Cyclic AMP and calcium interplay as second messengers in melatonin-dependent regulation of *Plasmodium falciparum* cell cycle

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The host hormone melatonin increases cytoplasmic Ca^{2+} concentration and synchronizes *Plasmodium* cell cycle (Hotta, C.T., M.L. Gazarini, F.H. Beraldo, F.P. Varotti, C. Lopes, R.P. Markus, T. Pozzan, and C.R. Garcia. 2000. *Nat. Cell Biol.* 2:466–468). Here we show that in *Plasmodium falciparum* melatonin induces an increase in cyclic AMP (cAMP) levels and cAMP-dependent protein kinase (PKA) activity (40 and 50%, respectively).

When red blood cells infected with *P. falciparum* are treated with cAMP analogue adenosine 3′,5′-cyclic monophosphate N6-benzoyl/PKA activator (6-Bz-cAMP) there is an alteration of the parasite cell cycle. This effect appears to depend on activation of PKA (abolished by the PKA inhibitors adenosine 3′,5′-cyclic monophosphorothioate/8 Bromo Rp isomer, PKI [cell permeable peptide], and H89). An unexpected cross talk was found to exist between the cAMP and the Ca^{2+}-dependent signaling pathways. The increases in cAMP by melatonin are inhibited by blocker of phospholipase C U73122, and addition of 6-Bz-cAMP increases cytosolic Ca^{2+} concentration, through PKA activation.

These findings suggest that in *Plasmodium* a highly complex interplay exists between the Ca^{2+} and cAMP signaling pathways, but also that the control of the parasite cell cycle by melatonin requires the activation of both second messenger controlled pathways.

Introduction

Plasmodium, the etiological agent of malaria and a major cause of death in the third world, is a unicellular parasite that in humans spends most of its life span within intact hepatocytes and red blood cells (RBCs) (Aravind et al., 2003; Bannister and Mitchell, 2003). Within the latter cells, in particular, *Plasmodium* multiplies and maturates in the forms ready to invade other erythrocytes. The increase in drug resistance of malaria parasites (Hall et al., 2003; Le Bras and Durand, 2003; Snow et al., 2005) is a dramatic and worrisome phenomenon that calls for an urgent elucidation of the mechanisms by which the parasite controls its developmental events (Bozdech et al., 2003). Although the *Plasmodium* genome sequencing is now complete, 60% of the proteins do not have sufficient similarity to any proteins in other organisms (Gardner et al., 2002) to allow comparative studies to be easily performed.

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Abbreviations used in this paper: 6-Bz-cAMP, adenosine 3′,5′-cyclic monophosphate N6-benzoyl/PKA activator; 8-BrcAMP-RP isomer, adenosine 3′,5′-cyclic monophosphorothioate/8 Bromo Rp isomer; H89 and PKI, PKA inhibitors; IBMX, 3-isobutyl-1-methylxanthine; PKA, cAMP-dependent protein kinase; RBC, red blood cell.

We have reported that Plasmodia have subverted the host endocrine system using the hormone melatonin to modulate its cell cycle (Hotta et al., 2000). The effect of melatonin appears to depend, at least in part, on the production of $InsP_3$, a well-characterized second messenger for Ca^{2+} mobilization from intracellular organelles (Pozzan et al., 1994; Berridge et al., 2003). We have also recently shown that the *Plasmodium,* once it has infected the RBCs, creates around itself a microenvironment, the parasitophorous vacuole, rich in Ca^{2+}, that is necessary to fully exploit the Ca^{2+} signaling pathway (Camacho, 2003; Gazarini et al., 2003). These and other data from different laboratories support the notion that Plasmodia, as most other eukaryotic cells, use the Ca^{2+} signaling pathway for the control of a number of vital functions (Passos and Garcia, 1998; Garcia, 1999; Garcia et al., 1996, 1998; Hotta et al., 2000; Marchesini et al., 2000; Alleva and Kirk, 2001; Varotti et al., 2003), primarily their progression throughout the cell cycle. Of interest, triptophane-related molecules could also induce Ca^{2+} release in *Plasmodium* and modulate its cell cycle (Beraldo and Garcia, 2005). The role of Ca^{2+} in *Plasmodium* cycle remains to be investigated although its transient rise was shown

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by internally quenched fluorescent peptides to activate parasite thiol proteases (Farias et al., 2005).

Relatively more scarce is the understanding of the importance of the other ubiquitous second messenger, cAMP, though evidence suggests that cAMP is also implicated in *Plasmodium* maturation and/or differentiation. cAMP has been in fact reported to promote in vitro gametocytogenesis (Kaushal et al., 1980; Trager and Gill, 1989; Dyer and Day, 2000) and to impair maturation of merozoite within RBCs (Inselburg, 1983). Furthermore an increase of both adenylyl cyclase and cAMP-dependent protein kinase (PKA) activities accompanies *Plasmodium falciparum* differentiation (Read and Mikkelsen, 1991a,b) whereas inhibition of PKA activity blocks parasite multiplication. The importance of cAMP in *Plasmodium* differentiation to gametocytes, the mosquito-infective form, has been known for a long time (Trager and Gill, 1989).

