Abstract

Compared with the impressive progress in understanding signal transduction pathways and mechanisms in mammalian systems, advances in protozoan signaling processes, including cyclic nucleotide metabolism, have been very slow. This is in large part connected to the fact that the components of these pathways are very different in the protozoan parasites, as confirmed by the recently completed genome. For instance, kinetoplastids have no equivalents to the mammalian Class I adenylyl cyclases (ACs) in their genomes nor any of the subunits of the associated G-proteins. The cyclases in kinetoplastid parasites contain a single transmembrane domain, a conserved intracellular catalytic domain and a highly variable extracellular domain – consistent with the expression of multiple receptor-activated cyclases – but no receptor ligands, agonists or antagonists have been identified. Apicomplexan AC and guanylyl cyclase (GC) are even more unusual, potentially being bifunctional, harbouring either a putative ion channel (AC) or a P-type ATPase-like domain (GC) alongside the catalytic region. Phosphodiesterases (PDEs) and cyclic-nucleotide-activated protein kinases are essentially conserved in protozoa, although mostly insensitive to inhibitors of the mammalian proteins. Some of the PDEs have now been validated as promising drug targets. In the following manuscript, we will summarize the existing literature on cAMP and cGMP in protozoa: cyclases, PDEs and cyclic-nucleotide-dependent kinases.

Introduction

Parasitic protozoa cause some of the most severe infectious human diseases worldwide, including malaria, Chagas’ disease, sleeping sickness, leishmaniasis, toxoplasmosis and many others. In addition, livestock infections such as trypanosomiasis, coccidiosis and babesiosis cause enormous economic damage to agriculture and threaten food security in developing countries. Although the disease burden is catastrophic, many of the diseases are limited to tropical and/or developing countries; hence, drug development efforts have been scarce, leading to a paucity of efficacious drugs. While there is no shortage of genetically validated drug targets in many of these organisms, the step towards actual drug development is an often insurmountable hurdle. Few organizations or pharmaceutical companies have the resources as well as the determination to develop a new class of compounds ab initio for these neglected diseases. Thus, the rare new clinical developments for these diseases are usually reformulations of existing drugs or improved compounds from existing antipROTOZOAL classes. Examples are the introduction by the Drugs for Neglected Diseases initiative of nifurtimox for African sleeping sickness (caused by Trypanosoma brucei spp.) – nifurtimox being the main treatment against American trypanosomiasis or Chagas’ disease (caused by Trypanosoma cruzi); the clinical trials of pafuramidine by the Consortium for Parasitic Drug Development against early sleeping sickness (Paine et al., 2010) – pafuramidine being an orally available analogue of pentamidine, a first-line drug since the 1930s; the introduction of a liposomal formulation AmBisome (Sundar et al., 2003) of the 1950s drug amphotericin B (Furtado, 1959) against visceral leishmaniasis – lipid amphotericin formulations having been developed originally for systemic mycoses associated with AIDS (Chavanet et al., 1992); and the development of various artemisinin combinations and formulations by the Medicines for Malaria Venture.
The above developments are very important and welcome; however, it is essential that new and unrelated compounds enter clinical development against parasitic disease. While it has often been argued that, for reasons of specificity, this should start from the many parasite-specific targets, actual drug development is much more feasible when an elaborate pharmacology and toxicology already exist for a targeted enzyme or pathway. The cyclic nucleotide signalling pathways are excellent examples of this paradigm. Although there are significant differences between host and parasite in some modules of the signalling cascade — for example G-protein-coupled receptors are apparently absent in these organisms (Fredriksson & Schiöth, 2005) — other aspects of the pathway such as the phosphodiesterases (PDEs) remain relatively conserved. As such, a number of the key proteins in these pathways have been genetically or pharmacologically validated as drug targets (Zoraghi & Seebeck, 2002; Yusa & et al., 2005; Oberholzer et al., 2007; Ono et al., 2008; Sudo et al., 2008) and drug development is focusing on inhibitors of protozoan PDEs (Shakur et al., 2011). In the current manuscript, we systematically review progress in understanding cyclic nucleotide metabolism and signalling in protozoan species.

Adenylyl cyclases (ACs) of kinetoplastids

**cAMP and the life cycle**

AC activity was first reported in trypanosomes in 1974 in *Trypanosoma gambiense* (Walter et al., 1974). This was quickly followed by the measurement of cyclic-AMP (cAMP) in different life-cycle stages of *Trypanosoma lewisi*, where it was observed that the cAMP concentration in the nonreproducing form was approximately twice that of the reproducing form (Strickler & Patton, 1975).

Further variations in the intracellular cAMP concentration were measured in T. brucei; as the parasitaemia in rats increased, the cAMP concentration per trypanosome increased with it (Mancini & Patton, 1981). In strains that caused a relapsing parasitaemia, the same initial change in cAMP was observed. However, as the proportion of trypanosomes in the long slender form decreased to give way to the short stumpy forms, the overall intracellular CAMP concentration also declined. When the next parasitaemic wave of long slender forms grew, the cAMP concentration in the trypanosomes also increased, suggesting a higher steady-state level of cAMP in long-slower than in short-stumpy life-cycle forms (Mancini & Patton, 1981). This could, however, be a reflection of the higher metabolic activity and the rapid proliferation of the former parasites, as opposed to the nondividing stumpy forms.

The emerging hypothesis of a link between cell density or life-cycle stage and cellular cAMP levels was reinforced with the identification of a putative ‘stumpy induction factor’ (SIF). A low-molecular-weight molecule or set of molecules, extracted from a medium taken from a bloodstream form (BSF) culture at maximal density, could induce the differentiation of long slender trypanosomes to stumpy forms. The cAMP analogue 8-(4-chlorophenylthio)-cAMP was demonstrated to have the same differentiation-inducing effect as SIF. In addition, trypanosomes incubated with a conditioned medium containing SIF displayed a two- to threefold increase in the intracellular concentration of cAMP compared with cells grown in a nonconditioned medium (Vassella et al., 1997; Breidbach et al., 2002). However, the exact identity of SIF has not yet been uncovered, nor has a SIF receptor been identified.

**Calcium-mediated AC regulation**

AC activity was found to be predominantly localized to the membrane portion of subcellular fractions of *T. cruzi* (Pereira et al., 1978) and *T. brucei* (Martin et al., 1978a). The AC activity in the membrane fraction was inhibited by Ca^{2+}; however, measurement of its effect in whole, intact cells showed that calcium actually stimulated AC activity, reaching a maximum effect at a concentration of 300 μM (Voorheis & Martin, 1980).

Calcium crossed the plasma membrane and equilibrated with the external concentration within just 30 s; however, there was a time lag of several minutes (depending on the Ca^{2+} concentration) before the AC activity was stimulated (Voorheis & Martin, 1981). The addition of the ionophore A-23187 hastened the Ca^{2+} stimulation of AC, possibly by allowing Ca^{2+} to access the lipid bilayer plasma membrane faster than on its own. This, along with evidence from other membrane-perturbing agents (Voorheis & Martin, 1982), suggests that the calcium receptor may also be located in the membrane with, or as part of, the AC.

The addition of calcium and the ionophore A-23187 also results in the shedding of the variant surface glycoprotein (VSG) coat by BSF trypanosomes (Bowles & Voorheis, 1982; Voorheis et al., 1982), a step necessary in the differentiation of BSFs to procyclic forms. Indeed, VSG shedding and AC stimulation appear to share numerous characteristics: the activation by calcium and ionophore; stimulation by the rupturing or the perturbation of the plasma membrane; and blocking of stimulation by the addition of Zn^{2+} (Voorheis et al., 1982). However, monitoring of AC activity and VSG shedding after triggering differentiation to the procyclic form showed that AC stimulation was not responsible for the release of VSG (Rolin et al., 1993).

Similarly, while circumstantial evidence indicated that VSG release by pH stress or glycosphatidylinositol phospholipase C (GPI-PLC) cleavage may stimulate AC activity (Rolin et al., 1996; Nolan et al., 2000), experiments with GPI-PLC null mutants showed that both effects were
simultaneous, but independent (Rolin et al., 1996). While one process does not trigger the other, it is still possible that a common stimulus initiates a signalling cascade further upstream, but that AC stimulation and VSG shedding are on separate, parallel branches.

**Isolation and biochemical characterization of AC genes and their proteins**

The first identification of a putative kinetoplastid AC gene occurred when the gene expression site of an active VSG was sequenced in *T. brucei* (Pays et al., 1989). The sequencing revealed that there were multiple genes in the site that were coexpressed with VSG. These genes were termed expression-site-associated genes (ESAGs), and one of them, ESAG4, showed homology with an AC from yeast. Hybridization with an ESAG4 probe to a procyclic cDNA library could not pick out a single clone identical to ESAG4, but detected several related sequences. Restriction mapping of the clones separated them into two categories, with the largest from each further characterized and named GRESAG4.1 and GRESAG4.2 (genes related to ESAG4). Related genes were also found in *T. gambiense*, *Trypanosoma congolense*, *Trypanosoma mega* and *Trypanosoma vivax* (Alexandre et al., 1990).

Specific antibodies against ESAG4 and GRESAG4.1 showed differential expression in transforming BSFs. Most of the ESAG4 AC was lost around 9 h after differentiation was triggered, paralleling the decline in the proportion of stumpy forms. The level of GRESAG4.1, however, remained constant from the stumpy form through to reproducing procycs (Rolin et al., 1993).

Proof that the putative AC genes actually coded for AC enzymes came when a *Trypanosoma equiperdum* gene highly homologous to ESAG4 was shown to complement AC-deficient yeast mutants (Ross et al., 1991). ESAG4 and GRESAG4.1 from *T. brucei* also complement AC-deficient yeast and it was shown that ESAG4 is stimulated by Ca\(^{2+}\), whereas GRESAG4.1 is not (Paindavoine et al., 1992). ESAG4 may be responsible for the calcium-mediated AC activity observed by Voorheis and Martin in intact BSF *T. brucei* (Voorheis & Martin, 1980, 1981, 1982), and its location within the VSG expression cassette may provide a link through the regulation of transcription between cAMP response and VSG release (see Calcium-mediated AC regulation).

Similar multigene families with high homology to ESAG4 and GRESAG4.1 have also been identified in *Leishmania donovani* (Sanchez et al., 1995) and *T. cruzi* (Taylor et al., 1999) and share the same predicted protein architecture. The regions responsible for the binding of the G\(_{S}\) and G\(_{i}\) subunits of the heterotrimeric G-proteins that regulate mammalian ACs are not conserved, suggesting that trypanosomal ACs are not regulated by G-proteins homologous to those of mammals. Indeed, while some characteristics of G-protein subunit activity have been reported in *T. cruzi* (Eisenchlos et al., 1986; Coso et al., 1992), no sequences homologous to the G-protein subunits have been identified in the genomes of any of the kinetoplastids sequenced to date.

Interestingly, the change of one residue, from serine in the mammalian ACs to aspartic acid in *TcADC1* or *TcADC4*, at the position corresponding to amino acid 942 of the C2 domain of rat Type II AC, predicts that the binding of forskolin to kinetoplastid ACs would be impeded. Forskolin is an activator of mammalian ACs and has sometimes been used uncritically in investigations of AC activity in protozoa, including kinetoplastids, on the unproven assumption that its effects in mammalian cells would be replicated in other systems. Functional characterization of three *T. cruzi* AC genes confirms the sequence-based prediction, as forskolin had no effect on *T. cruzi* AC activity (Taylor et al., 1999; D’Angelo et al., 2002). These results agreed with an early report on the activity of *T. cruzi* AC purified with the aid of monoclonal antibodies that also failed to see an effect from forskolin (Torruela et al., 1986). Previous work reporting the effects of forskolin on trypanosomes will need to be reappraised in the light that in these parasites, forskolin-mediated effects may be due to non-AC or non-cAMP signalling effects.

**AC protein structure**

As became apparent from the sequencing of numerous ACs from several species, the AC enzymes from kinetoplastids all share a similar overarching protein structure. They all have a large extracellular N-terminal domain with low homology between individual genes and/or species and a single membrane spanning region that connects to the catalytic domain. This is in stark contrast to mammalian ACs, which generally have 12 transmembrane domains, except for the soluble AC family (Dessauer, 2009).

