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cAMP signalling and its role in host cell invasion by malaria parasites

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Cyclic adenosine monophosphate (cAMP) is an important signalling molecule across evolution, but until recently there was little information on its role in malaria parasites. Advances in gene editing – in particular conditional genetic approaches and mass spectrometry have paved the way for characterisation of the key components of the cAMP signalling pathway in malaria parasites. This has revealed that cAMP signalling plays a critical role in invasion of host red blood cells by *Plasmodium falciparum* merozoites through regulating the phosphorylation of key parasite proteins by the cAMP-dependent protein kinase (PKA). These insights will help us to investigate parasite cAMP signalling as a target for novel antimalarial drugs.

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Introduction

Protozoan parasites of the genus *Plasmodium* are responsible for causing malaria, a disease with a huge global impact. The malaria parasite invades several cell types during the course of its complex life cycle in the vertebrate host and mosquito vector. Following the bite of an infected mosquito, injected sporozoites rapidly migrate to the liver, traversing several host cells before settling inside a hepatocyte. Here, each parasite divides and differentiates to produce thousands of merozoites which are released into the bloodstream [1]. All the clinical symptoms of malaria are caused by repeated cycles of red blood cell (RBC) invasion, modification and merozoite release (egress) with associated destruction of the host cell [2]. Some blood-stage parasites differentiate into sexual forms called gametocytes which mature and undergo fertilization in the female mosquito midgut following ingestion of an infected blood meal [3]. Motile ookinete forms then develop, which invade the epithelial layer, crossing the insect midgut [4] before developing into oocysts and eventually releasing thousands of new sporozoites. These migrate to and invade the mosquito salivary glands within which they become infectious before reintroduction into the vertebrate host [5]. Each of the cellular invasion events during the lifecycle are essential for the replication and transmission of malaria parasites, and as such are of great interest as targets of anti-malarial therapeutic agents [6].

Molecular characterisation of host cell invasion by *Plasmodium* has traditionally been very challenging; invasion occurs rapidly and invasive zoites are technically difficult to isolate and maintain for *in vitro* studies. However, increasingly sophisticated genetic and biochemical tools as well as recent breakthroughs in live cell imaging have allowed detailed dissection of the roles played by key proteins and signalling molecules, particularly in the invasion of human RBCs by merozoites of *P. falciparum*, the *Plasmodium* species that causes most malaria-associated fatalities. Very recent studies have highlighted a pivotal role for the second messenger cAMP in this process [7••,8••,9•]. Whilst cAMP is fundamental to a huge range of signal transduction processes, from human metabolism [10] to the behaviour of social amoeba [11], its role in malaria parasites was previously unclear. In this review we describe and summarise the newly elucidated crucial function of cAMP-dependent signalling in RBC invasion [7••,8••,9•].

Key components of *Plasmodium* cAMP signalling

Cellular cAMP concentrations are controlled by the interplay of two classes of enzymes, adenylyl cyclases (ACs) and phosphodiesterases (PDEs), that respectively synthesise and degrade cAMP. The *Plasmodium* genome encodes two ACs (ACα and ACβ) and four PDEs (PDEα, PDEβ, PDEγ and PDEδ). Transcription of *ACα* occurs only in the sexual and pre-erythrocytic life cycle stages [12,13] whereas *ACβ* expression is restricted primarily to mature intra-erythrocytic parasites (schizonts) as they approach egress, implicating ACβ as the essential source of cAMP production in blood stage parasites [13–16].

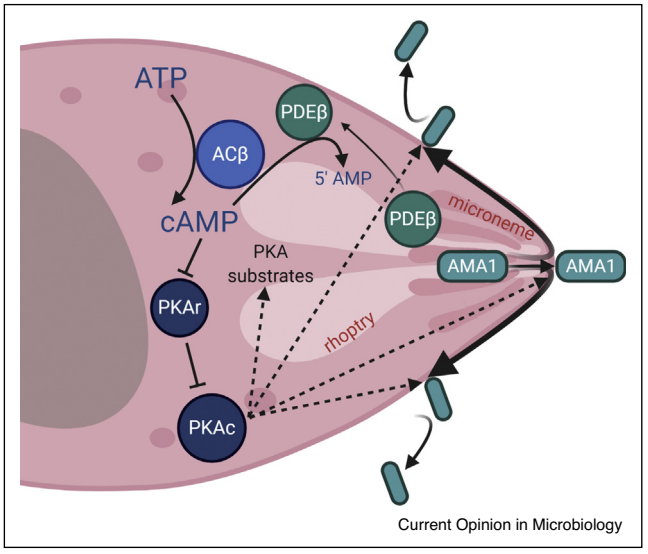
PDEγ and *PDEδ* are expressed in mature gametocyte and mosquito stages where the enzymes are thought to play roles that depend on their capacity to hydrolyse cGMP [17–21]. Both *PDEα* and *PDEβ* are transcribed maximally in mature blood stage schizonts. In an important distinction between these enzyme isoforms, PDEα hydrolyses cGMP only [22,23] whereas PDEβ is a dual-specific PDE able to hydrolyse both cGMP and cAMP [8••]. PDEβ is therefore the sole PDE that regulates cAMP levels in asexual blood stage parasites and is also the only essential PDE during this clinically important stage in the *P. falciparum* life cycle [8••,22].