The molecular machinery controlling cAMP production, degradation, and sensitivity of Plasmodia appear to be similar to that of higher eukaryotes. Thus a gene encoding the catalytic subunit of PKA (PKA-C) from the rodent and human malaria parasites has been cloned (Li and Cox, 2000; Ward et al., 2004); similarly, genes encoding a subunit resembling the mammalian PKA regulatory subunits, PKA-R, the cAMP-degrading enzyme phosphodiesterases and the adenylyl cyclase are all known to be present in the *Plasmodium* genome (Gardner et al., 2002). Last, but not least, PKA-C transcript levels are higher in intraerythrocytic stages, decreasing in gametocytes and gametes (Ward et al., 2004).

Given that in high eukaryotes there are complex synergistic and antagonistic effects between Ca^{2+} and cAMP (Bruce et al., 2003), we decided to investigate whether this could also be true in Plasmodia. In particular we addressed the question of whether melatonin affects not only the Ca^{2+} signaling pathway, but also that controlled by cAMP. Our results demonstrate that the host hormone not only regulates both second messengers, but also that they influence each other and both contribute to the control of the parasite cycle.

Results

Melatonin increase cAMP levels in *Plasmodium*: PLC inhibition impairs melatonin action

To investigate the effects of melatonin on cAMP levels in Plasmodia, isolated *P. falciparum* parasites, at the throphozoite stage, free of host cells to avoid interference from cAMP of RBCs, were treated with 100 nM melatonin. Fig. 1 shows that addition of the hormone leads to an increase of cAMP from 82.2 ± 5.0 fmoles/μg protein to 125.2 ± 3.0 fmoles/μg protein in the presence of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor (100 μM), although in the absence of IBMX the cAMP levels rose from 21.8 ± 0.1 fmoles/μg protein to 42.0 ± 5.0 fmoles/μg protein. Unexpectedly the increases in cAMP caused by melatonin are inhibited by blockers of phospholipase C such as U73122 (10 μM; Fig. 1), suggesting an important role of Ca^{2+} in the control of cAMP production/degradation. To investigate if the rise of Ca^{2+} per se could lead to a cAMP increase,

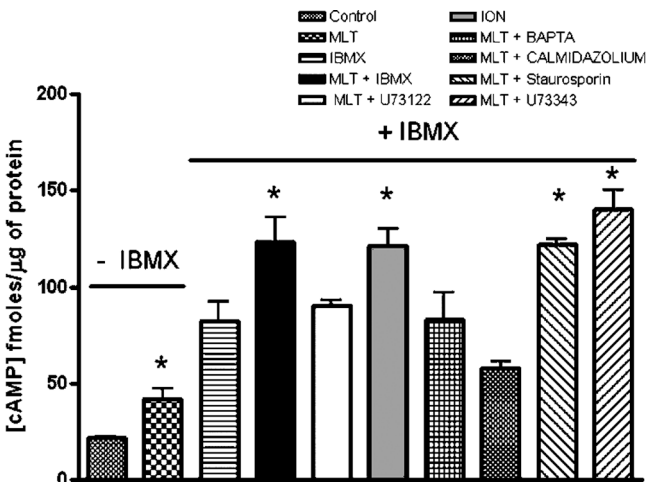

Figure 1. **Measurement of cAMP levels in *P. falciparum* parasites.** Experiments were performed by using isolated parasites at trophozoite stage treated with 100 nM melatonin in the presence or absence of 100 μM IBMX. Cells were incubated with: 10 μM U73122, 10 μM ionomycin, 25 μM BAPTA, 10 μM calmidazolium, 1 μM staurosporin, and 10 μM U73343. After treatment, samples were analyzed with cAMP enzyme immunoassay kit (Amersham Biosciences). There was a significant variation with melatonin addition (one-way analysis of variance vs. Newman-Keuls test, $P < 0.05$). Results represent duplicated data from three different experiments.

we added the Ca^{2+} ionophore ionomycin (10 μM) to the parasites. Fig. 1 shows that the Ca^{2+} ionophore causes an increase of cAMP similar to that observed with melatonin. Moreover the melatonin effect was blocked by the intracellular Ca^{2+} chelator (25 μM BAPTA) and by the calmodulin inhibitor (10 μM calmidazolium). No increase of cAMP by melatonin was observed by treating the parasites with the kinase inhibitor (1 μM staurosporin) or with the inactive analogue of the PLC inhibitor U73343 (10 μM; Fig. 1). As previously reported (Read and Mikkelsen, 1990; Muhia et al., 2003) forskolin has no effect on *Plasmodium* cAMP levels (unpublished data).