The C-terminal region of the ACs is highly conserved between kinetoplastids and is also highly related to that of the mammalian Class I AC catalytic domain, as well as to the receptor guanylyl cyclase (GC) family. A highly variable C-terminal extension, found after the catalytic domain, appears to be unique to kinetoplastids. The cytosolic domains of *T. brucei* AC genes GRESAG4.1 and GRESAG4.3, with the C-terminal extension removed, were crystallized and their structures were resolved by high-resolution X-ray analysis (Bieger & Essen, 2000; Bieger & Essen, 2001). As expected from the sequence analysis, the structures of the crystallized trypanosomal ACs were very similar to that of the C1A domain of dogs and the C2A domain found in rats.

The major structural difference between the *T. brucei* ACs and mammalian Class I ACs is the insertion of a 36-amino acid region into the catalytic domain, forming two extra helices and termed the Δ-subdomain. In Class I ACs, this
region, without the kinetoplastid insert, is involved in the interaction with G-protein subunits. The fact that trypanosomal ACs have such a variation at that particular point in the structure suggests that the Δ-subdomain may play a regulatory role and provides a structural reason for the inability of G-protein subunits to interact with kinetoplastid ACs. Further analysis of the crystal structure of GRESAG4.1 and GRESAG4.3 shows that the Δ-subdomain forms a cavity of 95 Å³. This is potentially large enough to form a binding pocket for a small molecule or protein cofactor that may exert an allosteric or a regulatory effect on the enzyme (Bieger & Essen, 2001).

Interestingly, a recent analysis of the kinetoplastid genomes published to date, investigating adaptive evolution in a number of large multigene families, identified 29 trypanosomal ACs as having sites of predicted positive selection (Emes & Yang, 2008). The majority were located in the N-terminal extracellular region, whereas only four codons were predicted to have been subject to positive selection in the catalytic domain. These four amino acid changes are all apparently located on the α4 helix, possibly impacting on the cavity enclosed by the Δ-subdomain and perhaps giving rise to a diversity of factors interacting with that domain.

The crystals of the catalytic domain of GRESAG4.1 and GRESAG4.3 were formed exclusively in a monomeric state. However, there is evidence to suggest that the active forms of GRESAG4.3 were formed exclusively in a monomeric state. A diversity of factors interacting with that domain.

The predicted substrate binding and active sites indicated that two molecules of ATP would be bound per catalytically active homodimer, in contrast to the one active site in mammalian Class I ACs. This was confirmed by heterodimerization with active site mutants of GRESAG4.1: a single site mutant in a dimer with the wild type resulted in a loss of ~60% of the activity, consistent with the loss of one active site, whereas a double mutant dimer results in the activity declining below 10%. Dimers made from complementary mutants could restore the activity of one active site (Bieger & Essen, 2001). Superimposition of the active sites of GRESAG4.1, GRESAG4.3 and the mammalian C1A, as well as a key-residue mutagenesis profile all suggest that the actual mechanism of catalysis of ATP to cAMP in trypanosomal ACs is identical to that of mammalian Class I ACs (Bieger & Essen, 2001).

**Why such diversity of AC?**

According to the published genome, *T. brucei* has over 60 putative AC genes (Berriman et al., 2005; http://www.tirtrypdb.org). The question that immediately springs to mind is ‘Why might a trypanosome need such a large panel of AC enzymes?’ Reviews by Seebeck et al. (2001, 2004), in particular, have highlighted the paradox of a relatively small cell size combined with an apparently overwhelming array of ACs expressed at any one time, which could quickly flood the cells with toxic levels of cAMP. This situation shows the absolute necessity for the strict regulation and modulation of the cAMP signal produced by the ACs.

Until recently, it was thought that the N-terminal extracellular domain of trypanosomal ACs had no similarity to any other protein domains, and it has been suggested that, because there were no G-protein-coupled receptors in the kinetoplastid genomes, this region may act as a receptor itself (Paindavoine et al., 1992; Seebeck et al., 2004; Laxman & Beavo, 2007). Indeed, a BLAST search combined with hidden Markov modelling recently revealed a relationship with an *Escherichia coli* l-leucine-binding protein (LBP) and the N-terminal of a representative AC from *T. brucei* (Emes & Yang, 2008). As a similar LBP acts as an amide receptor in *Pseudomonas aeruginosa* (O’Har et al., 2000), this finding lends support to the hypothesis that AC activity could be directly regulated by extracellular stimuli.

No putative ligand has been positively identified as yet, although a number of candidates have been uncovered. A preparation from the hindgut of the triatomine bug (the insect host for *T. cruzi*) was taken after the vector had been fed on chicken blood. This preparation activated AC in *T. cruzi* epimastigotes and stimulated differentiation to the metacyclic (mammalian infective) form (Fraidenraich et al., 1993). HPLC chromatography isolated a 10-kDa molecule, which was identified as part of chicken α3-globin, and the peptide was designated globin-derived factor. Synthetic peptides prepared from amino acids of chicken α3-globin also stimulated AC activity (Garcia et al., 1995). In the same way, the crude membrane fraction of the oesophagus of tsetse flies apparently activates the AC enzymes of both bloodstream and procyclic forms of *T. brucei* (Van Den et al., 1995). However, these observations do not appear to have been followed up. Similarly, as mentioned previously (see
cAMP and the life cycle), a low-molecular-weight molecule, SIF, probably secreted by the trypanosome itself, was inferred to trigger the differentiation of long slender bloodstream *T. brucei* to the nonreplicating stumpy form via the cAMP signalling cascade (Vassella *et al*., 1997).

The cellular localization of the ACs of kinetoplastids is also consistent with them acting as a receptor. Antibodies raised against ESAG4 showed a specific cellular region with which they react: the cell surface along the flagellum (Paindavoine *et al*., 1992). The localization of ACs in BSFs was apparently identical to that of procyclins. Similarly, in *T. cruzi* epimastigotes, the calcium-stimulatable AC was also found to be associated with the flagellum (D’Angelo *et al*., 2002).

In comparison with *T. brucei*, *Leishmania* and *T. cruzi* have a surprisingly low number of AC genes in their genomes: at the last count, *Leishmania* spp. had four to seven putative AC genes, depending on the species, with *T. cruzi* having nine or 10, again, dependent on the strain (http://www.tritrypdb.org).

One explanation for the much higher numbers in *T. brucei* is that in this species, the majority of AC genes are located in close proximity, along with a number of other genes, to genes encoding VSGs (see Isolation and biochemical characterization of AC genes and their proteins). These genes are found in telomeric regions termed VSG expression sites and are expressed as polycistronic units, but only one such site is active, and thus only one VSG is produced, at any one time. Periodical switching of VSG expression allows the BSF trypanosomes to evade the host immune response mounted to the previous version. In order for the parasites to persist in the mammalian host’s bloodstream for a prolonged time, a ready arsenal of hundreds of different VSG genes are encoded in the *T. brucei* genome (Pays *et al*., 2004). It could be that evolving the VSG diversity required to survive long term in the host also resulted in the duplication of the ESAGs, including ACs. Other forces of selection may then have resulted in alternative specificities for the individual receptor-cyclases to arise. A study investigating adaptive evolution in *T. brucei* identified 29 ACs as having regions of positive selection (Emes & Yang, 2008). The majority of these regions can be found in the N-terminal domains, specifically on the regions predicted to come into close contact with a putative ligand. Thus, the large numbers of ACs found in the *T. brucei* genome may allow more specific responses to the multiple ligands found in its extracellular environment, compared with the relatively sheltered intracellular lifestyle of *Leishmania* and *T. cruzi*.

**ACs of apicomplexans**

**Identification of non-mammalian AC in infected erythrocytes**

Early observations of AC activity in *Plasmodium*-infected red blood cells (RBCs) attributed the changes in cAMP concentration to the host cell cyclases (Hertelendy *et al*., 1979; Khare *et al*., 1984). However, a more detailed study of the membranes of infected erythrocytes, and of the isolated parasites, identified an AC activity with characteristics different from those displayed by the ACs of the host RBC (Read & Mikkelsen, 1991a). The erythrocyte membrane AC activity showed a marked stimulation on incubation with G-protein activators, whereas the parasites’ AC did not show any effect; forskolin also considerably stimulated erythrocyte AC activity, but did not significantly increase the activity of the parasites’ cyclase. When the AC profile of the isolated parasite was compared with that of the erythrocyte still infected with *Plasmodium*, it was found that the two profiles were highly similar, suggesting that it is the parasite ACs that predominantly contribute to the intracellular cAMP of infected RBCs and not those of the host cell.

**Effects of cAMP on gametocytogenesis**

Reports as to the effect of cAMP and its signalling cascade on the differentiation of blood-stage malaria to gametocytes are somewhat contradictory. cAMP (1 mM) added to 4-day-old cultures of *Plasmodium falciparum* in human erythrocytes resulted in 5–10 times more parasites transforming into gametocytes, with similar results for the cAMP analogue dibutyryl cAMP (Kaushal *et al*., 1980). In contrast, cAMP was shown to have an inhibitory effect on gametocytogenesis in a different strain of *P. falciparum*, as well as inhibiting asexual growth in the ring stage, but not maturation in the subsequent stages (Inselburg, 1983).

However, a third study, comparing two strains of *P. falciparum* with differing abilities to produce gametocytes, also implicated cAMP signalling. The parasites were isolated from their erythrocyte host cells and their AC- and cAMP-dependent protein kinase activities were assessed. Both strains had a similar AC activity, but the protein kinase activity in the strain that could produce gametocytes *in vitro* was threefold higher than in the strain that could not (Read & Mikkelsen, 1991b). The data suggest that it is the inability of the cAMP-dependent protein kinase of the latter strain to correctly respond to cellular cAMP levels that resulted in the impaired gametocytogenesis.

**cAMP and calcium cross-talk**

The symptomatic periodic fever of patients infected with malaria is triggered by the synchronized rupturing of parasitized erythrocytes and the release of merozoites into the bloodstream. The mammalian hormone melatonin, which is synthesized in the pineal gland when the host is in darkness, has been implicated in the synchronization of the maturation of parasites in the erythrocyte (Hotta *et al*., 2000). Melatonin was shown to increase the cytosolic concentration of Ca$^{2+}$ in saponin-isolated *Plasmodium* trophozoites,
PKA modulators also acting on the conducted with (Hotta et al., 2000). While the cell cycle experiments were necessarily potential difficulties in the interpretation of signalling, the fact that the same level of calcium stimulation can be achieved when melatonin and cAMP also increase the presence of the PKA inhibitor PKI. Furthermore, the melatonin stimulation can be completely abolished by the addition of PKA inhibitors, providing strong evidence of cAMP signalling regulating cell cycle synchrony alongside the calcium cascade (Beraldo et al., 2005).

Melatonin affects the maturation rate of blood-stage malaria by speeding up the transformation to the schizont stage (Hotta et al., 2000). This stimulation can be mimicked by the cAMP analogue and protein kinase A (PKA) activator N\(^6\)-benzoyl cyclic AMP (6-Bz-cAMP), as well as the PDE inhibitor IBMX. Furthermore, the melatonin stimulation can be completely abolished by the addition of PKA inhibitors, providing strong evidence of cAMP signalling regulating cell cycle synchrony alongside the calcium cascade (Beraldo et al., 2005).

The increase in cytosolic Ca\(^{2+}\) concentration of Plasmodium was observed to be a complex mechanism of cross-talk between the two signalling systems via PKA, summarized in Fig. 1. The increase in cytosolic Ca\(^{2+}\) due to melatonin exposure is independent of the extracellular Ca\(^{2+}\) concentration and also of PKA. This is inferred from the facts that the same level of calcium stimulation can be achieved when melatonin is assayed against the parasite in a Ca\(^{2+}\)-free medium as in a Ca\(^{2+}\)-supplemented medium, and also when assayed in the presence of the PKA inhibitor PKI. Furthermore, the cAMP analogue and PKA activator 6-Bz-cAMP also increase cytosolic Ca\(^{2+}\); however, unlike melatonin treatment, this can be blocked by PKI, suggesting that the cAMP-induced Ca\(^{2+}\) increase is mediated by PKA, but different from the melatonin response (Beraldo et al., 2005).

Evidence of the identity of the intracellular calcium store utilized by both melatonin and cAMP pathways came with the use of thapsigargin. Thapsigargin inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Pozzan et al., 1994), releasing Ca\(^{2+}\) from the organelle and resulting in the depletion of cytosolic Ca\(^{2+}\) stores in Plasmodium. Pretreatment with thapsigargin thus prevented the cytosolic Ca\(^{2+}\) increase caused by melatonin as the Ca\(^{2+}\) had already been released and dissipated from the cytosol (Hotta et al., 2000). 6-Bz-cAMP also could not increase cytosolic calcium after thapsigargin treatment when calcium was present in the assay medium (Beraldo et al., 2005). The data thus show that cAMP and melatonin utilize the same intracellular store of calcium, but also that cAMP can mobilize calcium from the external environment of the parasite while melatonin cannot.

