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An interplay between 2 signaling pathways: Melatonin-cAMP and IP_3–Ca^{2+} signaling pathways control intraerythrocytic development of the malaria parasite *Plasmodium falciparum*

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ABSTRACT

Plasmodium falciparum spends most of its asexual life cycle within human erythrocytes, where proliferation and maturation occur. Development into the mature forms of *P. falciparum* causes severe symptoms due to its distinctive sequestration capability. However, the physiological roles and the molecular mechanisms of signaling pathways that govern development are poorly understood. Our previous study showed that *P. falciparum* exhibits stage-specific spontaneous Calcium (Ca^{2+}) oscillations in ring and early trophozoites, and the latter was essential for parasite development. In this study, we show that luzindole (LZ), a selective melatonin receptor antagonist, inhibits parasite growth. Analyses of development and morphology of LZ-treated *P. falciparum* revealed that LZ severely disrupted intraerythrocytic maturation, resulting in parasite death. When LZ was added at ring stage, the parasite could not undergo further development, whereas LZ added at the trophozoite stage inhibited development from early into late schizonts. Live-cell Ca^{2+} imaging showed that LZ treatment completely abolished Ca^{2+} oscillation in the ring forms while having little effect on early trophozoites. Further, the melatonin-induced cAMP increase observed at ring and late trophozoite stage was attenuated by LZ treatment. These suggest that a complex interplay between IP_3–Ca^{2+} and cAMP signaling pathways is involved in intraerythrocytic development of *P. falciparum*.

1. Introduction

Malaria continues to be a worldwide public health problem causing significant morbidity and mortality, and its resistance to existing antimalarial drugs is an escalating worldwide health problem [1]. *Plasmodium*, the causative agent of malaria, is a unicellular parasite that spends most of its life span within intact human hepatocytes (during the liver stage) and erythrocytes (during the blood stage) [2,3]. In particular, analysis of signaling pathways necessary for parasite growth during the blood stage is an important therapeutic target for antimalarial drug development since *Plasmodium* pathogenesis occurs at this stage, causing morbidity and mortality.

Calcium (Ca^{2+}) is a universal second messenger that regulates contraction, secretion, neuronal plasticity, cell differentiation and other physiological functions in the cells [4,5]. In apicomplexan parasites such as *Plasmodium*, *Toxoplasma* and *Cryptosporidium*, Ca^{2+}-mediated signaling controls various vital functions such as protein secretion, motility, cell invasion and differentiation [6–14]. With regard to intracellular Ca^{2+} signaling in *Plasmodium* species, numerous studies have focused on calcium-dependent protein kinases, which are activated downstream of Ca^{2+} release from intracellular Ca^{2+} stores [8,11]. However, little is known about the role of Ca^{2+} signaling in intraerythrocytic development of *Plasmodium* species [15,16].

We have recently reported novel data showing that spontaneous Ca^{2+} oscillations can be observed in the intraerythrocytic stages of *Plasmodium falciparum* [17]. These stage-specific

Abbreviations: 2-APB, 2-aminoethyl diphenylborinate; IP_3, inositol 1,4,5-trisphosphate; LZ, luzindole; PLC, phospholipase C; AC, adenylyl cyclase; PKA, protein kinase A; MDL, MDL12330A; IBMX, 3-isobutyl-1-methylxanthine.

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spontaneous Ca^{2+} oscillations observed in the ring forms and early trophozoites can be blocked by the inositol 1,4,5-trisphosphate (IP_3) receptor inhibitor, 2-aminoethyl diphenylborinate (2-APB). Moreover, we found that blockage of Ca^{2+} oscillations at early trophozoite stage by 2-APB caused severe degeneration and breakdown of successive asexual reproduction in the intraerythrocytic parasites, resulting in their death [17].

Melatonin is a neuroendocrine hormone secreted by the pineal gland in association with the suprachiasmatic nucleus and peripheral tissues. Melatonin can regulate intracellular processes including the activity of second messengers such as cAMP, Ca^{2+} and IP_3 [18]. Previous studies suggested that *P. falciparum* responds to exogenous melatonin administration by modulating its proliferation cycle *in vitro* [19], and the effect of melatonin appears to at least partially depend on the production of IP_3 [20,21]. Exogenous melatonin administration increases cytoplasmic Ca^{2+} and cAMP concentrations and synchronizes the *Plasmodium* cell cycle. These effects were reported to be blocked by a melatonin receptor antagonist luzindole (LZ) [19]. However, effect of these second messengers and downstream signaling pathways of melatonin-sensitive receptors in *P. falciparum* are not fully understood.