Cell cycle development is modulated by cAMP signaling pathway

As the above data indicates that melatonin can increase cAMP levels, we investigated whether PKA inhibitors of cAMP action such as adenosine 3′,5′-cyclic monophosphorothioate/8 Bromo Rp isomer (8-BrcAMP-RP isomer), PKI, and H89 or PKA activators such as adenosine 3′,5′-cyclic monophosphate N6-benzoyl/PKA activator (6-Bz-cAMP), that can cross cell membranes, had any effect on parasite cell cycle. Experiments were performed by incubating the drugs (for 24 h) with asynchronous *P. falciparum* cultures followed by fixation of the cells and counting of the parasites and of their differentiation stages. Addition of 100 nM melatonin, as reported previously (Hotta et al., 2000), induced an increase of the schizont stage of 158% with a concomitant decrease of the ring and trophozoite stages of 50 and 13%, respectively (Fig. 2 A). When the *P. falciparum* cultures were treated with the PKA activator, 6-Bz-cAMP (20 μM) we observed an increase of the schizont stage similar to that observed with melatonin (Fig. 2 A). Addition of IBMX alone also induced an increase of the schizont stage of 76% (unpublished data). The

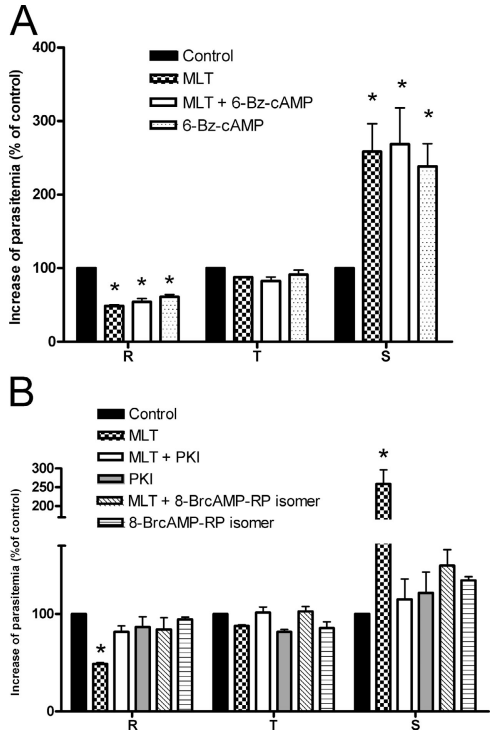

Figure 2. **Effect of cAMP signaling drugs on *P. falciparum* cell cycle development in vitro.** Incubation for 24 h at 37°C in 24-well plates with reagents: 100 nM MLT, 20 μM PKI, 20 μM 8-BrcAMP-RP isomer, and 20 μM 6-Bz-cAMP. Values are the percentage of parasitemia variation to control: (A) R, ring; T, trophozoite; and S, Schizont parasites stages; and (B) total parasitemia. The parasitemia (percentage of red cells infected) was determined by count of infected cells (1,000) from three different experiments. In these experiments the initial parasitemia was around 5% and the maximal parasitemia was around 15%. Data were compared by one-way analysis of variance and a Newman-Keuls test. *, Statistical significance with respect to control values $P < 0.01$.

effects of melatonin, in the increasing of schizont stage, were inhibited by the PKA inhibitors PKI (20 μM; increase of 14% in relative to control), 8-BrcAMP-RP isomer (increase of 49% relative to control; Fig. 2 B), and H89 (inhibition of 11% relative to control; unpublished data).

PKA activity in *Plasmodium* parasites

To further investigate the involvement of PKA as a transducer of melatonin action on *Plasmodium* cell cycle we measured PKA activity upon hormone treatment in cell extracts of *P. falciparum* (at the throphozoite stage). The percentage of PKA activity, as stimulated by each treatment, is compared with the maximal PKA activity, as obtained upon addition to the assay of 1 μM cAMP. Fig. 3 shows that activity of PKA in nonstimulated cultures of *P. falciparum* is 7.2 ± 1.2% of maximal activity. Treatment with 100 nM melatonin induced an increase a PKA activity to 39.3 ± 7.8% of maximum. Addition of 100 μM IBMX alone increased PKA activity to 26.5 ± 7.8%, consistent with the results obtained in intact cells demonstrating that this phosphodiesterase inhibitor alone increases parasite cAMP levels. Finally the treatment with 100 nM melatonin plus 100 μM IBMX increased PKA activity to 57.3 ± 7.4% of maximum. Addition to the assay of PKI, a specific PKA inhibitor, completely abolished cAMP induced phosphorylation of kemptide (unpublished data). A similar effect was observed when cells were treated with H89 before the measurements of PKA in the cell extracts (unpublished data).

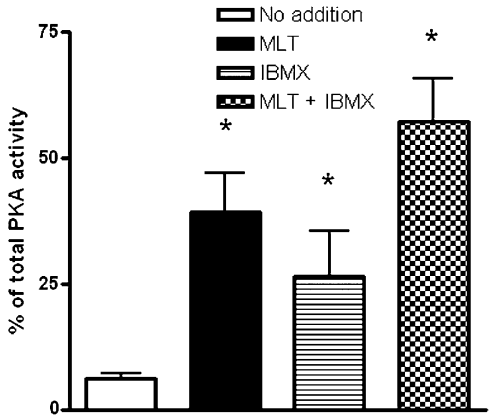

Figure 3. **PKA activity levels in *P. falciparum* parasites.** Isolated parasites at trophozoite stage treated with 100 nM MLT, 100 μM IBMX, or MLT and IBMX. Data was calculated from duplicated data from three different experiments assays, as a percentage of total PKA activity obtained with an additional 1 μM cAMP.