**Fig. 1.** Model for the cross-talk between calcium and cAMP signalling during sensing of melatonin by *Plasmodium falciparum* trophozoites. Melatonin (M) binds to the melatonin receptor (MR), leading to the activation of phospholipase C (PLC) and stimulation of the production of inositol 1,4,5-triphosphate (IP\(_3\)) from phosphatidylinositol 4,5 diphosphate (PIP\(_2\)). The IP\(_3\) triggers the release of calcium ions from the endoplasmic reticulum (ER), which activates cytosolic calmodulin (CaM), which in turn stimulates adenyl cyclase (AC), leading to the production of cAMP. This results in the activation of PKA, which leads to the opening of plasma membrane (PM) calcium channels. PKA and CaM appear to independently promote differentiation to trophozoites, presumably indirectly by modulating transcription factors in the nucleus (N).

**Isolation and characterization of Plasmodium ACs**

Two putative AC genes were identified by a BLAST search of the *P. falciparum* genome: one on chromosome 14 (PfAC\(_b\)) and the other on chromosome 8 (PfAC\(_a\)). PfAC\(_a\) contained 22 introns and multiple splice variants were identified by reverse-transcriptase (RT)-PCR, containing between two and six transmembrane domains (Muhia et al., 2003). PfAC\(_b\) appears to be quite different from PfAC\(_a\), having a double catalytic domain in comparison with the single catalytic domain of PfAC\(_a\); both the soluble and the membrane-bound forms of mammalian ACs also contain two catalytic domains (Sunahara et al., 1996).

Phylogenetic analysis of PfAC\(_b\) places it into a group containing all known soluble ACs including the mammalian bicarbonate sensor and a hypothetical protein from the mosquito *Anopheles gambiae*; PfAC\(_a\) clusters with putative
ACs from other apicomplexans as well as from prokaryotic organisms. The majority of conserved residues essential for catalytic function are present in both genes, with the substrate specificity residue being lysine for the two Plasmodium genes (Muhia et al., 2003) as in all other known ACs; the residue is glutamate in all GCs. Interestingly, both PfACα and PfACβ have amino acid inserts in the catalytic domain in the same position as the Δ-subdomain insert found in all kinetoplastid ACs (see AC protein structure), which has been hypothesized to have a regulatory ligand-binding role (Bieger & Essen, 2001). A similar insert is also present in P. falciparum GCs (Carucci et al., 2000).

Because Plasmodium genes have an extremely high AT base-pair percentage, it was necessary to resynthesize the entire gene to allow the correct heterologous expression and characterization of the protein. Expression in both X. laevis and Dictyostelium discoideum resulted in increased intracellular cAMP concentrations (Muhia et al., 2003), demonstrating that PfACα is a functional AC. Further characterization in Sf9 insect cells showed that GTP was not a substrate for the catalytic domain and that forskolin could not stimulate cyclase activity (Weber et al., 2004). Northern blot analysis showed that blood-stage expression is confined to the gametocyte forms, and PfACα mRNA was also detected in gametocytes stimulated to gametogenesis (Muhia et al., 2003).

Homologous AC genes were identified in the ciliates Paramecium and Tetrahymena, both of which are grouped into the superphylum Alveolata alongside Plasmodium. Sequence analysis combined with membrane topology predictions unveiled a potential voltage-gated ion channel in the N-terminal region of the AC in the two ciliates, made up of two blocks of transmembrane helices (Weber et al., 2004). The putative ion pore is connected to the AC catalytic domain by a short 18-amino acid linker region, suggesting the regulation of the cyclase activity by the ion channel. The ion pore region also contains the signature motif found in potassium channels, indicating the possible ion specificity reminiscent of the bifunctional AC/iion-channel activity reported earlier in Paramecium tetraurelia, which was blocked by potassium channel inhibitors (Schultz et al., 1992).

The domain structure of PfACα is predicted to be topologically identical to the ciliate ACs, with a high degree of conservation in the ion pore region and the AC catalytic domain. Some differences in the transmembrane domain may indicate a difference in the range of voltage sensitivity, however. Homologous genes were identified in a range of Plasmodium species genome databases as well as in that of Toxoplasma gondii and Cryptosporidium parvum; however, no such bifunctional ACs were spotted in other protozoa such as kinetoplastids, or in metazoa, eubacteria, archea or plants – suggesting that this protein arrangement may be unique to the Alveolata (Weber et al., 2004). Attempts to express a resynthesized version of the Paramecium AC/iion channel in heterologous systems did not result in any detectable cyclase or ion conductance activity, as did similar attempts to express the full-length resynthesized PfACα (Weber et al., 2004).

Knockout of the ACα gene in Plasmodium berghei revealed an essential role for this protein in exocytosis and hepatocyte infection by sporozoites. The active invasion of a host hepatocyte is brought about by apically regulated and expressed TRAP/SSP2 proteins. The expression of these proteins is stimulated by migration through the cytoplasm of other host cells on the way to the hepatocytes and can also be stimulated by uracil and its derived nucleotides UMP, UDP and UTP. This stimulation was considerably reduced in the ACα knockout mutants compared with the wild type and the mutant parasites were approximately 50% less infective in vitro and in vivo (Ono et al., 2008). Interestingly, in the wild-type strain, the uracil-induced stimulation of TRAP/SSP2 protein expression was inhibited when the assays were carried out in a potassium-free medium, as well as by incubation with K⁺ channel inhibitors, consistent with the hypothesis that the AC activity of PfACα can be regulated by an N-terminal potassium-specific ion channel (Ono et al., 2008).

**GCs of apicomplexa**

### Role of cGMP in exflagellation

Early reports of cyclic nucleotide signalling involvement in exflagellation from infected RBCs by the male Plasmodium gametocytes were somewhat equivocal (Martin et al., 1978b; Kawamoto et al., 1990). However, more clear-cut evidence of a role for cGMP signalling in exflagellation came with the identification of xanthurenic acid as the gametocyte-activating factor that triggered exflagellation in vivo. Purification of homogenates by HPLC, followed by analysis of exflagellation-inducing fractions using MS identified a molecule of composition C₁₀H₇NO₄ (Bilikker et al., 1998; Garcia et al., 1998). This formula is consistent with xanthurenic acid, which displayed an identical MS profile and induced exflagellation at concentrations in the same range as a purified gametocyte-activating factor. Purified membranes from gametocytes showed a markedly increased GC activity compared with the asexual blood stage. Xanthurenic acid also stimulated GC activity in gametocyte membranes in a dose-dependent manner, suggesting that the signal to exflagellate may be transduced by the cGMP signalling cascade (Muhia et al., 2001).

### Isolation and characterization of ciliate and Plasmodium GCs

A GC gene was identified from a Paramecium cDNA library coding for a large protein predicted to have 22
transmembrane domains (Linder et al., 1999). Topology modelling identified two main modules in the protein: an N-terminal P-type ATPase-like region and a C-terminal similar to mammalian membrane-bound ACs. Further analysis identified a double catalytic domain (C1a and C2a), similar to metazoan ACs, but the units appear to have switched position. Conserved motifs found in the C1a unit in mammals were found in the C2a unit in *Paramecium* and vice versa. Nevertheless, all residues essential for catalytic function were conserved, with the substrate specificity residues suggesting GC activity (Linder et al., 1999). Expression in S9 cells, after resynthesis for the respective codon bias, yielded a *Km* for MgGTP of 32 μM and specificity for GTP over ATP; however, some AC activity was observed in the presence of Mn2+. Expression in this system resulted in proteolytic cleavage of the protein in the region linking the cyclase to the ATPase unit, suggesting that GC activity is not dependent on the presence of the ATPase-like section (Linder et al., 1999).

Two GC genes homologous to that in *Paramecium* were identified, isolated and analysed from *P. falciparum* (Carucci et al., 2000). The N-terminal domains showed most sequence similarity to a Ca2+ ion pump, suggesting that calcium may regulate cGMP production. The cyclase catalytic domain is well conserved between genes and across the Alveolata, with both displaying the switched catalytic subunits of *Paramecium* and *Tetrahymena*. Interestingly, PfGCα has an amino acid insert in the catalytic domain in the same position as the Λ-subdomain of trypansomatids (see AC protein structure), whereas PfGCβ does not, possibly indicating differential allosteric regulation. Northern blot analysis of RNA from blood stages showed that both genes appear to be specific for the sexual stages, consistent with their role in gametocytogenesis. Immunoelectron microscopy localized PfGCα to either the parasitophorous vacuole or the plasma membrane of the gametocyte; extracellular gametes did not appear to show any reactivity to the specific antibodies (Carucci et al., 2000).

Resynthesis and expression of the catalytic domains in *E. coli* allowed limited characterization of the *Plasmodium* GCs (Carucci et al., 2000). No AC activity was found with either Mn2+ or Mg2+, but PfGCα showed no GC activity either. However, PfGCβ did show GC activity when the C1a and C2a catalytic domains were assayed individually; this was considerably increased when the domains were assayed in combination and was entirely dependent on the presence of Mn2+.

A *P. berghei* mutant with the GCβ gene knocked out showed normal development in mice, as well as normal exflagellation and zygote to ookinete transformation after fertilization in vitro. In mosquitoes, however, no oocysts could be found, indicating an essential role for PfGCβ in the insect vector stage of the parasite’s life cycle (Hirai et al., 2006). Further investigation using confocal microscopy showed that the number of knockout ookinetes in the midgut of mosquitoes was approximately the same as for the wild type, indicating that in vivo the mutants can correctly differentiate to that stage. However, while the knockout mutants could cross the peritrophic matrix of the midgut, invasion of the epithelial cells appeared to be severely inhibited. Motility of the ookinetes was also inhibited, with the knockout moving 10-fold slower than the wild type (Hirai et al., 2006). *In vitro*, the PbGCβ knockout parasites could form oocysts and transform into infective sporozoites, indicating that the GC is not involved in further development. It thus seems likely that the disruption of PbGCβ and the resultant inability to penetrate the epithelial cell layer of the mosquito midgut may be directly attributable to the parasite’s severely reduced motility, ultimately leading to an inability to complete its life cycle.

**PDEs of kinetoplastids**

The first PDE activities identified in kinetoplastids were observed in whole-cell lysates of *T. brucei*, *T. cruzi* and *Leishmania* (Walter et al., 1974; Goncalves et al., 1980; Walter & Opperdoes, 1982; al-Chalabi et al., 1989). Purification of the lysates showed that the vast majority of cAMP PDE activity was contained in the soluble fraction. Later investigators, however, found two distinct peaks of activity after further purification by affinity chromatography of *T. cruzi* epimastigote lysates. The purified PDE was activated by Ca2+/calmodulin and displayed two distinct activity peaks; however, the *Km* values for cAMP were orders of magnitude higher and could not be stimulated by Ca2+ (al-Chalabi et al., 1989; Rascon et al., 2000). Notably, in all of the studies carried out, inhibitors of mammalian PDEs were either ineffective or significantly less active against the kinetoplast PDEs.

The utilization of molecular techniques combined with the recent releases of the genome databases of a range of kinetoplastids provided a major spur in cyclic nucleotide signalling research. Many PDE genes were identified, cloned, sequenced, and relatively short space of time by multiple laboratories and collaborations. Unfortunately, each group adopted a different naming convention for the publication of their identified enzymes, resulting in a confusion of species abbreviations, numbers and letters threatening to slow and obfuscate scientific discourse on the subject. To rectify this, a unified nomenclature was published (Kunz et al., 2006), laying down a straightforward naming convention based on grouping PDEs by gene sequence homology and biochemical characteristics. Four families of Class I PDEs have been identified in kinetoplastids (PDEA–D), all of which are present as homologues in the genome databases of *T. brucei*, *T. cruzi* and *Leishmania*. A summary of the main characteristics of each PDE family in kinetoplastids can be found in Table 1.
PDEA

Trypanosoma brucei PDEA was identified by complementation screening of a cDNA library in a yeast mutant deficient in PDE enzymes and sensitive to heat shock (Kunz et al., 2004). The full sequence was determined, predicting a 620-amino acid protein. Further analysis indicated that the catalytic domain was well conserved between TbrPDEA and human PDEs, sharing approximately 30–40% identity with the 11 Class I PDEs in humans, conserving the key signature motif of all Class I PDEs (His-Asp-[Leu/Ile/Val/Met/Phe/Tyr]-X-His-X-[Ala/Gly]-X-X-Asn-X-[Leu/Ile/Val/Met/Phe/Tyr]). The almost complete absence of sequence identity in the N-terminal region outside the catalytic domain confirms TbrPDEA as being in a distinct family of Class I PDEs separate from all other PDE families identified (Kunz et al., 2004). Homologues of TbrPDEA have also been identified and characterized in Leishmania major and T. cruzi, having similar sequence characteristics (Alonso et al., 2007; Bhattacharya et al., 2009).