Thus, in this study, we investigated the link between melatonin-induced Ca^{2+} release and IP_3-dependent spontaneous Ca^{2+} oscillation at ring and trophozoite stages of the FCR-3 strain of *P. falciparum*. We found that Ca^{2+} oscillation at the ring form stage was blocked by LZ, whereas the compound could not completely inhibit Ca^{2+} oscillation at the early trophozoite stage, unlike 2-APB. However, LZ treatment produced severe degeneration and breakdown of successive asexual growth in the intraerythrocytic parasites, resulting in their death. In addition, melatonin could induce increases in cAMP concentration in the parasite cells at ring form and late trophozoite stage, and the effects of melatonin could be abolished by LZ treatment. Therefore, our present findings suggest that intraerythrocytic development of *P. falciparum* parasites depends on both a cAMP signaling pathway situated downstream of the melatonin-sensitive receptor, and the IP_3–Ca^{2+} signaling pathway.

2. Materials and methods

2.1. P. falciparum culture

P. falciparum (FCR-3 strain) parasites were maintained in cultures as previously described [22]. Briefly, *P. falciparum* were cultured in RPMI medium (Invitrogen) supplemented with 25 mM HEPES; 50 mg/l hypoxanthine; 24 mM sodium bicarbonate; 25 μg/ml gentamicin; 0.5% Albumax I (BSA; Invitrogen) and human erythrocytes at a hematocrit of 5% in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. Synchronization of the parasites in culture was achieved by 5% D-sorbitol treatment [23].

2.2. Fluorescence Ca^{2+} imaging

Cultures of infected erythrocytes were diluted 10-fold with BSA-free medium for Ca^{2+} imaging (RPMI 1640 medium without phenol red; Invitrogen) supplemented with 25 mM HEPES, 24 mM sodium bicarbonate, 0.5 g/l L-glutamine and 50 mg/l hypoxanthine. Parasite loading with Ca^{2+} indicator (Fluo-4-AM) and nuclei indicator (Hoechst 33342) was performed as previously described [17]. Sequential time-lapse imaging of Hoechst 33342, Fluo-4-AM and transparent images was performed using the Leica confocal microscope system (Leica TCS SP5; Leica Microsystems) with a 40× oil immersion objective lens and excitation at 410 nm (Diode laser) for Hoechst 33342 and 488 nm (Argon laser) for Fluo-4-AM and transparent images. Emissions were collected using the true spectral detection method developed by Leica Microsystems. Images were captured every 5–15 s until 300–600 s.

2.3. Inhibition of P. falciparum development by LZ and MDL12330A

The concentrations of LZ (Santa Cruz Biotechnology) and MDL12330A (MDL) (Santa Cruz Biotechnology) used in this study were determined by preliminary experiments with various concentrations ranging from 62.5 to 250 μM and from 25 to 50 μM respectively (data not shown). Effects of LZ and MDL on the intraerythrocytic development of the parasite were assayed using parasite cultures at ring form with initial parasitemia of approximately 0.5–1.5%. Cultures (500 μl) were placed in each well of a tissue culture plate (Corning). LZ and MDL were dissolved in DMSO Hybri-Max® (Sigma–Aldrich) at 100 mM. Stock solutions were diluted with RPMI 1640 medium and added to each well of the culture plate to give the specified concentration. DMSO diluted with medium served as control. After incubation, the effect of LZ and MDL was scored by microscopic examination of Giemsa-stained thin blood smears [17].

2.4. cAMP enzyme immunoassay

P. falciparum-infected erythrocytes were synchronized by D-sorbitol [23]. Parasites were released from infected erythrocytes by 0.05% (w/v) saponin treatment [24]. The parasite cells were resuspended in culture buffer at a concentration of 2×10^7 parasites/ml and incubated for 10 min with or without 250 μM LZ or 50 μM MDL. After the treatment, parasite cells were washed and resuspended in the culture buffer at the same concentration and incubated for 15 min with or without 100 nM melatonin (Nacalai Tesque, Kyoto, Japan) in the presence of 100 μM 3-isobutyl-1-methylxanthine (IBMX) (Wako, Osaka, Japan), a phosphodiesterase inhibitor, to prevent cAMP degradation. Samples were analyzed using the cAMP enzyme immunoassay kit (GE Healthcare), according to the manufacturer's instructions (protocol No. 4).