Most of the critical functions of cAMP in eukaryotes are mediated via the activity of the cAMP-dependent protein kinase (PKA). This enzyme exists as a protein heterodimer comprising a regulatory subunit (PKAr) and a catalytic subunit (PKAc). Binding of PKAr to PKAc inhibits kinase activity, but upon binding of cAMP, PKAr dissociates from PKAc, relieving the inhibition and allowing PKAc to phosphorylate protein substrates [24,25]. Whilst PKA is thought to be the main effector of cAMP-dependent signalling in *P. falciparum*, the parasite genome also encodes a protein previously designated as Epac (exchange protein activated by cAMP), with predicted cyclic nucleotide-binding domains [26]. The biochemistry, localisation and function of the *Plasmodium* proteins involved in cyclic nucleotide signalling are described in detail in a recent review [27] and summarised in Figures 1 and 2 (Table 1).

cAMP is critical for merozoite invasion of RBCs

Invasion of RBCs by *P. falciparum* merozoites is a multistep process which occurs following the generation and maturation of a new generation of daughter merozoites within an infected RBC. In preparation for invasion these intracellular merozoites must exit from their host RBC in a cGMP-dependent process known as egress [29,30], releasing proteins from apical secretory organelles called exonemes, micronemes and rhoptries that are critical for egress and invasion. Free merozoites associate tightly with a target RBC within seconds, pulling host RBC membrane around themselves as they drive into the cell powered by an actinomyosin motor [31]. A role for cAMP signalling in invasion was initially suggested by the ability of pharmacological inhibitors of mammalian cAMP regulatory and responsive proteins to interfere with invasion [14,26,32] as well as phosphoproteome data suggesting that PKA is highly active in mature schizonts [33,34]. A more detailed analysis of cAMP signalling was recently made possible by novel genetic techniques [35–37] that allowed robust, inducible disruption of *ACβ* [7••], *PKAc* [7••,9•] and *PDEβ* [8••] in *P. falciparum*. These studies showed unambiguously that in the absence of either cAMP synthesis or PKA activity, merozoites form normally but are completely unable to invade RBCs [7••,9•].

Figure 1



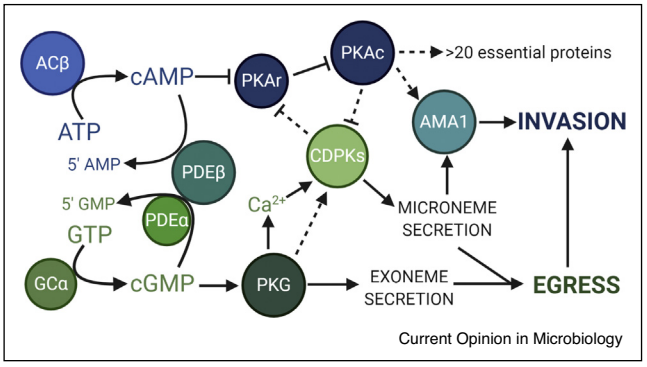
Key components of cAMP signalling in the *Plasmodium* merozoite. ACβ is thought to associate with the cytosolic surface of the rhoptry membrane. Here it produces cAMP that regulates the activity of PKA, which has a diffuse localisation within the parasite cytoplasm. PDEβ is trafficked via the ER to an apical location, from which it appears to re-localise to the parasite plasma membrane in mature schizonts. AMA1 is stored in micronemes before it is discharged onto and across the merozoite surface just before egress. Most of this AMA1 is then proteolytically removed except for a small fraction at the apical end that binds to RON proteins on the RBC surface during invasion. These processes are regulated in a poorly understood manner by PKA-dependent phosphorylation of the cytoplasmic tail of AMA1.