Northern blots and RT-PCR identified the expression of TbrPDEA mRNA in all life-cycle forms of T. brucei cultured in vitro (Kunz et al., 2004). Similarly, PDEA was also identified in both mammalian and insect forms of L. donovani, with relative quantification showing the highest expression in promastigotes during log-phase growth (Bhattacharya et al., 2009). In spite of being able to complement PDE-deficient yeast, an active form of PDEA from T. brucei, T. cruzi or L. major could not be purified, suggesting low expression in yeast (Kunz et al., 2004; Johner et al., 2006; Alonso et al., 2007). Kinetic characterization of PDEA was therefore conducted on recombinant protein expressed and purified from E. coli.

PDEA required a divalent metal ion, such as Mg$^{2+}$, for full activity, yielding an unexpectedly high $K_m$ for cAMP of between 160 μM for PDEA from L. donovani and over 600 μM for the T. brucei homologue (Kunz et al., 2004; Bhattacharya et al., 2009) (Table 1). Most Class I PDEs characterized from other organisms have a much lower $K_m$, usually well below 50 μM. cGMP neither inhibited hydrolysis of cAMP nor stimulated it, clearly demonstrating that PDEA is specific for cAMP (Kunz et al., 2004; Alonso et al., 2007; Bhattacharya et al., 2009).

A double knockout of PDEA demonstrated that the gene was nonessential for T. brucei, with in vitro differentiation of BSFs to procyclics or procyclic infestation of the tsetse fly midgut completely unaffected (Gong et al., 2001). The total PDE activity was reduced by approximately 20–30% in the mutant procyclic and BSFs compared with the wild type, with the steady-state level of intracellular cAMP increasing by approximately one-third. The morphology and motility of the knock out mutants were indistinguishable from the wild type; however, the procyclic generation time was increased by around 2.5 h (Gong et al., 2001).

In L. donovani promastigotes, overexpression of PDEA produced a 3–3.5-fold increase in PDEA mRNA and protein levels. The resultant reduction in intracellular cAMP concentration was concomitant with a reduced upregulation of oxidative stress-protection genes during differentiation to the amastigote form (Bhattacharya et al., 2008). What is not clear is whether the overexpression of any other L. donovani PDE would have had the same effect or whether it is solely limited to that of PDEA. Curiously, the inhibition of gene upregulation during amastigote differentiation is entirely reversed on the addition of 20 μM IBMX to the overexpressing promastigotes. This is surprising, considering that IBMX does not inhibit recombinant T. cruzi or T. brucei PDEA up to 1 mM (Kunz et al., 2004; Alonso et al., 2007), suggesting that the effects of PDEA overexpression could be compensated for by the inhibition of a different, IBMX-sensitive PDE, or by non-PDE off target effects of the compound.

PDEB

Basic gene features and enzyme characterization

The PDEB family contains two tandemly arranged genes, termed PDEB1 and PDEB2. Both PDE enzymes were first cloned and sequenced from T. brucei and share 99.6% amino acid identity in the catalytic core region (Rascon et al., 2002; Zoraghi & Seebeck, 2002). The ORFs predict proteins of 930 amino acids in length and contain the canonical Class I PDE signature motif in the catalytic domains. Highly homologous genes were identified in T. cruzi (D’Angelo et al., 2004; Diaz-Benjumea et al., 2006) and Leishmania (Johner et al., 2006). Interestingly, when the catalytic domains of LmjPDEB1 and LmjPDEB2 were compared, a small stretch of 24 divergent amino acids was observed in the otherwise almost perfectly conserved domain. The position of this nonconserved region is the same as a similar divergent sequence in T. brucei and T. cruzi (Johner et al., 2006). The functional significance of this variable patch in the middle of the catalytic domain is unclear and the authors speculate that this stretch might confer specific regulatory or functional differences in the isoenzymes (Johner et al., 2006).

In agreement with the extent of the sequence similarity, the kinetic characteristics of PDEB1 and PDEB2 are also very similar (Table 1). Both enzymes are specific for cAMP, with $K_m$ values ranging from 1 to 11 μM in T. brucei, T. cruzi and L. major, with cGMP having no effect on PDE activity (Rascon et al., 2002; Zoraghi & Seebeck, 2002; D’Angelo et al., 2004; Diaz-Benjumea et al., 2006; Johner et al., 2006).

The mammalian PDE inhibitors assayed against kinetoplastid PDEB proteins displayed IC$_{50}$ values orders of magnitude above those against mammalian PDEs (Wentzinger & Seebeck, 2006). This suggests that in spite of the
Table 1. Summary of the characteristics of kinetoplastid phosphodiesterase enzyme families

<table>
<thead>
<tr>
<th>Organism</th>
<th>T. brucei</th>
<th>T. cruzi</th>
<th>Leishmania</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Current name</td>
<td>Former names</td>
<td>( K_m ) cAMP (( \mu \text{M} ))</td>
</tr>
<tr>
<td>PDEA</td>
<td>TbPDE1</td>
<td>&gt; 600</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>PDEB1</td>
<td>TbPDE2C</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>PDEB2</td>
<td>TbPDE2B</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>PDEC</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>PDED</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*Kunz et al. (2006).
1Kunz et al. (2004).
2Gong et al. (2001).
3Alonso et al. (2007).
4Bhattacharya et al. (2009).
5Zoraghi & Seebeck (2002).
6Oberholzer et al. (2007).
7Diaz-Benjumea et al. (2006).
8Johner et al. (2006).
9Laxman et al. (2002).
10Laxman et al. (2005).
11Angelo et al. (2004).
12Alonso et al. (2006).
13Gong et al. (2001).
14Kunz et al. (2005).
15Bhattacharya et al. (2008).
16Kunz et al. (2009).
ND, not determined.
conserved catalytic domain, there is sufficient divergence from host enzymes to potentially allow the engineering of compounds specific for kinetoplastid PDE active sites and, in the light of the essential nature of PDEB genes in trypanosomes [see Effects of RNAi of the PDEB family], form the basis of novel chemotherapies.

**GAF domains of PDEB**

The N-terminal regions of all known kinetoplastid PDEB1 and PDEB2 contain two regions known as GAF domains. These domains have been shown to function as regulatory, small ligand-binding entities; in mammalian PDE families 2, 5 and 6 they bind cGMP to modulate enzyme activity (Aravind & Ponting, 1997). Since the characterization of the recombiantly expressed PDEB1 and PDEB2 proteins ruled out modulation by cGMP (see Table 1), the properties of the two GAF domains (GAF-A and GAF-B) were investigated further. The N-terminal section of TbrPDEB2 up to the end of the GAF-A domain was expressed and immunoprecipitated with [3H]-cAMP, clearly demonstrating binding of the nucleotide. Affinity purification and assay with labelled and unlabelled cAMP in a competition binding of the nucleotide. Affinity purification and assaying with concentrations of the substrate (Laxman binding cAMP, resulting in more hydrolysis at lower concentrations) and tryomastigote forms in *T. cruzi* (Zoraghi & Seebeck, 2002; Diaz-Benjumea et al., 2006; Johner et al., 2006; Oberholzer et al., 2007). Subcellular localization experiments were first conducted in *T. cruzi* showing that TcrPDEB2 had the same fraction distribution as paraflagellat rod protein from the flagellum (D’Angelo et al., 2004). Confirmation of the flagellar localization of PDEB1 and PDEB2 in *T. cruzi* came from immunofluorescent microscopy (D’Angelo et al., 2004).

Similar results were also observed in *T. brucei*: immunofluorescence microscopy of TbrPDEB1 in procyclines showed that the protein was localized along the flagellum, but only where the flagellum had exited the cell body. TbrPDEB2 had a minor fraction colocalized with TbrPDEB1, but the majority was distributed throughout the cytoplasm. RNA interference (RNAi) of PFR2, a major structural protein of the paraflagellat rod, results in proteins normally associated with the structure amassing at the tip of the flagellum in a droplet shape. Tagged TbrPDEB1 accumulates in the tip of the flagellum on induction of RNAi against PFR2, as does a small portion of TbrPDEB2, clearly indicating that both are integrated into the paraflagellat rod structure. The remainder of the TbrPDEB2 is distributed normally in the cytoplasm showing dual localization for this isoform (Oberholzer et al., 2007).

Two alleles for TbrPDEB2 were identified in the strain used in the protein localization studies, but allelic variation was found not to be responsible for the dual localization (Kunz et al., 2009). Instead, the N-terminal 70 amino acids of the two proteins appear to contain the signal for localization. Studies with recombinant enzymes indicated that PDEB1 is responsible for the dual localization in specific transport of a green fluorescent protein (GFP) fusion protein to the PFR, whereas the same section from PDEB2 yielded localization mainly in the cell body (Luginbuehl et al., 2010). Mutating the specific leucine residue to cysteine, and vice versa, resulted in a switching of the localization of the GFP fusion proteins.

**Effects of RNAi of the PDEB family**

RNAi against TbrPDEB1, TbrPDEB2 or both had no effect on the survival or the proliferation of procyclic *T. brucei*. In contrast, RNAi of the TbrPDEB family demonstrated that these enzymes are essential in BSF trypanosomes (Zoraghi & Seebeck, 2002; Oberholzer et al., 2007). Induction of interference simultaneously against TbrPDEB1 and TbrPDEB2 in either the bloodstream or the procyclic form resulted in a strong reduction of both genes’ mRNA. No compensatory upregulation of genes from the other trypanosomal PDE families (PDEA, PDEC and PDED) was observed. The effect on the phenotype of the BSFs is emphatic, with the double-RNAi construct resulting in the cells rounding up and becoming...
multinuclear, multikinetoplastic and multiflagellar. RNAi against TbrPDEB1 and TbrPDEB2 individually had no apparent phenotypic effect. The ratio of the multiple nuclei to kinetoplasts in the double-RNAi induced cells was maintained at one, suggesting that DNA replication and segregation occur as normal, but that separation of the mother and daughter cells is blocked, apparently as a result of a cytokinesis defect. Normal proliferation of the BSFs was completely disrupted, resulting in eventual cell death approximately 50 h after double-RNAi induction (Oberholzer et al., 2007). The effect on intracellular cAMP mirrored that of the morphological phenotype in that RNAi against either individual TbrPDEB gene did not result in a change in the cAMP level relative to the control, but knockdown of both genes produced a 100-fold increase in cAMP in the BSFs. In procyclics, the double induction only resulted in an approximately 10–30-fold increase in intracellular cAMP (Zoraghi & Seebeck, 2002; Oberholzer et al., 2007). It would thus seem that BSF trypanosomes can compensate for the loss of either PDEB activity, but not for the loss of both simultaneously. One of the clear conclusions from this study is that BSFs are highly sensitive to increased levels of cAMP, whereas the insect forms are relatively resistant. This is consistent with the much higher sensitivity of BSFs to membrane-permeable cAMP analogues (Oberholzer et al., 2007).

Further investigation into the effects of inducing RNAi against PDEB genes in infected mice elegantly highlighted the essential nature of these proteins in vivo. Mice infected with BSF trypanosomes transfected with the double-TbrPDEB1/B2 RNAi construct produced high parasitaemias after 3–4 days postinoculation. However, mice pretreated with doxycycline in the drinking water from 2 to 3 days before infection displayed no sign of parasitaemia for the entire duration of the experiment (35 days). The same effect was seen when the mice were chemically immunocompromised, ruling out the necessity for an intact immune system to clear the trypanosomes with the double-RNAi construct (Oberholzer et al., 2007). This study, in conjunction with the earlier RNAi experiments, clearly shows that TbrPDEB1 and TbrPDEB2 together are absolutely essential for the proliferation of BSF trypanosomes, in vitro and in vivo, and that the inhibition of both proteins results in cell death, validating the genes as excellent drug targets for future chemotherapies.