3. Results

3.1. Melatonin receptor antagonist LZ blocks Ca^{2+} oscillation in P. falciparum at ring stage

As demonstrated in our previous study [17], spontaneous Ca^{2+} oscillations were observed in early ring forms and early trophozoites (Fig. 1A and C). Treatment of the early ring form with 250 μM LZ completely blocked Ca^{2+} oscillation (Fig. 1B). In contrast, LZ did not have a substantial effect on Ca^{2+} oscillation at the early trophozoite stage (Fig. 1D). DMSO used as solvent control did not affect the Ca^{2+} oscillation seen in early ring forms and early trophozoites (Fig. 1A and C). To investigate the effects of LZ in detail, we performed quantitative analysis of the amplitude of periodic Ca^{2+} fluctuations in early ring form and early trophozoite stages (Fig. 1E). A statistically significant effect of LZ was observed in the ring form ($p < 0.05$), strongly suggesting that at the ring form stage, regulation of Ca^{2+} oscillation lies downstream of a melatonin-sensitive receptor.

3.2. LZ and MDL block intraerythrocytic development of P. falciparum

The effect of LZ on intraerythrocytic development of *P. falciparum* FCR-3 strain was examined. Synchronized parasite cultures at ring form with initial parasitemia of approximately 1% were used for this assay. Treatment of the culture with LZ at 250 μM delayed intraerythrocytic development of the parasites as compared to that in the DMSO-treated control culture (Figs. 2A and S2A).