Artificially increasing cAMP levels by disruption of PDEβ also significantly impaired invasion and moreover prevented further development of those parasites that did go on to invade [8••]. Taken together, these findings demonstrate that finely tuned regulation of intracellular cAMP levels is crucial to control the invasion process and subsequent parasite development.

Key events before invasion do not depend on cAMP

It has been suggested that before RBC invasion the production of cAMP causes an Epac-dependent rise in merozoite cytosolic Ca^{2+} and that this in turn leads to the secretion of critical proteins from micronemes [26]. However, recent conditional genetic knockout studies unequivocally show that Ca^{2+} signalling, microneme secretion and egress do not require cAMP [7••], PKA [7••,9•] or Epac [7••,15,28]. Instead, chemical genetic approaches suggest that these processes critically depend on cGMP production and the activity of the parasite cGMP-dependent protein kinase (PKG) [29,30,38]. Epac does not play an essential role at any point in merozoite invasion *in vitro* [7••]; indeed, it appears to be completely dispensable in asexual blood stages and is readily lost or mutated in laboratory isolates [7••,15,28].

Figure 2



cAMP signalling and its interactions with cGMP signalling in mature schizonts.
ACβ catalyses the conversion of ATP to cAMP, which binds to the PKA regulatory subunit, PKAr, releasing inhibition of PKAc kinase activity. PKAc then phosphorylates a broad range of substrates, including the cytoplasmic tail of AMA1. This induces a conformational change in AMA1 that is important for merozoite invasion. cGMP, thought to be produced by GCα, activates PKG. This leads to the intracellular release of Ca^{2+}, the activation of calcium-dependent protein kinases (CDPKs), secretion of exoneme and microneme proteins (including AMA1), and ultimately egress. cAMP and cGMP are both hydrolysed by PDEβ. CDPK1 is phosphorylated following PKG activation and is thought to enhance the activity of PKAc, which may in turn negatively regulate the function of CDPK1. Dotted lines indicate phosphorylation events.

Table 1

Key proteins involved in cAMP signalling in asexual blood stages of *Plasmodium*

Protein	Name	Identifier (PlasmoDB)	Null phenotype
ACβ	Adenylyl cyclase β	PF3D7_0802600	Essential for merozoite invasion [7••]
Epac	Exchange protein activated by cAMP	PF3D7_1417400	Non-essential in asexual blood stage parasites [7••,15,28]
PDEβ	Phosphodiesterase β	PF3D7_1321500	Essential for efficient merozoite invasion and subsequent ring-stage development [8••]
PKAc	cAMP-dependent protein kinase catalytic subunit	PF3D7_0934800	Essential for merozoite invasion [7••,9•]
PKAr	cAMP-dependent protein kinase regulatory subunit	PF3D7_1223100	No data available

Observation of cAMP signalling-defective merozoites by time-lapse and electron microscopy immediately following egress has shown that, despite an inability to invade, the parasites are still able to interact with RBCs [7••,9•]. ACβ-null and PKA-null merozoites induce deformations of the RBC surface [7••,9•], indicating that the parasite actinomyosin motor is active [31,39] and can drag membrane around the merozoite to some degree as it attempts to initiate invasion. The mutant merozoites can also induce echinocytosis [7••,9•], a transient, striking change in RBC architecture that is indicative of successful discharge of parasite rhoptries [40]. Collectively, these observations suggest that cAMP-dependent signalling is not essential for early stages in the invasion pathway but is required for a relatively late step in the process.

PKA mediates a large number of phosphorylation events, many of which may be critical for invasion

One plausible model that might explain why the cAMP dependency of merozoite invasion is restricted to a late stage in the process came from the identification of the PKA-dependent phosphorylation of a merozoite protein called apical membrane antigen 1 (AMA1) [7••,9•,41], a type I integral membrane protein that is secreted from micronemes onto the merozoite surface just before egress. Much of this AMA1 is rapidly shed from the merozoite surface upon egress, but some is retained (Figure 1). Upon interaction with a target RBC, the AMA1 ectodomain binds to parasite rhoptry neck (RON) proteins that are also discharged into the RBC membrane, forming a tight interaction between the two cells before the merozoite enters. Phosphorylation of the short cytoplasmic tail of AMA1 at Ser_{610}, mediated directly by PKA [7••,9•,41], causes a significant conformational change in the tail structure [7••] and is critical for invasion [41]. Interactions between PKAc-null or ACβ-null merozoites and RBCs show similarities to those observed when AMA1-RON interactions are blocked [7••,9•,40–44]. Furthermore, parasites deficient in cAMP signalling do not shed AMA1 efficiently from the merozoite surface [7••] and fewer merozoites attach to RBCs in the absence of *PKAc* [9•]. By contrast, PDEβ-null parasites in which cAMP levels are elevated show premature shedding of AMA1 [8••]. Together these observations present the possibility that the invasion defect observed in the absence of cAMP signalling could be largely or wholly explained by a lack of PKA-dependent regulation of AMA1 function.