Pharmacological validation of TbrPDEB as a drug target

The genetic validation of the T. brucei PDEs by RNAi prompted a drug discovery programme at Altana Pharma and Nycomed involving the screening of compound libraries for inhibitors of TbPDEB1 and B2. One potent inhibitor, BYK54826, was shown to increase intracellular cAMP levels within minutes (Shakur et al., 2011) – further confirmation of the high constitutive rate of AC activity in trypanosomes. Despite the rapid and sustained increase to very high levels of cAMP, BYK54826-induced cell death was slow in onset. The effect of the drug was, in fact, completely consistent with the observations of the TbPDEB1/2 double knockdown: defects in cytokinesis during cell division resulting in multinucleated cells that were ultimately not viable (Shakur et al., 2011).

The crystal structure of PDEB1 catalytic domain of L. major

A major advance in the quest for specific inhibitors of kinetoplastid PDEB genes came with the resolving of the crystal structure of the catalytic domain of L. major PDEB1 in complex with the nonspecific inhibitor IBMX (Wang et al., 2007). The PDEB1 fragment forms an apparent dimer on crystallization, with the catalytic domain made up of 16 α-helices and no β-sheets. A low root mean squared deviation for the position of the Cα atoms of selected amino acid residues indicates similar folding of the polypeptide compared with those of human PDEs; however, four regions have significant differences. Two of these are in positions that have divergent insertions or deletions in almost every Class I PDE characterized to date, but the other two (residues 729–752 in the H9 helix of the H-loop and residues 858–882 in the M-loop) result in a positional shift compared with human PDEs of up to 3 Å. This is predicted to significantly alter interactions with inhibitors (Wang et al., 2007), which is of obvious pharmacological interest.

The 3D structure of LmjPDEB1 was superimposed over human PDE crystal structures from eight different gene families in order to identify aspects of the catalytic domain unique to the parasitic PDE that may be utilized in the development of chemical inhibitors specific to the parasite. Some subtle and not so subtle differences were identified, with a glutamine (residue 887) displaying the largest positional displacement compared with representatives of all 11 human PDEs. This invariant glutamine is conserved across the Class I PDEs and plays an essential role in substrate and inhibitor binding: large positional changes may allow the structural engineering of inhibitors to capitalize on this distinctive difference and confer selectivity for that compound over human PDEs.

The most unique feature of the crystal structure of LmjPDEB1 is the identification of a subpocket within the active site essentially forming a pore or channel through the enzyme. The pocket (dubbed the ‘L-pocket’ by the paper’s authors) is gated by the residues Thr854, Met874 and Gly886. The latter amino acid can bend to accommodate any conformation due to its flexible backbone and is conserved in four out of the five identified L. major PDEs, but found only in PDE10 of the human PDEs. Met874 and
Gly886 are separated by 7.5 Å to leave the pocket open at the active site. The L-pocket has hydrophobic as well as hydrophilic characteristics and is large enough to accommodate a structure the size of a five-membered ring (Wang et al., 2007). A very similar pocket is observed in the T. brucei PDEB1 crystal structure (H. Ke, pers. commun.).

**PDEC**

The TcrPDEC gene has some unusual characteristics for a kinetoplastid PDE enzyme. The ORF predicts a polypeptide of 924 amino acids with the catalytic domain situated in the middle of the protein (Thr291–Ser657), whereas all other Class I PDEs contain a C-terminal catalytic domain (Kunz et al., 2005). The catalytic core contains the canonical Class I PDE signature motif, confirming its grouping in spite of its nonconforming functional domain arrangement. Amino acids important for catalytic function are all conserved. However, one of the residues conferring substrate specificity (Asn395) is substituted for alanine, which, as is the case for human PDEs 5 and 6, might allow it to hydrolyse cGMP instead of cAMP. Southern blot analysis of restriction digested genomic DNA indicates that TcrPDEC is a single-copy gene. However, two different alleles were detected with 38-amino acid residues different between them: 21 conservative substitutions and 17 nonconserved. Although six amino acid differences were located in the catalytic domain, none of them were predicted to be essential for the catalytic function of the enzyme (Kunz et al., 2005).

The N-terminal contains an FYVE-type domain (Pro10–Gly73), along with two coiled-coil regions (Asp144–Asp179 and Lys207–Glu264) (Kunz et al., 2005; Alonso et al., 2006). Classical FYVE domains bind to phosphatidylinositol-3-phosphate [PtdIns(3)P] embedded in the membrane, but the FYVE-like domain of TcrPDEC has significant changes, making it unlikely that it binds to PtdIns(3)P. The recombinant FYVE domain of TcrPDEC does not bind to PtdIns(3)P or to a series of other phospholipids in a dot-spot assay (Kunz et al., 2005). Investigation of the coiled-coil regions indicates that they may be important to the quaternary structure of the protein, although expression of a truncated TcrPDEC catalytic domain, minus the FYVE and coiled-coil regions, complemented PDE-deficient yeast (Kunz et al., 2005). Not only does this confirm that TcrPDEC is an active PDE enzyme that can hydrolyse cAMP but also that the FYVE domain and the coiled-coil regions are not essential for catalytic activity.

Two independent characterizations of PDEC in T. cruzi yielded $K_m$ values for cAMP of 20–32 μM (Kunz et al., 2005; Alonso et al., 2006). Kunz et al. (2005) reported that cGMP was also a substrate for the enzyme, with a $K_m$ of ~80 μM – a unique feature for kinetoplastid PDEs characterized to date, but predicted from the sequence analysis. In contrast, Alonso et al. (2006) reported that cGMP did not compete with cAMP up to a concentration of 2 mM. It must be noted that the recombinant PDEC was purified from different fractions of yeast cell lysates, with Alonso et al. (2006) characterizing PDE activity from the membrane fraction and Kunz et al. (2005) investigating soluble cell lysates. This dual localization and kinetic activity may represent the characterization of the two distinct alleles, although further investigation is required to ascertain whether this is the case.

Inhibitors of TcrPDEC have been identified by homology modelling and the virtual screening of a compound library originally synthesized with the aim of inhibiting the mammalian PDE4 family (King-Keller et al., 2010). A number of compounds tested inhibited recombinant TcrPDEC with $IC_{50}$ values < 10 μM and also inhibited amastigote growth with similar potencies. Intracellular cAMP concentrations were increased on incubation with the test compounds as well as the ability to recover from hypo-osmotic stress. This supports findings from earlier work where it was noted that cAMP levels were increased in epimastigotes on incubation under hypo-osmotic conditions and that the nonhydrolysable analogue dibutylryl-cAMP mimicked the induction of aquaporin translocation seen under hypo-osmotic stress (Rohlff et al., 2004). Interestingly, an aquaporin from T. cruzi was demonstrated to interact with a cAMP-dependent protein kinase (Bao et al., 2008), providing more circumstantial evidence of the cAMP cascade mediating the response to osmotic stress. If the inhibitors identified by King-Keller and colleagues are specific for PDEC over the other kinetoplastid PDEs, they could provide useful tools in teasing apart the osmoregulatory pathway as well as provide the basis for potential new chemotherapies.

PDEC has also been identified in the genomes of T. brucei and L. major, although apart from a cursory analysis of the predicted amino acid sequence confirming its belonging to the Class I PDEs, no in-depth characterization has been published as yet. It was noted, however, that the L. major version, at least, had conserved the ‘dual-specificity’ alanine residue, indicating that in *Leishmania*, the protein might also be able to hydrolyse cGMP (Johner et al., 2006).

**PDED**

So far, PDED has not been cloned and recombinantly characterized from any of the kinetoplastids, although it does appear in all the kinetoplastid genomes published. It is predicted that PDED is a single-copy gene coding for a protein of approximately 700 amino acids, with its catalytic domain containing the conserved Class I PDE signature motif and being C-terminally located (Johner et al., 2006; Kunz et al., 2006). In an investigation focusing on the role of PDEA in L. donovani (see PDEA), it was noted that PDED was expressed in both the insect and the mammalian infective forms (Bhattacharya et al., 2009).
PDEs of apicomplexans

Characterization of apicomplexan PDE enzymes had been relatively limited in the pregenomic era, other than to infer their modulatory presence being necessary due to the observation of adenyllyl and GC activity, as well as that of cyclic nucleotide-specific effector-proteins PKA and protein kinase G (PKG). However, four putative P. falciparum PDEs have now been identified, containing the Class I signature motif and sharing approximately 40% amino acid identity (Yuasa et al., 2005; Wentzinger et al., 2008). A general naming convention was adopted and the four PIPDEs are now termed PIPDEα, β, γ and δ (Wentzinger & Seebeck, 2006). All four genes are predicted to contain between four and six transmembrane domains, unique among the Class I PDEs, suggesting that they are all integral membrane proteins (Wentzinger et al., 2008). As predicted, cytosolic and membrane fractions purified from mixed blood-stage cultures showed that the vast majority of cGMP-PDE activity localized in the membrane fraction (Yuasa et al., 2005; Wentzinger et al., 2008), with an identical profile for cAMP-PDE activity (Wentzinger et al., 2008).

PDEα

In P. falciparum, PDEα is represented by at least two splice variants: PIPDEαA and PIPDEαB. The first investigation found an ORF made from two exons and predicted to code for a protein of 884 amino acids (Yuasa et al., 2005), whereas a second study identified the ORF as starting 210 bp upstream, yielding a protein of 954 amino acids (PIPDEαA) with a splice variant of 892 amino acids (PIPDEαB) (Wentzinger et al., 2008). PIPDEαA is predicted to have six transmembrane domains in the N-terminal, while PIPDEαB has helices 4 and 5 removed to yield four membrane-spanning domains. Although no other functional domains were identified, apart from the catalytic domain, a potential phosphorylation motif was found that could potentially be phosphorylated by PKA, casein kinase II and/or tyrosine kinase, as well as a casein kinase II-specific motif after the last transmembrane helix and before the catalytic domain. These motifs are also present in Plasmodium vivax, Plasmodium yoelii and Plasmodium knowlesi, possibly indicating a conserved regulatory function (Wentzinger et al., 2008).

Relative expression levels in the intraerythrocytic stages were investigated using RT-PCR. The abundance of PIPDEα transcripts mirrored the proportion of ring stages in the cultures, inferring stage-specific regulation of PIPDEα production (Yuasa et al., 2005).

Recombinant expression of a hexahistidine-tagged PIPDEα catalytic domain in E. coli as well as a catalytic domain resynthesized using bacterial codon preferences yielded similar kinetic data. Divalent cations were necessary for proper function, with PIPDEα displaying a $K_m$ for cGMP between 0.65 and 2 μM; no cAMP-hydrolysing activity was observed (Yuasa et al., 2005; Wentzinger et al., 2008). Of the mammalian PDE inhibitors tested, the most active was zaprinast, with an IC$_{50}$ value of ~3.5 μM. Mutation of a conserved glycine residue implicated in zaprinast binding in bovine PDE5A resulted in no change to the IC$_{50}$ for the compound by PIPDEα, suggesting a divergence in the catalytic domain that may allow chemical engineering of a specific inhibitor (Yuasa et al., 2005).

The endogenous cGMP-hydrolysing activity of the membrane fraction from mixed asexual blood-stage parasites could be inhibited by zaprinast with an IC$_{50}$ of 4.1 μM, similar to the inhibition of the recombinant PIPDEα catalytic fragment. In vitro treatment of asexual blood-stage P. falciparum with zaprinast resulted in growth inhibition of the parasite, with an ED$_{50}$ value of ~35 μM. Assuming that this effect is mediated by PIPDEα, this implies that its activity is essential for the development and proliferation of blood-stage Plasmodium parasites (Yuasa et al., 2005). However, knockout mutants of PDEα in P. falciparum were derived with no obvious morphological changes or disruption of the blood-stage asexual cell cycle. The only discernable effect was that ring stages always appeared slightly sooner after synchronization of the culture compared with the parental strain (Wentzinger et al., 2008).

RT-PCR determined that the other three PIPDEs were not upregulated to compensate for the loss of PDE activity and, also, that PIPDEγ and PIPDEδ are only expressed at very low levels in blood stages (Wentzinger et al., 2008). These data suggest that PIPDEα is not an essential enzyme in blood-stage Plasmodium, at least during in vitro culture, and that the other PDE that is highly expressed in intraerythrocytic stages, PIPDEβ, may be more important for controlling cyclic nucleotide levels.