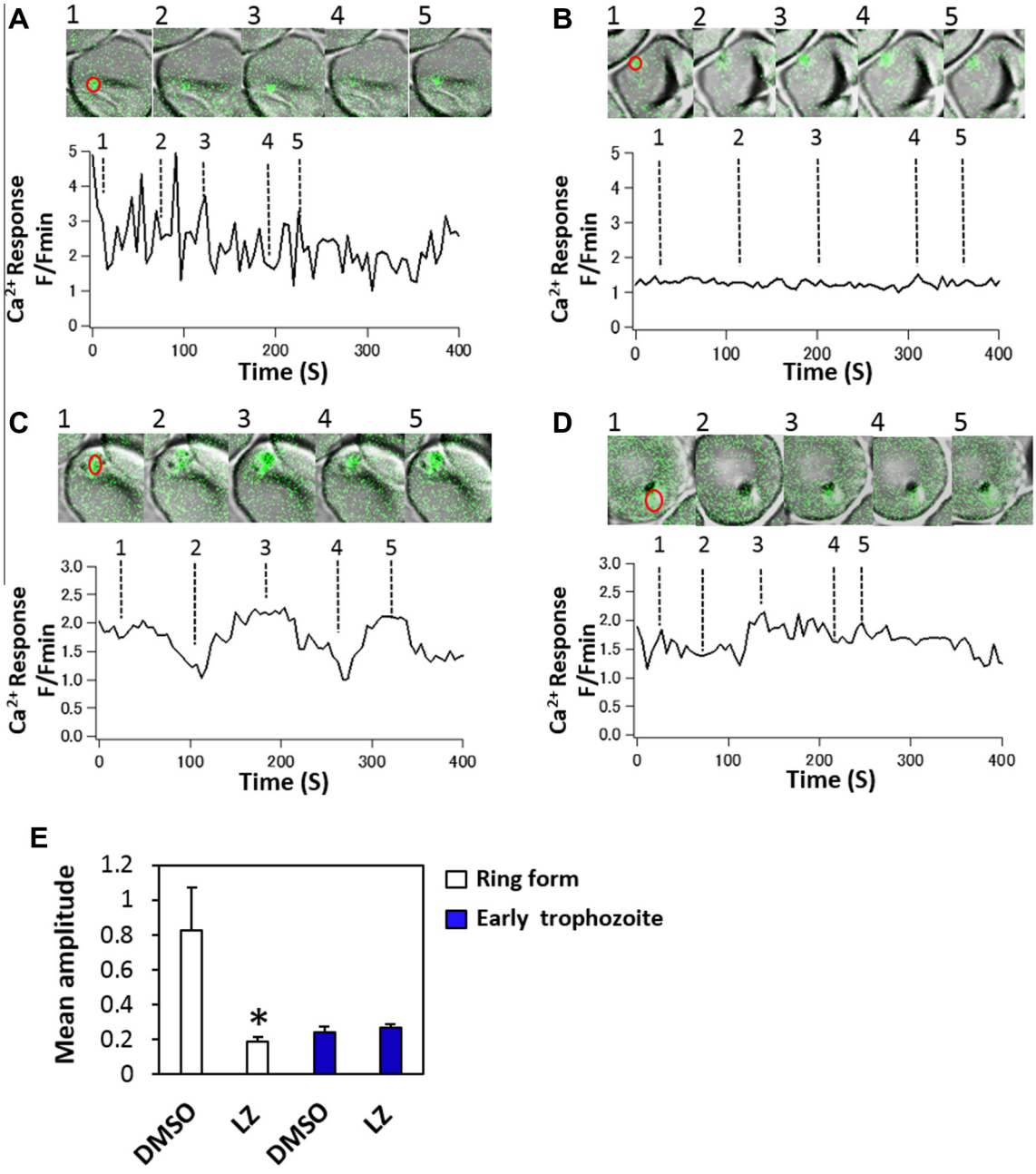

Fig. 1. Effects of luzindole (LZ) on cytosolic Ca^{2+} dynamics in parasite cells. Figures represent cytosolic Ca^{2+} dynamics in the ring (A, B) and early trophozoite forms (C, D). The cytosolic Ca^{2+} dynamics were acquired from individual parasites in the presence (B, D) or absence (A, C) of 250 μM LZ. Ca^{2+} imaging of parasites was performed in culture chambers at 37 °C with 5% O_2 and 5% CO_2. The Fluo-4 fluorescence in parasite cytoplasm (F) was calculated by subtraction of the background fluorescence and normalized to the minimum fluorescence during the imaging period (F_{min}). In each experiment, more than 3 independent parasites were observed and representative data is shown. The data for statistical analysis were obtained at five points indicated by dotted lines (1–5). Images (1–5) above each graph show the fluorescence/differential interference contrast (DIC) time-lapse images at these timings in cytoplasm. The red circle represents the region of interest (ROI), defined for data acquisition. Panel E shows effects of LZ on mean amplitude of periodic Ca^{2+} fluctuations as calculated by the difference of the mean minimal and maximal value of F/F_{min}. Representative data was shown as mean + standard deviation (SD) ($n = 3$, $^*p < 0.05$, Two-tailed Student t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Parasites cultured with DMSO developed into early schizonts (parasites with fewer than 8 nuclei) at the 20-h time point of the assay. These schizonts developed into late schizonts (parasites with at least 8 nuclei) and produced ring forms in the next developmental cycle at the 40-h time point. In contrast, parasites cultured with 250 μM LZ either remained in ring form (parasites with smaller cell size than trophozoite) or developed into trophozoites (parasites with a single nucleolus and hemozoin deposits) both with abnormal morphology (shrinkage of the parasite and nuclear concentration) at 20 h of the assay. These abnormal ring forms and trophozoites could not develop further and remained as those stages when examined at 40 h and 70 h of the assay. Parasites could develop normally in LZ-pre-treated erythrocytes similar to that in cultures with DMSO-pre-treated erythrocytes (Fig. S1). Next we examined the effect of MDL, an inhibitor of adenylyl cyclase, on intraerythrocytic development of the parasite to investigate signaling pathways that lie downstream of the melatonin-sensitive receptor. It has been reported in *Plasmodium* sporozoites that