Comparative phosphoproteomic studies have shown that, in addition to AMA1, a large number of proteins are phosphorylated in mature schizonts in response to cAMP-dependent signalling [7••,8••,9•]. At least 60 phosphorylation sites within ~40 schizont proteins can be confidently identified as cAMP-dependent, based on their significantly decreased phosphorylation in PKAc-knockout and ACβ-knockout parasites and significantly enhanced phosphorylation in PDEβ knockouts [7••,8••]. At least 20 of these proteins are thought to be essential for blood stage parasite development [15], but further investigation of the effect of PKA-dependent phosphorylation

of these sites will be required to determine what contribution they make to merozoite invasion.

Unanswered questions and avenues for further study

What are the roles of cAMP signalling in RBC invasion and subsequent parasite development?

Loss of cAMP signalling has no observable impact on merozoite maturation or function until after egress and interaction of merozoites with a new host RBC [7••,9•]. Around this stage of the invasion pathway we know that PKA-mediated phosphorylation of AMA1 modulates its proteolytic shedding from the merozoite surface. However, the mechanistic link between these two processes, and the function of AMA1 shedding remain unclear. It is plausible that a phosphorylation-dependent conformational change in the cytosolic domain of AMA1 marks the protein for efficient proteolysis which may in turn be required for correct tight junction formation and entry of the parasite into the RBC. However, the critical role of cAMP at and beyond this point in the erythrocytic life cycle is challenging to dissect with current experimental tools. The large number of cAMP-dependent phosphorylation events in merozoites [7••,8••], coupled with the observation that PDEβ-deficient merozoites that enter RBCs cannot develop further [8••], make it tempting to speculate that PKA plays an important role in regulating the function not just of AMA1 but also of other key components of the invasion machinery as well as proteins involved in early intracellular development.

How do cGMP and cAMP signalling interact?

The precise temporal regulation of cyclic nucleotide-dependent signalling events in mature schizonts and merozoites appears critical for successful egress and invasion; blocking these signalling events prevents invasion and/or egress [7••,9•,29,30] whilst artificially elevating cyclic nucleotide levels leads to premature egress and severe impairments to invasion [8••,29]. Current knowledge suggests that cGMP-dependent processes are important for controlling key events just before egress and that cAMP then becomes important just before invasion. However, it is still unclear precisely how the transition from cGMP-dependent to cAMP-dependent control is timed and co-ordinated. Tight control of the expression of the cyclases, PDEs and kinases likely plays an important role but it is possible that post-translational modifications, such as the phosphorylation of PKA [45], PDEβ, ACβ and GCα [46] or the sensing of small molecules also contribute to the timely regulation of their activities. The homology between ACβ and the bicarbonate-sensitive mammalian soluble adenylyl cyclase has led to the suggestion that bicarbonate ions may activate cAMP production in asexual blood stage parasites, but this has yet to be conclusively demonstrated [26].

Is cAMP signalling important for invasion events in mosquito and pre-erythrocytic life cycle stages?

Almost all of our insights into the role of cAMP in invasion apply to asexual blood stage malaria parasites, for which genetic tools and culture systems are most advanced. The role of cAMP may be quite different during sporozoite invasion of hepatocytes; early work suggested that ACα-mediated cAMP production is required for this process [13] but more recent findings indicate that PKAc is non-essential at this life cycle stage [47•]. Whilst little is known about the role of cAMP signalling in mosquito stage invasion events, we are hopeful that this situation will soon change since it has recently become possible to apply similar conditional genetic approaches that have transformed our understanding of merozoite invasion to all stages of the *Plasmodium* life cycle [48•].

Could components of cAMP signalling make good drug targets?

The essentiality of cAMP signalling for malaria parasite development in the human hosts makes this pathway an attractive target for therapeutics. Alongside its role in invasion, regulation of cAMP signalling is also critical for gametocyte survival [49], raising the attractive prospect that pharmacological targeting of cAMP signalling could impact both blood stage replication and transmission to the mosquito vector. The cyclases, PDEs and PKA are all potential target enzymes that are being explored, although designing drugs with sufficient selectivity over human isoforms will be an important challenge to overcome during development.

Conflicts of interest statement

Nothing declared.

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