The effect of the PIPDEα knockout on the overall PDE activity showed an approximately 20% reduction in cGMP hydrolysis, with no effect on cAMP-PDE activity. This is consistent with the data obtained from the characterization of the recombinant catalytic domain, which showed cGMP specificity, and also implies that at least one other PDE is capable of cGMP-PDE activity. Furthermore, when cAMP was present in excess, cGMP-PDE activity was reduced by 20% in the membrane fraction of wild-type parasites compared with > 60% in the PIPDEα knockout, suggesting that at least one of the remaining PDEs is inhibitable by cAMP. When cGMP was present in excess, cAMP-PDE activity was also reduced in both wild type and PIPDEα knockout strains. Taken together with the fact that PIPDEα is cGMP-specific, the above data suggest that at least one out of PIPDEβ, γ or δ is a dual-substrate PDE enzyme (Wentzinger et al., 2008). Because PIPDEβ is the only other PDE highly expressed in asexual blood stages, this makes it the most likely candidate for dual specificity.
PDE\(\beta\)

Sequencing of PfPDE\(\beta\) revealed a gene of 3420 bp coding for a protein of 1139 amino acids. The catalytic domain is located at the C-terminal end of the polypeptide and the N-terminal contains six transmembrane domains, suggesting that it is an integral membrane protein. Interestingly, hidden Markov modelling of the transmembrane regions predicts that PfPDE\(\beta\) is oriented in the membrane so that the catalytic domain is not in the parasite cytosol, but is projecting into the parasitophorous vacuole; this may allow the parasite to modulate the cyclic nucleotide milieu of the host erythrocyte. RT-PCR of total RNA extracted from blood stages, in combination with knocking out PfPDE\(\alpha\), indicates that it is the predominantly expressed PDE in these stages. So far, recombinant expression of PfPDE\(\beta\) has proved unsuccessful in numerous systems and with different methodologies (Wentzinger et al., 2008).

PDE\(\gamma\)

PDE\(\gamma\) is found on chromosome 13 of \textit{P. falciparum}, approximately 3000 bp downstream of PfPDE\(\beta\). Sequencing of PfPDE\(\gamma\) identified an ORF of 2310 bp made from nine exons and coding for a protein of 769 amino acids, a slightly larger gene than published in the \textit{Plasmodium} genome database (Wentzinger et al., 2008). In an investigation into gametogenesis, PfPDE\(\gamma\) was knocked out as an experimental control. No aberrant phenotype was observed in gametocytes, with normal rounding up and exflagellation upon stimulation by xanthurenic acid, and unchanged PDE activities for cGMP and cAMP. It was concluded that this protein is not essential in gametogenesis (Taylor et al., 2008).

PDE\(\delta\)

PDE\(\delta\), made from six exons and coding for a protein of 815 amino acids, is located on chromosome 14 of \textit{P. falciparum} and also contains six N-terminal transmembrane regions (Wentzinger et al., 2008).

Previous work had observed that cGMP-PDE inhibitor zaprinast could stimulate rounding up and exflagellation of gametocytes in the absence of xanthurenic acid, implicating the cGMP signalling pathway in male gametocyte exflagellation (McRobert et al., 2008). However, knocking out GC\(\beta\) in \textit{P. berghei} failed to produce any significant change in gametocyte phenotype (see Isolation and characterization of ciliate and \textit{Plasmodium} GCs), leading to the hypothesis that cGMP degradation rather than production was critical in gametogenesis. Because of the high level of PfPDE\(\delta\) mRNA expression, compared with the other PDEs, in developing and mature gametocytes, PfPDE\(\delta\) knockouts were generated in order to investigate the involvement of cGMP metabolism further (Taylor et al., 2008).

Analysis of the particulate fractions of lysed stage V gametocytes showed an approximately 50% reduction in cGMP PDE activity in the knockout mutants compared with the wild type, indicating that PfPDE\(\delta\) does hydrolyse cGMP and also that at least one other cGMP-PDE is expressed in this parasite stage. Zaprinast reduced the wild-type PDE activity by approximately 75%, whereas in the knockout parasites, the activity was removed completely, indicating that PfPDE\(\delta\) is at least partly resistant to the inhibitor and also that the residual PDE activity is made up of zaprinast-sensitive enzymes, probably PfPDE\(\alpha\) (Taylor et al., 2008).

Phenotypically, the PfPDE\(\delta^-\) mutants generated normal gametocytes. However, their ability to round up and exflagellate on stimulation by xanthurenic acid was significantly reduced. A similar effect was observed on treatment with zaprinast. On examination of the ability of the gametocytes to release themselves from infected erythrocytes, < 20% of the PfPDE\(\delta^-\) mutants emerged after stimulation with XA compared with nearly 60% emergence by the control strain (Taylor et al., 2008). This indicates that PfPDE\(\delta\) is important for both male and female gametogenesis. The authors of this study propose that xanthurenic acid stimulates GC activity, thereby increasing the cGMP level, which, in turn, activates PKG to trigger gametogenesis. PfPDE\(\delta\), in conjunction with other minor PDEs in this stage, degrade the cGMP signal, allowing further gametocyte development, eventually resulting in full gametogenesis (Fig. 2a). A sustained increase in cGMP, for example by inhibiting PfPDE\(\delta\), retards development once gametogenesis has been initiated (Taylor et al., 2008).

On the other hand, knocking out PDE\(\delta\) in the rodent parasite \textit{P. berghei} suggests an essential role for cGMP signalling in ookinete motility and infection of the mosquito, rather than exflagellation. PbPDE\(\delta\) knockout mutants did not differ from the wild type with respect to their ability to produce gametocytes and to exflagellate on stimulation with xanthurenic acid; however, after 24 h of \textit{in vitro} culturing, almost all ookinetes had become spherical in shape and midgut infection of mosquitoes was massively reduced (Moon et al., 2009). Injection of PbPDE\(\delta\) knockout ookinetes into the haemocoel of mosquitoes resulted in the production of mouse infective parasites in the salivary gland, demonstrating that the mutants are still viable for continued differentiation when the necessity of crossing the midgut epithelium is bypassed. Closer inspection of the mutant ookinetes showed that they were still capable of rotating, but were unable to glide forward in the normal way. This phenotype is very similar to the GC\(\beta\) cell line described earlier (see Isolation and characterization of ciliate and \textit{Plasmodium} GCs), where the mutants were unable to invade midgut epithelium cells after crossing the peritrophic matrix and ookinet motility was disrupted (Hirai et al., 2006), suggesting that both the production of
cGMP and its subsequent degradation are necessary for epithelium invasion.

The addition of the PKG-specific inhibitor Compound 1 (see Apicomplexan PKG; *Plasmodium* spp.) prevented the formation of the PbPDE6 knockout morphology phenotype, although motility was still impaired, as did the creation of a double knockout cell line, removing both GCβ and PbPDE6 (Moon *et al*., 2009). This clearly shows that GCβ, PDE6 and PKG are all on the same pathway and regulate the motility of the ookinetes. Overactivation of PKG by cGMP (e.g. by blocking PDE6) results in rounding up and loss of motility; no stimulation of PKG (e.g. by blocking GCβ or inhibiting PKG) just results in the loss of motility and cell invasion (see Fig. 2b).

**Kinetoplastid PKA**

*T. brucei*

cAMP-dependent protein kinase (PKA) activity has been associated with a number of processes in *T. brucei*, but mostly by inference from the presence of ACs and cAMP-specific PDEs in the parasite, as well as by analogy with mammalian systems that use PKA as the main effector protein in cAMP signalling. The first actual measurement of cyclic nucleotide-dependent protein kinase activity in *T. brucei* came with the identification and sequencing of a trypanosomal PKA-regulatory subunit. In mammalian organisms, the PKA holoenzyme is found in an inactive state, with two catalytic subunits (PKA-C) bound in a heterotetramer to two regulatory subunits (PKA-R). When cAMP binds to the regulatory subunits, a conformational change activates the phosphorylating catalytic domain by releasing the PKA-C subunit from the holoenzyme. In *T. brucei*, a 499-amino acid protein with high homology to eukaryotic regulatory subunits of PKA was identified and named *TbRSU* (Shalaby *et al*., 2001). The protein has the usual two cyclic nucleotide-binding domains, which are predicted to retain all the conserved residues necessary for function, as well as a pseudoinhibitor site, which interacts with the catalytic subunit.

Despite this broad homology with type I mammalian PKA regulatory subunits, some sequence deviations were apparent, the most intriguing of which was a substitution at residue 319 in the nucleotide-binding region. In all cAMP-binding domains, this residue is alanine, whereas in cGMP-
Cyclic-nucleotide signalling in protozoa


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dependent protein kinases (PKG), this amino acid is either a serine or a threonine. Uniquely, this residue is a valine in TbRSU (Shalaby et al., 2001).

Antibodies to full-length recombinant TbRSU were raised and immunoblots detected the protein in both bloodstream and procyclic forms, with higher levels in the latter (Shalaby et al., 2001). Immunoprecipitation of the native TbRSU coprecipitated a protein of approximately 40 kDa, indicating that the trypanosomal catalytic subunit elutes bound to its regulatory subunit. The coprecipitates displayed phosphorylating activity, transferring a phosphate to classical PKA-specific substrates, as well as being inhibited by the peptide inhibitor PKI (also specific for PKA). Surprisingly, however, cAMP did not stimulate the phosphorylating activity of the purified lysates. Even more unexpected was the finding that cGMP stimulated kinase activity three to four times that of controls, with 20 μM of the nucleotide yielding the maximum activity, an observation unique to T. brucei out of all the eukaryotic PKA homologues characterized at that point (Shalaby et al., 2001).

To investigate the cyclic nucleotide regulation specificity further, the nucleotide-binding domains of TbRSU were individually expressed in Drosophila S2 cells and purified. Domain A had a dissociation constant (Kd) for cGMP of 7.5 μM, while domain B had a Kd of 11.4 μM. cAMP did not compete with cGMP up to concentrations 100-fold in excess (Shalaby et al., 2001). It thus appears that T. brucei expresses a PKG activity, but no classical cAMP-stimulatable PKA activity has been demonstrated to date.

Evidence of PKA activity has also been uncovered in Trypanosoma evansi. Intriguingly, cAMP also had no effect on the phosphorylating activity of whole-cell lysates or particulate fractions, but a doubling of kinase activity was recorded on the addition of cAMP in the soluble fraction (Galan-Caridad et al., 2004). This stimulation could be inhibited almost completely by the addition of PKI. These results could be interpreted as evidence for the presence of kinase inhibitory factors that are not sensitive to cAMP in whole-cell lysates, but have been removed by fractionation of the soluble extracts.

Trypanosoma cruzi

PKA activity was characterized in T. cruzi epimastigotes after affinity chromatography with histone H1A and cAMP. A cAMP-stimulatable protein kinase fraction was identified with a half-maximal effect at approximately 1 nM cAMP; the activity was not affected by cGMP and phosphate-acceptor preferences were as expected for a cAMP-dependent protein kinase (Ulloa et al., 1988). The apparent holoenzyme was then separated into the catalytic and regulatory subunits by further chromatography in the presence of cAMP. The catalytic subunit was only stimulated by cAMP when in the presence of the regulatory fraction (Ulloa et al., 1988). Estimates of the size of the individual subunits, as well as that of the reconstituted holoenzyme, suggest a tetrameric conformation similar to that of mammalian PKA (Ochatt et al., 1993). An explanation is that because the TcPKA catalytic subunit was purified from transiently transfected mammalian 293T cells, the endogenous PKA regulatory subunit from the expression system may be recognizing and binding to TcPKA-C and coprecipitating with the catalytic subunit during purification. This would allow cAMP regulation of the purified TcPKA without the presence of the parasite’s regulatory subunit. The alternative scenario of cAMP...
directly interacting with TcPKA-C to stimulate kinase activity would be entirely unique among eukaryotes, although because cyclic nucleotide-binding sites have not been identified in TcPKA-C, this hypothesis is extremely unlikely.

Nonetheless, the importance of PKA activity in T. cruzi was clearly demonstrated by the transfection of the PKA-specific peptide-inhibitor PKI into epimastigotes, which displayed a delayed lethal phenotype (Bao et al., 2008). Inhibition of PKA activity using the inhibitor H89 also resulted in slow cell death, with 10 μM of the inhibitor killing all parasites in 10 days.