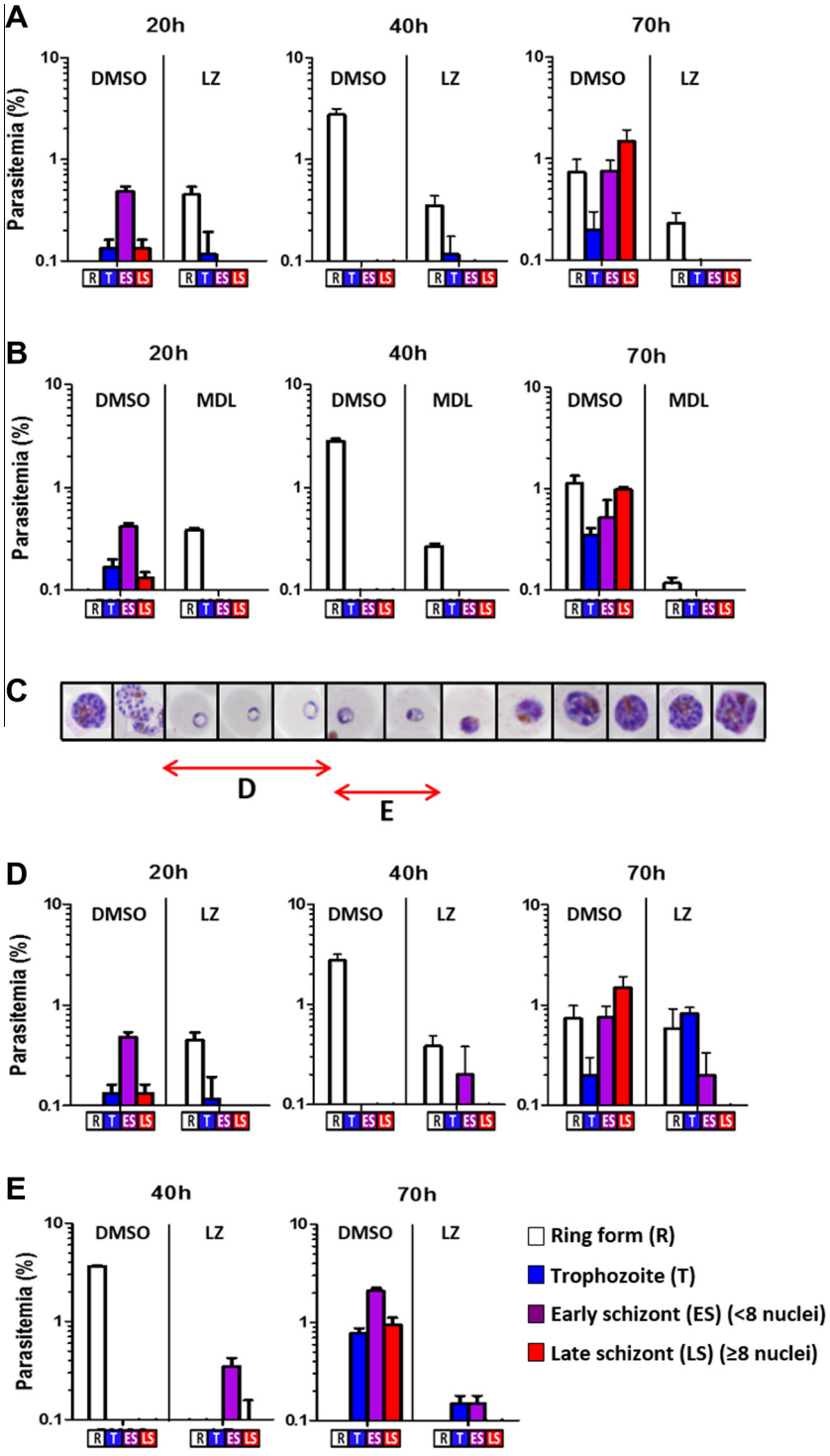

Fig. 2. Inhibition of intraerythrocytic *P. falciparum* development by luzindole (LZ) and MDL12330A (MDL). The FCR-3 strain was cultured for 40 h of the intraerythrocytic cycle, synchronized at ring forms, and terminated at assay time points 20, 40, and 70 h post-synchronization. Thin smears were prepared for parasite counting and parasitemia of each developmental stage is shown as mean + SD. Stages with parasitemia of less than 0.1% are not shown. DMSO represents solvent control. (A) Parasites in the presence or absence of 250 μM LZ. (B) Parasites in the presence or absence of 50 μM MDL. Culture medium with DMSO or the compounds was replaced at 40 h. (C) LZ administration time points; each box represents one stage in the intraerythrocytic life cycle of *P. falciparum*. (D) LZ was removed at 20 h from the assay. (E) Cells were treated with LZ (250 μM) from 20 to 30 h. Representative results are shown as mean + SD of 3 independent counts of 3 wells. Stages with parasitemia of less than 0.1% are not shown. DMSO represents solvent control. R, ring form; T, trophozoite, ES, early schizont; LS, late schizont.

MDL inhibits cAMP production [25]. Parasites cultured with 50 μM MDL remained as abnormal ring forms and trophozoites at 20 h of the assay; these parasites could not develop into the schizont stage (Figs. 2B and S2B). The effect of MDL on parasite development was quite similar to that of LZ (Fig. 2A and B). Fig. 2 depicts the inability of LZ- and MDL-treated parasites to develop into the late trophozoite form.

To determine which stages of parasite development are affected by LZ treatment, the compound was removed from, or added to, cultures at various time points (Fig. 2C). When LZ was removed

from the culture at the ring stage (20-h assay time point), parasite development was delayed compared with that of the control culture (Fig. 2D, 20 h). However, after removal of LZ, these parasites developed into trophozoites with normal morphology and could develop into the ring stage in the next developmental cycle (Fig. 2D, 40 h and 70 h). When LZ was added at the trophozoite stage (20 h of the assay) and removed 30 h into the assay, the parasites developed into early schizonts with abnormal morphology at the 40-h time point of the assay, and they did not develop into the late schizont stage (Fig. 2E). When LZ was removed from culture at the 40-h time point of the assay, the ring forms and trophozoites with abnormal morphology, similar to that observed in the growth inhibition experiment (Fig. 2A), did not develop further and were incapable of producing ring forms in the next developmental cycle (Fig. S2C). These results clearly demonstrate that LZ affected both the ring form and the trophozoite stage, resulting in delayed development into trophozoite and inhibition of development into late schizont, respectively.