Ca^{2+} and cAMP interplay

Fig. 4 shows that addition of 100 nM melatonin to isolated *P. falciparum* parasites at trophozoite stages, loaded with calcium indicator Fluo-4, induced a rapid increase of cytoplasmic Ca^{2+} concentration. We observed the same rise when melatonin was added in a medium containing 2 mM $CaCl_2$ (Fig. 4 A), in a Ca^{2+} free medium (+ 100 μM EGTA; Fig. 4 B) or when parasites were incubated with the PKA inhibitor PKI (Fig. 4 C). These results indicated that the increase of Ca^{2+} induced by melatonin is independent of extracellular Ca^{2+} or PKA activation.

Addition of the cAMP analogue 6-Bz-cAMP to the parasites, in a medium with 2mM $CaCl_2$, induced a cytoplasmic [Ca^{2+}] increase similar to that observed with melatonin (Fig. 4 D). However, in Ca^{2+}-free medium the increase of cytoplasmic [Ca^{2+}] promoted by 6-Bz-cAMP was transient, unlike observed in Ca^{2+} medium (Fig. 4 E). To investigate if cAMP itself or PKA activation were responsible for the increases of citosolic [Ca^{2+}] we added the cAMP analogue, 6-Bz-cAMP in *P. falciparum* loaded with Fluo-4 in the presence of the PKA inhibitor PKI. Under these conditions, 6-Bz-cAMP did not promote an increase of cytoplasmic Ca^{2+}, thus indicating that the Ca^{2+} increase caused by cAMP (Fig. 4 F) depends on the activation of PKA. Finally 6-Bz-cAMP was added to cells pretreated with the PLC inhibitor U73122 (Fig. 4 G). Unlike the results obtained with melatonin (where the Ca^{2+}increase is completely blocked by the drug) in the case of 6-Bz-cAMP the increase in cytoplasmic Ca^{2+} was insensitive to the PLC inhibitor, suggesting that either PKA is able to promote an increase of cytosolic Ca^{2+} independently of PLC or that the kinase activates a PLC isoform insensitive to U73122.

To better understand the origin, mechanism and possible interactions of the cytoplasmic Ca^{2+} increases caused by melatonin and PKA activators, in the experiments presented in Fig. 5