Investigations into the potential PKA interacting proteins in T. cruzi were conducted using a yeast two-hybrid system. 38 candidates were identified; of these, 18 could not be named or given even a hypothetical function; 12 genes were tentatively identified from putative domains, allowing the prediction of their function (including a putative AG); and eight out of the 38 candidate proteins had a known and defined function or were deemed to have important functions for the parasite based on putative domains (Bao et al., 2008). The authors speculate that three of the latter proteins may be involved in adaptation to environmental stress: osmoregulation may be aided by PKA activation of the T. cruzi aquaporin and also with an ATPase; DNA excision repair protein could regulate the rate of glycolysis and thus energy production (Bao et al., 2008). Members of the trans-sialidase family have also been demonstrated to interact with, and be phosphorylated by T. cruzi PKA (Bao et al., 2010), with the location of putative PKA phosphorylation sites beside endoplasmic reticulum retention motifs suggesting that PKA may help regulate the translocation of these proteins.

The components of a negative-feedback system also appear to be in place in T. cruzi. A putative mitogen-activated extracellular signal-regulated kinase (ERK) and TcPDEC-2 were demonstrated to interact with each other, as well as with TcPKA-C (Bao et al., 2008). In Dictostelium, ERK activity has been shown to activate AGs and inhibit PDEs. The resulting increase in cAMP activates PKA, which inhibits ERK and stimulates PDE activity, thereby lowering the cAMP concentration again (Loomis, 1998) – a scenario that might be replicated in T. cruzi.

The localization of the TcPKA holoenzyme was further investigated, this time using the regulatory instead of the catalytic subunit of TcPKA as ‘Bait’ in the yeast two-hybrid system. Three P-type ATPases were shown to interact with TcPKA-R. Furthermore, immunocomplexes precipitated from trypomastigote lysates using anti-TcPKA-R monoclonal antibodies were recognized by anti-ATPase antibodies and vice versa, confirming the interaction between the TcPKA regulatory subunit and the P-type ATPases (Bao et al., 2008). Immunofluorescence demonstrated that TcPKA-R was associated with the flagellum and plasma membrane of trypomastigotes, as well as in the cytosol of all three T. cruzi life-cycle stages (Bao et al., 2009), in good agreement with previous investigations (Huang et al., 2006).

Because Na\(^+\)-ATPases are also upregulated in trypomastigotes (Iizumi et al., 2006), a pool of TcPKA-R may be localized to the plasma membrane and flagellum in this life cycle form by binding to this or other P-type ATPases already located there. This would then recruit the PKA catalytic subunit to the same location and could serve as a useful reservoir of effector proteins compartmentalized in and around the flagellum. PKA would then be capable of responding rapidly, and perhaps more specifically than cytosolically located PKA, to any sensory signals induced by the changed environment found after being released from the host cell (Bao et al., 2009).

**Leishmania spp.**

A Leishmania catalytic subunit of PKA was first isolated and characterized in the early 1990s from *L. donovani* promastigotes by column chromatography (Banerjee & Sarkar, 1992). The purified molecule was constitutively active and shared peptide phosphorylation preferences with that of mammalian PKA. The fact that cAMP, cGMP, calcium and calmodulin had no effect on the kinase activity, combined with the size of the eluted molecule (~34 kDa), indicated that the catalytic subunit of the *Leishmania* PKA had probably been purified on its own without a regulatory subunit. The addition of a bovine regulatory subunit inhibited the *Leishmania* kinase activity and the kinase activity returned to the basal level on the addition of cAMP. As with *T. brucei* and *T. cruzi*, the activity of LdPKA was inhibited by PKI, indicating that the kinetoplastid enzymes are likely to be structurally related and topologically similar to mammalian PKA (Banerjee & Sarkar, 1992).

A gene encoding a protein of approximately 38 kDa, with high homology to other PKA genes, was cloned and sequenced from *L. major* and designated *LmPKA-C1* (Siman-Tov et al., 1996). All the important conserved features involved in ATP and phosphate-acceptor binding are present. However, some deviations from the classic PKA-C sequence were observed, with the LmPKA-C1 protein having a relatively short N-terminal region of just 11 residues before the catalytic core, and lacking a site for potential myristoylation. In addition, LmPKA-C1 has an eight-amino acid, C-terminal extension beyond the usually final FXXF motif, compared with most other PKA-C subunits (Siman-Tov et al., 1996, 2002).

Northern blot analysis showed that expression of *LmPKA-C1* by promastigote forms is essentially abolished on a change of temperature from 26 to 35 °C, with hybridization levels declining to < 30% after just 1 h and...
undetectable after 4 h. The change in temperature also triggers differentiation from promastigote to amastigote forms, indicating that the expression of LmPKA-C1 is downregulated on initiation of the transformation process and the kinase appears to play no role in the amastigote form (Siman-Tov et al., 1996).

According to Genestra et al. (2004), cAMP-dependent kinase activity is the highest in metacyclic-enriched promastigote cultures of Leishmania amazonensis, whereas kinase activity was low in amastigotes and noninfective promastigotes. A role for cyclic nucleotide-regulated protein kinase activities in promastigote proliferation and infectivity was confirmed as PKA inhibitors PKI and H89, as well as the PDE inhibitors dipyridamole, rolipram and IBMX, all affected both replication and infection (Malki-Feldman & Jaffe, 2009). However, the addition of cAMP did not affect kinase activity in promastigote lysates as measured by kemptide phosphorylation, although it was stimulatable by CPT-cAMP, and cGMP increased kemptide phosphorylation to nearly 250% of the control. This increase could not be inhibited by PKI, although PKI inhibited the base-line activity by > 50% (Malki-Feldman & Jaffe, 2009), suggesting that the cGMP-stimulated kinase activity is independent of the PKI-inhibitable kinase, consistent with an earlier report of cGMP-dependent kinase activity in L. amazonensis promastigotes (Geigel & Leon, 2003). The stimulation of kinase activity by nonhydrolysable analogues, but not by cAMP itself indicates high cAMP-specific PDE activity in the lysates.

Two further isoforms of PKA-C were later identified, cloned and sequenced in L. major. While LmPKA-C2a and LmPKA-C2b share 96.6% identity to each other overall and 100% identity in the catalytic core, LmPKA-C1 only shares ~60% amino acid identity to the other two catalytic subunits (Siman-Tov et al., 2002). Three-dimensional models of the LmPKA-Cs based on mammalian PKA-Cx crystal structures indicate only minor topological differences, with the majority of nonconserved substitutions found on the protein surface and not in the catalytic core. The N-terminals of the LmPKA-C2 isoforms are predicted to form an α-helix-like mammalian PKA-Cs, whereas the truncated region in LmPKA-C1 does not. In addition, LmPKA-C1 has an extra C-terminal loop compared with typical eukaryotic PKA-Cs, due to its eight-amino acid extension (Siman-Tov et al., 2002).

Apicomplexan PKA

Toxoplasma gondii

The effects of cyclic nucleotide signalling in T. gondii parasites provide conflicting results, but show some connection to proliferation and to differentiation from tachyzoite to bradyzoite stages. Incubation of tachyzoites with cAMP, and analogues of the nucleotide, resulted in an increase in growth as well as increased development of bradyzoite pseudocysts (Choi et al., 1990, 1994).

Conversely, the cAMP analogue CPT-cAMP appeared to inhibit differentiation to the bradyzoite stage, whereas CPT-cGMP stimulated it (Kirkman et al., 2001). It was hypothesized that it is the duration of cAMP increase that is important in triggering differentiation, because all conditions and compounds that result in differentiation also result in an increase in the intracellular cAMP concentration, including incubation with cGMP and CPT-cGMP. Similar measurements using a strain that does not differentiate well to bradyzoite form showed little or no increase in intracellular cAMP under the same conditions, confirming the role of cAMP signalling in the differentiation process (Kirkman et al., 2001).

However, a more recent study using a genetically engineered strain of T. gondii transfected with stage-specific reporter genes clouds the issue yet again. CPT-cAMP and CPT-cGMP did not initiate differentiation. In contrast, the PKA inhibitor H89 as well as the PKG-specific inhibitor Compound 1 (see Apicomplexan PKG) did result in bradyzoite formation (Eaton et al., 2006). These data suggest that it is the inhibition of the cyclic nucleotide effector-proteins that triggers differentiation from the tachyzoite to the bradyzoite form, rather than the activation of PKA by increased concentrations of cAMP.

Almost all of the studies on T. gondii PKA were observed in cultures of trophozoites within mammalian host cells. Until species-specific inhibitors are developed, it remains unclear whether the effects of modulators of cyclic nucleotide concentrations are as a result of the direct action of compounds on the parasites or on the host cells or a combination of both.

Plasmodium spp.

cAMP-dependent kinase activity was extracted from lysates of P. falciparum (Read & Mikkelsen, 1990). Further purification by affinity chromatography resolved two proteins of approximately 108 and 53 kDa molecular weights, respectively. Photo-affinity labelling with the cAMP analogue 8-azido-cAMP associated with just the 53-kDa protein, which was inhibited by 50 µM unlabelled cAMP and coeluted with a protein capable of cAMP-dependent histone II-A phosphorylation, consistent with the possibility that it was the regulatory subunit of Plasmodium PKA-C (Read & Mikkelsen, 1990).

A follow-up study (Read & Mikkelsen, 1991b) directly implicated cAMP-dependent protein kinase activity with the production of gametocytes in P. falciparum. Two strains were investigated: LES, which could produce gametocytes, and T9/96, which was considerably impaired in gametocytopogenesis. There was no significant difference in AC activity between the two strains; however, cytosolic extracts from
erythrocyte-free parasite lysates revealed strain T9/96 to have an approximately threefold lower cAMP-dependent kinase activity than LE5, apparently due to a reduced $V_{\text{max}}$ as the half-maximal activation by cAMP for the kinase activity for both the strains was the same. Analysis of cAMP-binding proteins revealed the previously reported 53-kDa protein in both strains, plus an additional 49-kDa protein in the gametocytes of the asexual blood stages than in gametocytes of PfPKA-R, in the asexual blood stages than in gametocytes of PfPKA-C, Northern blots detected higher expression levels of PfPKA-C. In tandem with no dimerization domain was located. In tandem with ing motifs were found in the amino acid sequence; however, PfPKA-C, homologous PKA-C from P. falciparum, expressed by two independent groups. PfPKA-C, an apicomplexan PKA-C was first cloned and sequenced from P. yoelii (Saito-Ito et al., 1995). The gene appeared to be a single-copy gene and contained all invariant residues necessary for function. However, alignment with PKA-Cs from other organisms reveals two deviations in the region predicted to interact with the regulatory subunit. The protein also lacks the DDYEEEE motif in the C-terminal region, which, in mammalian PKA-C, is necessary for binding PKI (Saito-Ito et al., 1995). Expression of the highly homologous PKA-C from P. falciparum was only detected in the asexual stages (Li & Cox, 2000), with the highest expression in the schizont stage (Wurtz et al., 2009a), suggesting involvement in stage-specific events.

The P. falciparum PKA catalytic subunit was recombinantly expressed by two independent groups. PfPKA-C, fused with GST and expressed in a wheat germ cell-free protein synthesis system, was assessed for the effects of mammalian PKA inhibitors. H89 yielded around 60% inhibition at 100 $\mu$M and PKI resulted in < 20% inhibition at the same concentration (Sudo et al., 2008). However, expression of PfPKA-C with a hexahistidine tag in E. coli yielded somewhat different results: just 1 $\mu$M H89 resulted in ~70% inhibition of the kinase activity, with the same concentration of PKI inhibiting by approximately 50% (Wurtz et al., 2009b). Attempts to generate a PKA-C knock-out cell line in the rodent malaria P. berghei were unsuccessful, indicating that the protein may be essential for the parasite (Sudo et al., 2008).

A putative PfPKA regulatory subunit was identified in the P. falciparum genome database. Two cyclic nucleotide-binding motifs were found in the amino acid sequence; however, no dimerization domain was located. In tandem with PfPKA-C, Northern blots detected higher expression levels of PfPKA-R in the asexual blood stages than in gametocytes (Merckx et al., 2008). PfPKA-R was expressed in E. coli as a fusion protein with maltose-binding protein (MBP) and purified. When recombinant MBP-PfPKA-R was added to parasite cell extracts, cAMP-dependent phosphorylation of the synthetic peptide kemptide was significantly reduced (Merckx et al., 2008).