3.3. Melatonin increases cAMP levels in P. falciparum

The effect of melatonin on cAMP levels in *P. falciparum* at ring form and early and late trophozoite stages was evaluated with isolated parasites liberated from host erythrocytes (Fig. 3A). Addition of 100 nM melatonin to the isolated ring form and late trophozoite stage led to a significant increase in cAMP concentration compared with untreated controls ($p < 0.05$). However, cAMP levels at the early trophozoite were not increased by the presence of melatonin ($p > 0.05$). Further, LZ treatment abolished the increase in cAMP concentration in both ring form and late trophozoite stage induced by melatonin ($p < 0.05$). The increase in cAMP concentration was abolished by MDL treatment ($p < 0.05$) (Fig. 3B). These results indicate that the cAMP signaling pathway at the ring form and trophozoite stage lies downstream of the melatonin-sensitive receptor.

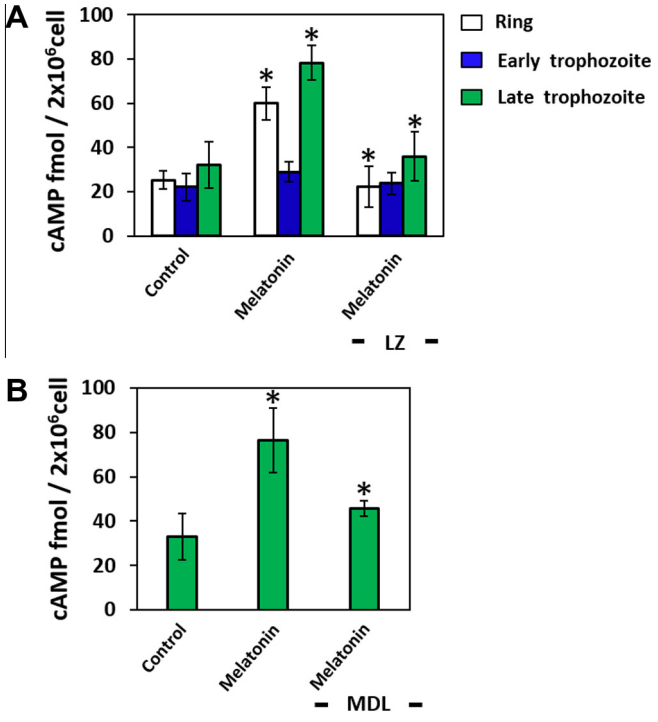

Fig. 3. Measurement of cAMP concentration in *P. falciparum* parasites. Experiments were performed at ring and trophozoite stages. The parasite cells were treated with 100 nM melatonin in the presence of 100 μM IBMX. Cells were incubated with 250 μM luzindole (LZ) (A) and 50 μM MDL12330A (MDL) (B) at 10 min. Data in (A) are mean of duplicates of 3 independent experiments ± SD whereas in (B) are mean ± SD of triplicated experiments with late trophozoite stage (*$p < 0.05$, Two-tailed Student *t*-test).

4. Discussion

The elucidation of signal transduction pathways in *P. falciparum* is fundamental in facilitating development of new anti-malarial strategies. Recent studies have begun to identify components of intracellular signaling cascades in *P. falciparum* [17,19,26–28]. However, it remains unclear when and how these signaling molecules act to trigger the parasite's maturation, division, differentiation and reinvasion during the asexual stage within erythrocytes. There is substantial evidence that *P. falciparum* possesses the molecular machinery for IP_3-dependent signaling [19,26]. Additional reports indicate that host melatonin is able to induce Ca^{2+} release from cultured *P. falciparum* [27]. However, our previous study has shown IP_3-dependent spontaneous Ca^{2+} oscillation in *P. falciparum* in the absence of exogenous stimulation [17]. In the present study, we therefore investigated the possible connection between melatonin-induced Ca^{2+} release and IP_3-dependent spontaneous Ca^{2+} oscillation.