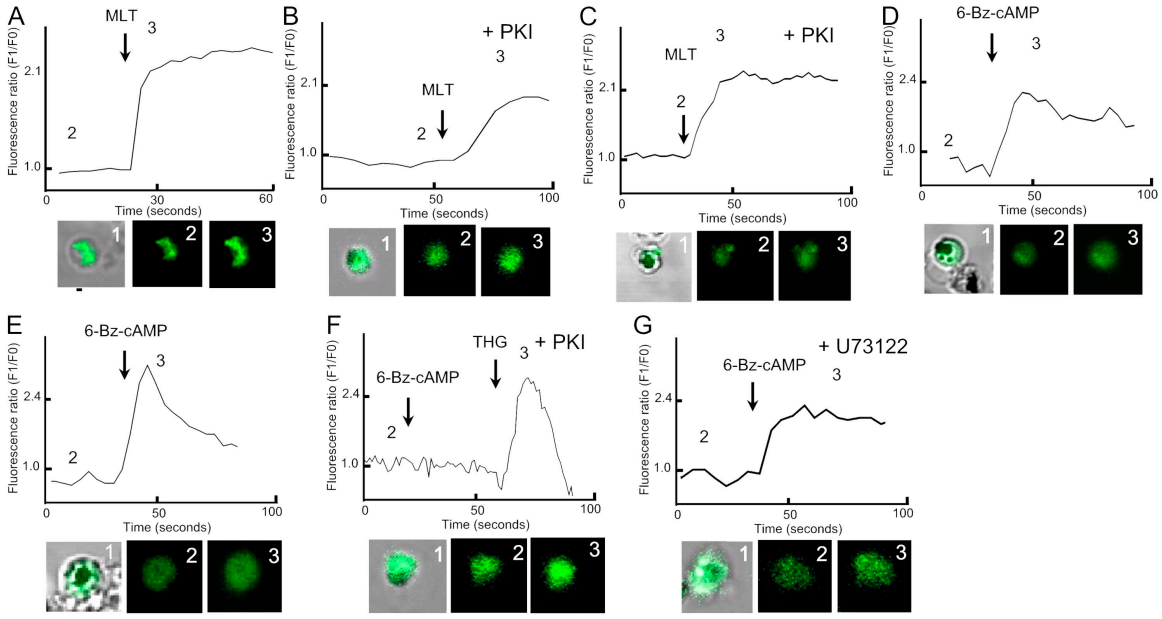

Figure 4. **Ca^{2+} fluorescence measurement in *P. falciparum* parasites analyzed by confocal microscopy.** Parasites at trophozoite stage were loaded with Fluo-4 AM. (see Materials and methods) and its fluorescence ratio (F1/F0) intensity was plotted as a function of time. (A–C) Addition of 100 nM melatonin in buffer with 2 mM Ca^{2+} promotes an increase of 1.4 ± 0.4 (n = 18) and similar results was observed with melatonin 100 nM in the presence of PKI in a Ca^{2+} medium or in a Ca^{2+}-free medium, respectively. (D) 20 μM 6-Bz-cAMP resulted in 1.5 ± 0.1 (n = 6). (E) 20 μM 6-Bz-cAMP was added in Ca^{2+}-free medium (EGTA containing) and causes a transient fluorescence increase of 1.36 ± 0.2 (n = 4). (F) 20 μM 6-Bz-cAMP did not induce an increase of Ca^{2+} when added in the presence of PKA inhibitor PKI. (G) 20 μM 6-Bz-cAMP induced an increase of Ca^{2+} of 1.41 ± 0.3 (n = 3) in the presence of PLC inhibitor U73122 (10 μM).

the effects on cytoplasmic Ca^{2+} of the combination of these treatments are presented. Addition of 6-Bz-cAMP after melatonin (Fig. 5 A) was without effect and, similarly, the addition of melatonin after 6-Bz-cAMP (Fig. 5 B) did not promote a further increase of intracellular Ca^{2+}, suggesting that the two pathways converge on the same mechanism. We finally tested the effect of the inhibitor of the sarcoendoplasmic reticulum Ca^{2+} ATPase thapsigargin, THG. This drug, when added to cells (either in

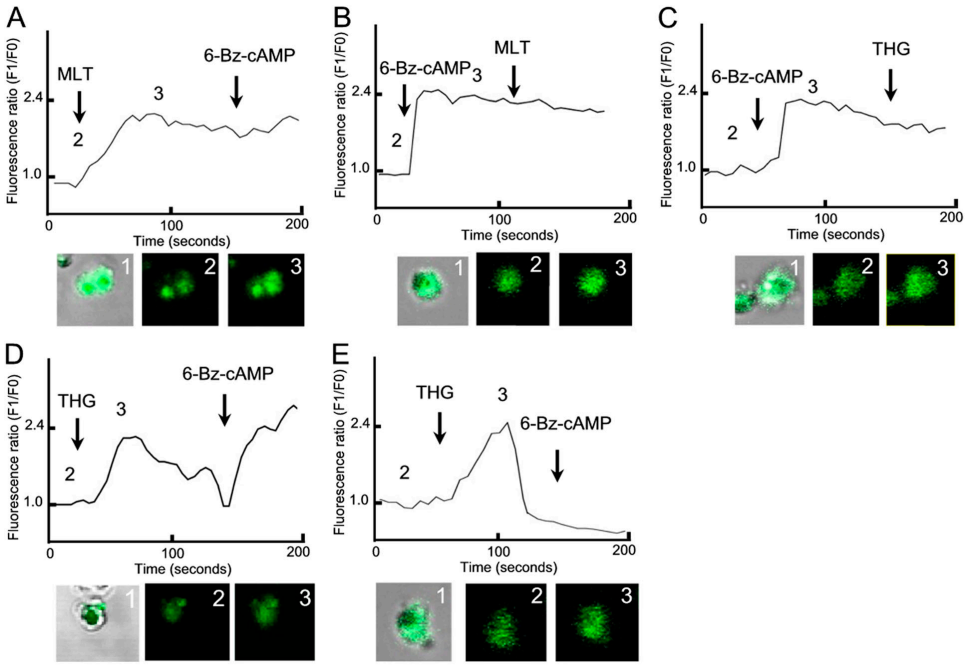

Figure 5. **Ca^{2+} fluorescence measurement in *P. falciparum* parasites analyzed by confocal microscopy.** Parasites at trophozoite stage were loaded with Fluo-4 AM (see Materials and methods) and its fluorescence ratio (F1/F0) intensity was plotted as a function of time. (A) Addition of 100 nM melatonin in buffer with 2 mM Ca^{2+}: the mean increase of fluorescence ratio was 1.32 ± 0.2 (n = 11) no effect was observed with a further addition of 20 μM 6-Bz-cAMP. (B) Addition of 20 μM 6-Bz-cAMP caused an average an increase in fluorescence ratio of 1.40 ± 0.3 (n = 5) and no effect was observed with a further addition of melatonin. (C) Same conditions as above. When indicated 10 μM THG was added. (D and E) Where indicated THG and 6-Bz-cAMP were added. In D the medium contained 2 mM $CaCl_2$ whereas in E no Ca^{2+} was added and 100 μM EGTA was included instead.

