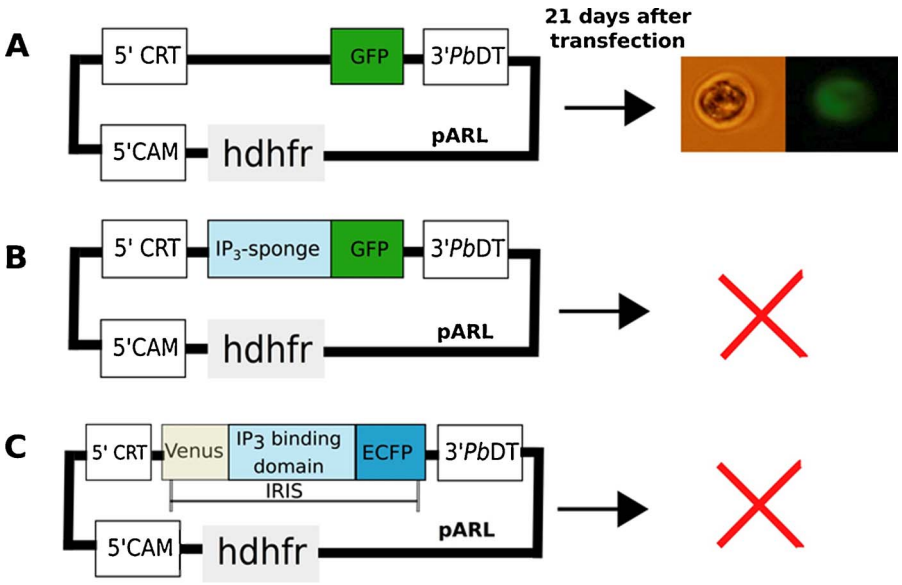

Fig. 6. Constitutive expression of IP_3-sponge and IRIS with an episomal transfection on *P. falciparum*. Synchronized ring-stage Dd2 strain cultures of *P. falciparum* were transfected with (A) pARL plasmid that contains GFP protein under CRT promoter, (B) pARL-IP_3-sponge and (C) pARL-IRIS. On the left side contains all the schematic representation of the plasmids: CRT (Chloroquine Resistance Transporter promoter region), PbDT (*Plasmodium berghei* dihydrofolate reductase-thymidylate synthase 3′-transcription terminator sequence), CAM (Calmodulin promoter region) and hdhfr (the selectable marker for human dihydrofolate reductase). Right side reveals the outcome of transfection after 21 days under positive selection with WR99210. The red cross indicates that no parasite was observe after selection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DPB163-AE showed a bimodal effect similar to 2-APB whereas DPB162-AE had only an inhibitory effect, but importantly, both derivatives were 100-fold more potent than 2-APB as inhibitors of SOCE in mammalian cells while being similarly low potency inhibitors with respect to IP_3-induced Ca^{2+} release [31,34].

We have shown previously that melatonin elicits a $[Ca^{2+}]_{cyt}$ signal in blood-stage malaria parasites that depends on IP_3 generation [6,21,26] and can be blocked by 2-APB [37]. In the present study we investigated whether the melatonin-induced $[Ca^{2+}]_{cyt}$ signal also depends on SOCE, and tested the effects of the 2-APB derivatives as a means to distinguish the roles of intracellular Ca^{2+} release and SOCE in the parasite. We found that DPB162-AE and DPB163-AE at 100 μM can block the $[Ca^{2+}]_{cyt}$ increase in response to melatonin. At lower concentrations where these 2-APB derivatives have been reported to be selective for SOCE in mammalian cells, they had no effect on the $[Ca^{2+}]_{cyt}$ response to melatonin in *P. falciparum*. However, while we show that SOCE is present in the blood-stage malaria parasite, this process does not show the high sensitivity to DPB162-AE and DPB163-AE that was observed in mammalian cells. Thus, even when activated by thapsigargin-induced emptying of intracellular Ca^{2+} stores, the SOCE in blood-stage *P. falciparum* also required the highest (100 μM) concentrations of the 2-APB derivatives, similar to the parent 2-APB compound. Interestingly, melatonin does not seem to activate Ca^{2+} entry in *P. falciparum*. The parasite SOCE activated by thapsigargin was only partially inhibited even at the 100 μM concentrations of 2-APB, DPB162-AE and DPB163-AE, whereas melatonin-induced $[Ca^{2+}]_{cyt}$ increase was totally ablated in the presence or absence of extracellular Ca^{2+}. Taken together, the data suggest that the primary effect of 2-APB and the derivatives is to interfere with intracellular Ca^{2+} release *via* the IP_3-mediated pathway.