Our previous study demonstrated that *P. falciparum* exhibited stage-specific spontaneous Ca^{2+} oscillations in ring forms and early trophozoites, which in the latter was essential for parasite development [17]. Blockade of spontaneous Ca^{2+} oscillation by LZ in early ring forms suggests that Ca^{2+} oscillation at this stage lies downstream of a melatonin-sensitive receptor. However, Ca^{2+} oscillation in trophozoites could not be blocked by LZ. This result indicates that Ca^{2+} oscillation in early trophozoites, which is indispensable for parasite development, is mediated by an unknown LZ-insensitive receptor. In contrast, measurements of parasitemia revealed that LZ inhibits *P. falciparum* development, suggesting that downstream of the melatonin sensitive receptor, another signaling pathway, in addition to the IP_3–Ca^{2+} signaling pathway, may be involved in parasite growth. This notion was proposed in recent studies showing that melatonin regulates multiple intracellular second messengers including cAMP, IP_3 and Ca^{2+} in *P. falciparum* [19,26–28]. It is also well demonstrated that cAMP is the major effector of adenylyl cyclase in *P. falciparum* [28]. We therefore tested whether an AC inhibitor, MDL, could block the parasite's development. The results indicated that the mode of MDL-induced growth arrest was similar to that observed with LZ. These results strongly suggest that intraerythrocytic development of *P. falciparum* depends on melatonin sensitive receptor – AC signaling pathways.

When LZ was added during the ring form (0–20 h), the parasite's growth was delayed compared to control, whereas when LZ was added during the trophozoite stage (20–30 h), early schizonts could not mature into late schizonts. These results indicate that LZ has stage-specific effects on the parasite development, namely, arrest of the parasite's development at the ring form stage and inhibition of maturation of early schizonts into late schizonts. Our experiments demonstrate that melatonin is able to increase cAMP levels during the ring form stage and late trophozoite stage and that LZ blocks these effects, suggesting that the cAMP signaling pathway in both ring form and late trophozoite stage was mediated by a melatonin-sensitive receptor. While a recent study provides evidence that melatonin can increase cAMP levels during the trophozoite stage [28], to our knowledge, the current study represents the first report of melatonin-induced cAMP upregulation at the ring stage.

Intraerythrocytic development of *P. falciparum* parasites occurs via a series of complex signaling pathways in which second messengers such as IP_3, Ca^{2+} and cAMP appear to play key, yet largely