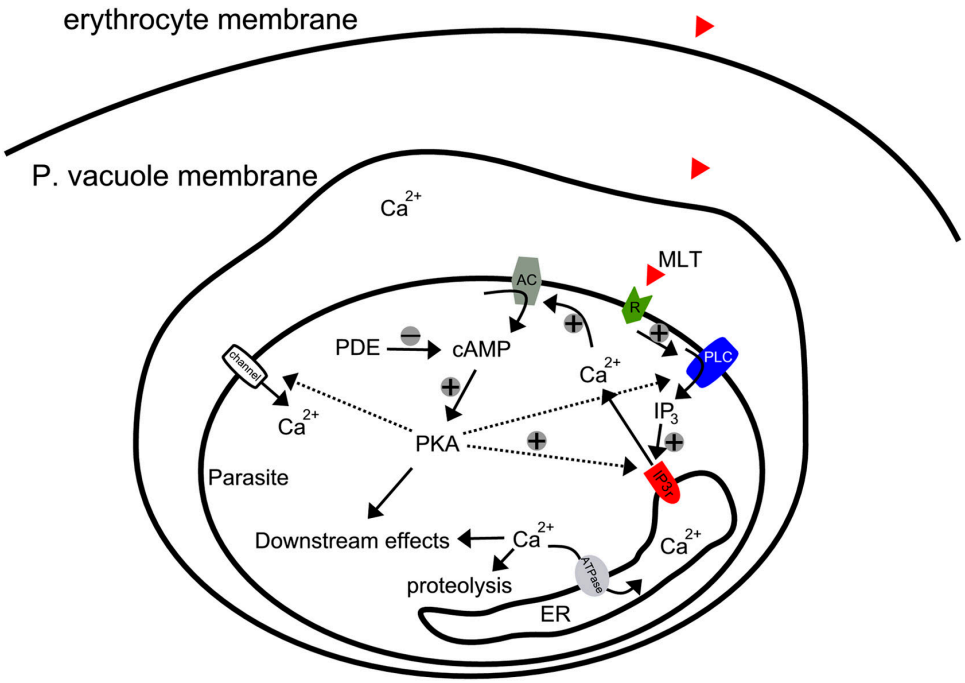

Figure 6. **Schematic model of signaling events in *Plasmodium* with data provided from literature and our results.** AC, adenylyl cyclase; PLC, phospholipase C; PDE, phosphodiesterase; PKA, protein kinase A; ER, endoplasmic reticulum; R, hypothetical melatonin receptor.

Ca^{2+}-containing or Ca^{2+}-free medium) causes a transient increase in cytoplasmic [Ca^{2+}], due to Ca^{2+} release from intracellular stores (Hotta et al., 2000). When the parasites, incubated in Ca^{2+}-free medium, were treated first with THG and then with 6-Bz-cAMP no increase in [Ca^{2+}] was observed (Fig. 5 E). Similar results, i.e., no rise in response to the second stimulus, were obtained when the order of drug addition was reversed, suggesting that 6-Bz-cAMP acts on a Ca^{2+} store endowed with a THG-sensitive Ca^{2+} ATPase (unpublished data). However, in Ca^{2+}-containing medium, although addition of THG after 6-Bz-cAMP resulted in no further increase of cytosolic Ca^{2+} (Fig. 5 C), THG pretreatment, though inducing the usual transient increase of cytosolic Ca^{2+}, did not prevent the rise in [Ca^{2+}] caused by 6-Bz-cAMP (Fig. 5 D). The latter result suggests that, in addition to mobilizing Ca^{2+} from intracellular stores 6-Bz-cAMP promotes an influx of Ca^{2+} from the extracellular medium. Taken together these data demonstrate that: (a) an increase in cAMP causes the mobilization of Ca^{2+} from intracellular stores endowed with a THG-sensitive Ca^{2+} ATPase; (b) the Ca^{2+} stores mobilized by melatonin, through PLC produced $InsP_3$, are the same as those mobilized by a cAMP increase; and (c) the cAMP rise also activates an influx of Ca^{2+} from the extracellular medium.

Discussion

Intraerythrocytic development of *Plasmodium* parasites depends on a series of complex signaling pathways in which second messengers such as Ca^{2+} and cAMP appear to play a key, yet largely mysterious, role. In the present work we have studied in details the involvement of cAMP and of its classical target enzyme PKA on the development within RBCs of *P. falciparum* and on the Ca^{2+} signaling mechanisms, in particular in relation to the modulatory effects on the cell cycle elicited by the host-derived hormone melatonin.