The finding that the malaria parasite has a robust SOCE pathway that is relatively insensitive to DPB162-AE and DPB163-AE suggests that it may operate in a different manner from SOCE in mammalian cells. It is well established that a major mechanism for SOCE in mammalian cells involves the STIM and Orai proteins, but our efforts to identify homologs of STIM and Orai in the *P. falciparum* genome have proved negative. This may reflect the fact that the counterparts of many mammalian proteins are not easily identified in *Plasmodium* on the basis of sequence homology. It is also possible that the SOCE in malaria uses a totally different mechanism. This may explain the lack of a role for SOCE in the melatonin-induced $[Ca^{2+}]_{cyt}$ increases observed in the malaria parasite. However, since melatonin mobilizes Ca^{2+} from a thapsigargin-sensitive intracellular store in P. falciparum [6,21,26], the lack of melatonin-activated SOCE is somewhat unexpected. One possible explanation is heterogeneity in the thapsigargin-sensitive Ca^{2+} store, since IP_3 releases only a subfraction of the Ca^{2+} that can be released by thapsigargin [26]. It is also noteworthy that the $[Ca^{2+}]_{cyt}$ increases following melatonin addition are relatively slow and, in contrast to thapsigargin, may not lead to complete emptying of the Ca^{2+} stores, as is generally required for full activation of SOCE.

Although there is no high affinity site for inhibition of SOCE by the 2-APB derivatives in the malaria parasite, the compounds 2-APB, DPB162-AE and DPB163-AE clearly affected parasite development during its intraerythrocytic life cycle at higher concentrations. As a result we observed a reduction in parasitemia after 24 and 48 h of treatment when compared with the control group. Beraldo et al. [37] reported that treatment of asynchronous *P. falciparum* (Palo alto strain) with 75 μM 2-APB abolished the effect of melatonin to stimulate progression through the intraerythrocytic life cycle, but did not see a reduction in parasitemia after 24 h. In our experiments, we used a synchronous culture of *P. falciparum* 3D7 strain, and all three compounds tested (2-APB, DPB162-AE and DPB163-AE) were also able to interfere with parasite life cycle, causing a delay or blocking parasite development, with the 2-APB derivatives being the most effective in this respect.

Both expression of IP_3-sponge and IP_3 sensor IRIS resulted in lethal outcome during the intraerythrocytic stage of *P. falciparum*. Despite the ability of both proteins to trap the cytosolic IP_3 of the cell, they have different binding affinity towards IP_3 [24]. Buffering the native IP_3 of the cell might compromised both inositol turnover as well as IP_3-Ca^{2+} dependent pathway, that might explain the vulnerability of malaria parasites in the presence of IP_3-sponge and IRIS. Our data suggests that constitutive expression of those proteins under a strong promoter is not an ideal strategy to investigation of IP_3/IP_3R dynamic in malaria parasites instead, the expression of IP_3-sponge and IRIS under an inducible system might be a better approach.

Taken together, the findings provide additional evidence that IP_3-dependent Ca^{2+} signaling is critical for parasite development in the intraerythrocytic stage, and for the control of this pathway by the host hormone melatonin. In addition, there is a mechanism for activation of SOCE in *P. falciparum* that does not show the typical mammalian cell pharmacology, but may also be linked to depletion of Ca^{2+} from intracellular stores. At the higher concentrations where they typically affect IP_3-induced Ca^{2+} release, 2-APB derivatives, DPB162-AE and DPB163-AE, were able to block the melatonin-induced $[Ca^{2+}]_{cyt}$ increase and SOCE in *P. falciparum*. These results are consistent with our

previous work suggesting that melatonin acts *via* an IP_3 receptor signaling mechanism in malaria parasites. The observation that the compounds impaired parasite development and invasion of erythrocytes, demonstrates the importance of Ca^{2+} signaling in the intraerythrocytic cycle of *P. falciparum*. These results reinforce the concept that the melatonin signaling pathway is a potentially interesting target for the development of antimalarial drugs.

Author contributions

C.R.S.G., A.T. and K.M. designed research and suggested the experiments. M.F.P., L.B.-P, J.L.-G., A.B. and E.A. performed the experiments and analyzed the data. C.R.S.G., A.T and K.M. discuss the results. All the authors contributed in writing the paper. Declaration of interest: none.

Conflict of interest

The authors declare that there are no conflict of interest.

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