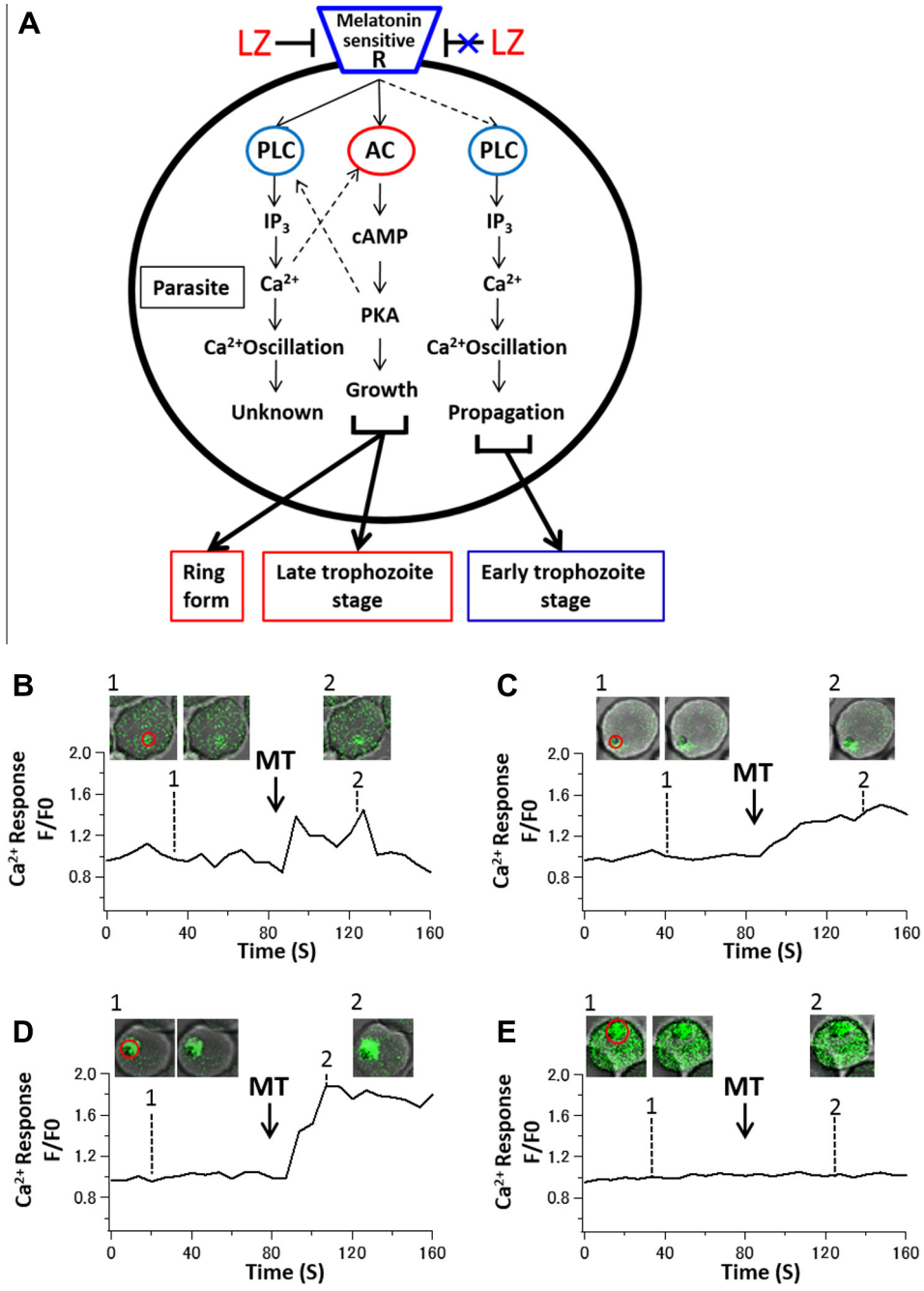

Fig. 4. Schematic model of the signaling events in intraerythrocytic *P. falciparum* development. Panel A represents the signaling events in intraerythrocytic *P. falciparum* development. LZ, luzindole; R, receptor; PLC, phospholipase C; AC, adenylyl cyclase; PKA, protein kinase A; IP_3, inositol 1,4,5-trisphosphate. Panels B–E show the effect of melatonin (MT) on cytosolic Ca^{2+} concentration in ring form (B), early trophozoite (C), late trophozoite (D), and schizont (E). Cytosolic Ca^{2+} elevation was induced by 100 nM MT in ring form, early trophozoite or late trophozoite but not in schizont. Ca^{2+} imaging was performed as described at the Materials and methods section and analyzed as follows: the Fluo-4 fluorescence in parasite cytoplasm (F) was calculated by subtraction of the background fluorescence and normalized by the average fluorescence obtained before MT was added (F0). Images (1, 2) above each graph show the fluorescence/DIC time-lapse images in parasite cytoplasm at the time points indicated (dotted lines). Red circle represents ROI set for data acquisition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unidentified, roles. In the present study we investigated cAMP signaling mechanisms in the context of the modulatory effects of host-derived melatonin on the cell cycle. Exogenous melatonin stimulation to the parasite cells appears to act via at least two signaling pathways (IP_3–Ca^{2+} and cAMP), which act in concert to control parasite development during the trophozoite stage in *plasmodium* species [29].

Taken together with our previous results [17], the results of the present study suggest that the parasite's cell cycle is controlled by melatonin-sensitive receptor via two second-messenger pathways: the cAMP signaling pathway, which regulates the parasite's growth in the ring form and late trophozoite stages, and the IP_3–Ca^{2+} signaling pathway, which modulates parasite proliferation during the early trophozoite stage (Fig. 4A).

Exogenous melatonin induced Ca^{2+} release at ring form and trophozoite stage, but not schizont stage (Fig. 4B–E). These results indicate the presence of a melatonin-sensitive receptor at these stages. However, spontaneous Ca^{2+} oscillation was not observed in our previous study of the late trophozoite stage [17]. Furthermore, a recent study showed that the Ca^{2+} rise in the parasites

initiates an amplification loop via cAMP and PKA that then further modulates the Ca^{2+} signal [28]. Taken together, Ca^{2+} release induced by melatonin at the late trophozoite stage might be attributed to cross-talk between IP_3–Ca^{2+} and the cAMP signaling pathway downstream of the melatonin-sensitive receptor. In contrast, weaker melatonin-induced Ca^{2+} release was observed at the early trophozoite stage (Fig. 4C), possibly indicating the presence of a melatonin-sensitive Ca^{2+} release pathway in this stage. However, this was not a major pathway generating Ca^{2+} oscillation at this essential stage in the development of the parasite [17], since the Ca^{2+} oscillation was not blocked by LZ. Our results suggest that LZ-resistant IP_3–Ca^{2+} signaling pathway downstream of melatonin-sensitive receptor or IP_3–Ca^{2+} signaling pathway downstream of an unknown LZ-insensitive receptor in the early trophozoite stage may instead have an important role in parasite development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.070.

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