Our data show that: (a) melatonin increases cAMP levels and activates PKA; (b) inhibition of PKA strongly reduces the effects of melatonin on *P. falciparum* cell cycle; (c) a cAMP increase, as induced either by inhibiting the cAMP phosphodiesterases with IBMX or by adding the membrane permeable cAMP analogue N6cAMP mimics the effects of melatonin on cell cycle. Taken together these data make a strong case in favor of a key role played by cAMP and PKA as key modulators of the cell cycle in this malaria parasite. As to the mechanisms through which melatonin increases cAMP levels in *P. falciparum* the observations that (a) the inhibition of PLC by U73122 prevents the cAMP increase induced by melatonin and (b) a rise in cAMP induced by artificially increasing the cytosolic Ca^{2+} concentration with the Ca^{2+} ionophore ionomycin, suggest that the increase in cytosolic Ca^{2+} concentration caused by melatonin, rather than a direct coupling of the melatonin receptors to adenylyl cyclase is involved in the augmentation of the parasite cAMP levels. Whether this rise is due to activation by Ca^{2+} of an adenylyl cyclase (Cooper et al., 1995), or whether it is due to inhibition of a phosphodiesterase remains to be established. However, the observation that the melatonin-induced cAMP increase is observed also in the presence of the rather unspecific phosphodiesterase inhibitor IBMX suggests that the first possibility is more likely.

The interactions between the cAMP and the Ca^{2+} signaling pathways are not however limited to the Ca^{2+}-dependent activation of cAMP production; in fact we also observed that the PKA activator 6-Bz-cAMP is capable of inducing a cytosolic Ca^{2+} increase in the intact parasites, in part by mobilizing Ca^{2+} from intracellular stores and in part by stimulating Ca^{2+} influx from the medium. It is worth noting that the stores that are mo-

bilized by the rise in cAMP and those sensitive to $InsP_3$ produced by melatonin receptor stimulation appear to be the same, in as much as: (a) once Ca^{2+} has been released by cAMP, melatonin had no further effect (and vice versa); and (b) Ca^{2+} mobilization by cAMP or melatonin both occur from an organelle endowed with a THG-sensitive Ca^{2+} ATPase (i.e., presumably the endoplasmic reticulum). However, the mechanisms through which cAMP or melatonin cause the release of Ca^{2+} from the endoplasmic reticulum are clearly different. In particular, Ca^{2+} mobilization by melatonin is insensitive to PKA inhibition (and thus it is independent of the cAMP rise), but it is sensitive to the PLC inhibitor U73343 (and thus presumably depends on $InsP_3$ production); on the contrary cAMP-induced Ca^{2+} mobilization is sensitive to PKA inhibition, but it is insensitive to U73343, indicating that either the cAMP rise induce the activation of another PLC isoform or that it causes the release of Ca^{2+} through an $InsP_3$-insensitive pathway. Whether PKA activates a yet unknown endoplasmic reticulum channel different from the $InsP_3$ receptor or whether it activates the $InsP_3$ receptors at basal level of $InsP_3$ remains to be investigated.

In the light of the present results we thus propose the following model: (a) melatonin promotes a cytosolic [Ca^{2+}] rise by increasing $InsP_3$ production; (b) the rise in [Ca^{2+}] stimulates cAMP production, as parasites treated with the phospholipase C blocker, U73122 do not show any increase in cAMP upon melatonin treatment; (c) changes in intracellular cAMP concentration induce further Ca^{2+} release (by an as yet unknown mechanism).

Last, but not least, the cAMP increase induces in *P. falciparum* the activation of a Ca^{2+} influx pathway whose nature and mechanism of activation is yet to be determined.

In conclusion, the present and previous results suggest that the modulation of Plasmodia cell cycle by the host hormone melatonin is mediated by two second messengers acting in concert: on the one hand, melatonin is directly coupled to a classical Ca^{2+} signaling pathway, via phospholipase C–dependent $InsP_3$ production and Ca^{2+} mobilization from stores; on the other, the Ca^{2+} rise initiates an amplification loop via cAMP and PKA that further modulates the Ca^{2+} signal.

In mammalian cells recent work has demonstrated that not all the effects of cAMP are mediated by PKA, as several other cAMP binding proteins exist and appear to mediate, PKA-independent, cAMP-activated cellular processes (Stork and Schmitt, 2002; Kopperud et al., 2003). However, this appears to not be the case for the melatonin effects on cell cycle of *P. falciparum* as all the hormone effects on the development of the intracellular parasites described here are strongly inhibited by PKA inhibitors such as H89, PKI, and 8-BrcAMP-RP isomer. Whether Ca^{2+} has other effects on the parasite development, independent of its capacity to modulate PKA downstream signaling remains to be established. Fig. 6 summarizes melatonin signaling pathways in *Plasmodium*.

Materials and methods

Parasites

P. falciparum, Palo Alto strain, were cultured in flasks with RPMI 1640 medium as previously described (Trager and Jensen, 1976). Parasitemias were determined from Giemsa-stained smears.

Plasmodium cell cycle

These experiments were performed with *Plasmodium*-infected erythrocytes. *P. falciparum*–infected cells incubation was performed for 24 h at 37°C on plates in a closed chamber with controlled atmosphere in stationary condition. The proportion of different parasite forms as well as parasitemia was determined by analyzing 1,000 RBCs on Giemsa-stained slides after the following incubations: 100 nM melatonin; 20 μM PKA inhibitors/PKI (cell permeable), 20 μM H89 and 20 μM 8-BrcAMP-RP isomer; 100 μM IBMX; 20 μM PKA activator/6-Bz-cAMP. Control with solvent blank (DMSO, ethanol) used in the dilutions of the reagents were assayed.

cAMP enzyme immunoassay

P. falciparum–infected cells at trophozoite stage, synchronized with sorbitol (Lambros and Vanderberg, 1979), were submitted to the melatonin treatment 18 h after synchronization. *P. falciparum* parasites, isolated at the trophozoite stage, as described previously (Hotta et al., 2000), were washed twice with PBS, resuspended at a concentration of 5×10^6 cells ml^{-1} in the same buffer and incubated for 15 min with 100 nM melatonin, 20 μM phospholipase-C inhibitor (U73122), 1 μM staurosporin (kinase inhibitor), calmidazolium (calmodulin inhibitor), 10 μM ionomycin, or 25 μM BAPTA in the presence or absence of 100 μM IBMX to prevent cAMP degradation. After treatment, samples were analyzed with cAMP enzyme immunoassay kit (Amersham Biosciences) following protocol No. 4. Parallel experiments with no treatment were also carried on as control. Controls with solvents did not show an increase of cAMP.

PKA activity

P. falciparum malaria parasites were isolated from RBCs as described above and resuspended in PBS. Parasites (5×10^6 cells in 200 μl) were incubated by 15 min in the presence of 100 nM melatonin and/or 100 μM IBMX. After incubation the parasites resuspended in 500 μl of extraction buffer (25 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol) supplemented with protease inhibitors (leupeptin, pepstatin A, antipain, and chymostatin at 20 μg ml^{-1} and benzamidine at 0.5 mM). Parasites kept on ice bath were then broken by sonication (15W/15 s) and the lysates were centrifuged (14,000 *g*, 4°C for 5 min). Protein concentration in the supernants was determined by BCA Protein Assay Kit (Pierce Chemical Co.) using bovine serum albumin as standard. PKA activity was assayed by ^{32}P phophorylation of kemptide (Sigma-Aldrich) as previously described (Goueli et al., 1995). The assay was performed in a total volume of 100 μl containing 50 μl of parasites lysates supernatant (120–140 μg of protein) and 50 μl of PKA reaction buffer (40 mM Tris/HCl, pH 7.4, 50 μM kemptide, 25 μM [$\gamma^{32}P$]ATP (100 mCi/$mmol^{-1}$), 25 μM ATP, 10 mM $MgCl_2$, 0.2 mg/ml^{-1} BSA). After 5 min at 30°C, the reaction was stopped by addition of 25 mM of guanidine and the ^{32}P incorporation was measured by spotting 25 μl of the reaction mixture onto duplicate phosphocellulose filters (GIBCO BRL). After air drying for 20 min, the filters were washed five times for 5 min in 75 mM H_3PO_4 and dried before scintillation counting. Specificity of the PKA assay was assessed by performing the reaction in the presence of 4 ng (200 nM) of PKI (PKA-specific inhibitor peptide). Unspecific ^{32}P incorporation was measured in the absence of kemptide. PKA assay conditions (protein amount and reaction time) were adjusted to ensure linear reaction rates.

Ca^{2+} measurements and data analysis

Parasite loading with Ca^{2+} indicators was performed as described in Garcia et al. (1996) and Hotta et al. (2000). RBCs infected *P. falciparum* were washed three times at room temperature by centrifugation at 1,500 *g* for 5 min in PBS. Isolated parasites were obtained by lysing infected cells (10^8 cells) with 10 mg/ml^{-1} saponin in PBS. After pelleting to remove red cell membrane material, the parasites were washed twice in PBS by centrifugation at 2,000 *g* at room temperature. Parasites were then washed twice in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 50 mM Mops, pH 7.2, 2 mM $CaCl_2$), and resuspended in the same buffer containing probenecid, an inhibitor of organic anion transport (Di Virgilio et al., 1990). A stock solution of Fluo-4 AM in DMSO (1 mg/ml^{-1}) was added to the parasite suspension to reach a final concentration of 5 μM and left for 50 min at 37°C. At the end of the incubation, the cells were washed three times with buffer A to remove extracellular dye and placed in the microscopy dishes. The experiments were performed at room temperature. The data acquisition was performed as described in Gazarini et al. (2003) with a Zeiss confocal microscope (LSM 510; MicroImaging, Inc.); excitation 488 nm (Argon laser) and emission collected with band pass filter 505–530 nm. The Axiovert 100M microscope is equipped with 63× water immersion objective. In the ex-

periments with Fluo-4 AM the fluorescence was normalized (F1 − maximal fluorescence after drug addition/F0 − fluorescence before drug addition). Software-based analysis (LSM 510 software, version 2.5; Carl Zeiss MicroImaging, Inc.) allowed fluorescence imaging in the whole field of view (or in a selected cell) as function of time. Experiments were performed with at least three different cell preparations, and 10–20 cells were monitored in each experiment. Traces represent typical single cell responses, unless otherwise indicated.

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