The role of PfPKA was investigated by analysing the effect of gene silencing following transfection with double-stranded RNA corresponding to a fragment of the PfPKA catalytic subunit (Wurtz et al., 2009a). The mRNA of PfPKA-R was reduced by ~70% after transfection; surprisingly, mRNA of the regulatory subunit was also decreased by a similar extent, suggesting self-regulation of transcription of the PfPKA holoenzyme components. The decrease in PfPKA transcripts resulted in defects in the development of the schizont stage and eventual cell cycle arrest, highlighting the importance of the kinase in progression of the asexual cell cycle. Microarray analysis identified changes to the regulation of 329 genes after PfPKA-C gene silencing (Wurtz et al., 2009a), including a group of upregulated nuclearily encoded genes that have mitochondrial signal sequences, suggesting a role for PKA modulation of mitochondrial protein traffic in the parasite. Six reticulocyte-binding-like genes, which may be involved in invasion of erythrocytes, were also downregulated after electroporation. Taken in combination with a recent report that suggests PfPKA phosphorylates AMA1 (Leykauf et al., 2010), a protein crucial for tight-junction formation during RBC invasion, these findings imply a potential role for cAMP signalling in cell invasion by merozoites.

The recombinant regulatory subunit was used to investigate anion conductances in infected erythrocytes. These anion channels, also known as new permeation pathways (NPPs), become gradually activated after Plasmodium invasion and are essential for the salvage of nutrients by the intracellular parasite (Kirk, 2001). In the presence of ATP, the addition of bovine PKA to uninfected erythrocytes resulted in an inwardly rectified whole-cell current similar to that found in Plasmodium-infected RBCs. The current was completely inhibited in infected erythrocytes by alkaline phosphatase, indicating the regulation of the current by phosphorylation (Decherf et al., 2004). The addition of recombinant MBP-PfPKA-R resulted in lowering conductance by around 40%, but the inhibitory effect on the current could be almost completely blocked by the presence of 5 mM cAMP (Merckx et al., 2008). Transgenic parasites overexpressing PKA-R displayed a much-reduced anion conductance and a growth defect that was reversed by high levels of cAMP induced by the addition of the PDE inhibitor IBMX or by adding the cell-permeable cAMP analogue 8-bromo-cAMP. These results suggest that the NPPs are also regulated by Plasmodium PKA (Merckx et al., 2008).

**Apicomplexan PKG**

The cGMP-dependent protein kinase (PKG) from the apicomplexan parasites Eimeria and Toxoplasma has been extensively characterized. This has been both instigated and facilitated by the identification of the specific PKG inhibitor Compound 1 as a lead compound for the chemotherapy of coccidiosis in chickens, which is caused by Eimeria, as well as of toxoplasmosis (Donald et al., 2002; Gurnett et al., 2002).
2002; Nare et al., 2002). Independently, the PKG from *P. falciparum* was also cloned and characterized at around the same time, with broadly similar findings, if differing slightly in detail (Deng & Baker, 2002; Deng et al., 2003).

**Eimeria and Toxoplasma**

Compound 1 showed *in vitro* activity against a wide range of apicomplexan parasites, as well as being orally active against *Eimeria tenella* and *Eimeria acervulina* in chickens and effective against murine toxoplasmosis. These findings spurred the Merck Research Laboratories to identify the intracellular target of Compound 1 and to characterize it biochemically and molecularly.

Affinity chromatography with *E. tenella* extracts identified a 120-kDa protein with homology to *Drosophila melanogaster* PKG that bound with high affinity to Compound 1 (Gurnett et al., 2002). The 120-kDa protein was assayed for kinase activity in the presence of cAMP and cGMP. cAMP yielded a two- to threefold stimulation, whereas cGMP stimulated kinase activity 500–1000-fold, which could be inhibited by Compound 1 with an IC₅₀ of around 0.6 nM (Gurnett et al., 2002). *Eimeria tenella* PKG was kinetically characterized, producing a *Kₘ* for ATP of 12 μM and a *Kₘ* for a synthetic peptide phosphate acceptor of 19 μM. Compound 1 was shown to be a competitor for ATP binding (Gurnett et al., 2002).

An *E. tenella* oocyst cDNA library was screened for the 120-kDa protein. The ORF predicted a protein of 1003 amino acids with an approximate molecular weight of 113 kDa. Sequence alignment with human PKG shows that the parasite protein is much larger, by around 300 amino acids found mostly in a single stretch between the catalytic and the nucleotide-binding domains, which was predicted to contain a third cGMP-binding site – a feature shared by other apicomplexan PKGs (Gurnett et al., 2002). Mutation of this site markedly reduces cGMP-dependent kinase activity, whereas mutation of the other two sites had less effect on kinase activity. Mutating all three putative cGMP-binding sites simultaneously resulted in only background effect on kinase activity. The PKG inhibitor also blocked the secretion of proteins associated with parasite motility, thereby inhibiting gliding motility of sporozoites and tachyzoites (Wiersma et al., 2004). This suggests that inhibition of PKG by Compound 1 inhibits cell invasion more than cell growth. On the way to their ultimate host cell, apicomplexan parasites also migrate through other cells in the host to reach their final destination.

Immunofluorescent microscopy showed that the larger isoform was localized to the plasma membrane, whereas the short isoform was concentrated in the cytosol (Donald & Liberator, 2002). The differential localization of the two isoforms could be explained by the presence of a dual acylation motif in the extreme N-terminal seven residues of both EtPKG and TgPKG. Myristoylation has been shown to be responsible for the localization of other mammalian PKGs to membranes. Consequently, the N-terminally truncated second isoform, no longer containing the acylation motif, would be prevented from translocation to the plasma membrane, thereby yielding a cytosolic distribution.

Inhibition of PKG in *T. gondii* tachyzoites with Compound 1 results in growth suppression with an IC₅₀ of approximately 200 nM (Nare et al., 2002). However, the growth inhibition can be removed by replacing the culture medium, demonstrating that Compound 1 is cytostatic rather than lethal. Treatment of *T. gondii*-infected mice for 10 days with Compound 1 resulted in cure, with the observed numbers of parasites in organs declining to undetectable levels after 50 days. Immunosuppressed mice showed recrudescence, with 100% of infected mice succumbing just 5 days after the termination of treatment, revealing the necessity for an intact immune system for chemotherapy with the Compound 1 to be successful (Nare et al., 2002).

It has been observed during the course of whole-cell *in vitro* efficacy experiments that the timing of addition of Compound 1 to the assay, in relation to the inoculation of the host cell layer with *T. gondii* tachyzoites or *E. tenella* sporozoites, was crucial. If Compound 1 was added to the assay 4 h after the host cell layer was inoculated with parasites, there would be essentially no effect on *Eimeria* sporozoite intracellular growth and only a limited impact on *Toxoplasma* tachyzoites (Wiersma et al., 2004). This suggests that inhibition of PKG by Compound 1 inhibits cell invasion more than cell growth. On the way to their ultimate host cell, apicomplexan parasites also migrate through other cells in the host to reach their final destination. Compound 1 blocked this type of cell invasion by *E. tenella* sporozoites in a dose-dependent manner. The PKG inhibitor also blocked the secretion of proteins associated with parasite motility, thereby inhibiting gliding motility of sporozoites and tachyzoites of *E. tenella* and *T. gondii*, respectively, on a glass slide (Wiersma et al., 2004). The data indicate that PKG plays a role in the regulation of adhesin secretion and host cell invasion by apicomplexan parasites.

**Plasmodium spp.**

The homologous PKG gene was also identified in *P. falciparum*. The deduced amino acid sequence is 853 residues long, with the regulatory domains at the N-terminal end and, like *Toxoplasma* and *Eimeria* PKG, containing three cyclic

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**Cyclic-nucleotide signalling in protozoa**

2002; Nare et al., 2002). Independently, the PKG from *P. falciparum* was also cloned and characterized at around the same time, with broadly similar findings, if differing slightly in detail (Deng & Baker, 2002; Deng et al., 2003).

**Eimeria and Toxoplasma**

Compound 1 showed *in vitro* activity against a wide range of apicomplexan parasites, as well as being orally active against *Eimeria tenella* and *Eimeria acervulina* in chickens and effective against murine toxoplasmosis. These findings spurred the Merck Research Laboratories to identify the intracellular target of Compound 1 and to characterize it biochemically and molecularly.

Affinity chromatography with *E. tenella* extracts identified a 120-kDa protein with homology to *Drosophila melanogaster* PKG that bound with high affinity to Compound 1 (Gurnett et al., 2002). The 120-kDa protein was assayed for kinase activity in the presence of cAMP and cGMP. cAMP yielded a two- to threefold stimulation, whereas cGMP stimulated kinase activity 500–1000-fold, which could be inhibited by Compound 1 with an IC₅₀ of around 0.6 nM (Gurnett et al., 2002). *Eimeria tenella* PKG was kinetically characterized, producing a *Kₘ* for ATP of 12 μM and a *Kₘ* for a synthetic peptide phosphate acceptor of 19 μM. Compound 1 was shown to be a competitor for ATP binding (Gurnett et al., 2002).

An *E. tenella* oocyst cDNA library was screened for the 120-kDa protein. The ORF predicted a protein of 1003 amino acids with an approximate molecular weight of 113 kDa. Sequence alignment with human PKG shows that the parasite protein is much larger, by around 300 amino acids found mostly in a single stretch between the catalytic and the nucleotide-binding domains, which was predicted to contain a third cGMP-binding site – a feature shared by other apicomplexan PKGs (Gurnett et al., 2002). Mutation of this site markedly reduces cGMP-dependent kinase activity, whereas mutation of the other two sites had less effect on kinase activity. Mutating all three putative cGMP-binding sites simultaneously resulted in only background effect on kinase activity. The PKG inhibitor also blocked the secretion of proteins associated with parasite motility, thereby inhibiting gliding motility of sporozoites and tachyzoites of *E. tenella* and *T. gondii*, respectively, on a glass slide (Wiersma et al., 2004). The data indicate that PKG plays a role in the regulation of adhesin secretion and host cell invasion by apicomplexan parasites.

**Plasmodium spp.**

The homologous PKG gene was also identified in *P. falciparum*. The deduced amino acid sequence is 853 residues long, with the regulatory domains at the N-terminal end and, like *Toxoplasma* and *Eimeria* PKG, containing three cyclic
nucleotide-binding domains, as well as a fourth degenerate binding site between sites 2 and 3 (Deng & Baker, 2002). Expression of an N-terminally truncated PiPKG in *E. coli* showed that it was cGMP specific. Western blots using anti-PPKG antibodies detected an approximately 95-kDa band only in the ring stage. No corresponding bands were identified in trophozoite, schizont or gametocyte stages (Deng & Baker, 2002); however, *P. berghei* has been shown to express PKG in the liver stages (Falae et al., 2010). In agreement with the studies in *E. tenella* and *T. gondii*, the third cGMP-binding site has the most influence on the activity of PiPKG, although the fourth, degenerate binding site may play a role in conformational stability of the protein, as truncated mutants with this domain removed were much less active, even though the degenerate domain was shown not to bind cGMP (Deng et al., 2003).

Compound 1 was shown to inhibit a recombinant, synthetic version of PiPKG expressed in *T. gondii* with an N-terminal FLAG epitope tag to allow affinity purification (Diaz et al., 2006). In agreement with a number of previous studies, Compound 1 disrupted gametogenesis regulation (see PDEs of apicomplexans – PDEs); however, as a means of further dissecting the process of gametogenesis, mutant *P. falciparum* PKG were generated with a key threonine mutated to glutamine. The mutant *Plasmodium* PKG was rendered > 3000-fold less sensitive to Compound 1 than the wild type (McRobert et al., 2008). Allelic replacement of the wild-type PKG for the glutamine-substituted PKG in *P. falciparum* generated a Compound 1 refractory cell line. The wild type and the PKG mutant parasites displayed similar asexual growth rates and gametocyte development, demonstrating that the mutant PKG is fully functional. Treatment with Compound 1 resulted in only the wild-type parasites being insensitive to gametogenesis triggering. This not only demonstrated that PKG is the primary target for Compound 1 in *Plasmodium* but also that PKG is directly involved in gametogenesis and that the effects of xanthurenic acid and zaprinast are mediated solely through this kinase (McRobert et al., 2008). Furthermore, wild-type gametocytes treated with Compound 1 showed no evidence of DNA synthesis, axoneme formation or emergence from erythrocytes – all signs of gametogenesis – confirming that cGMP signalling is involved at the very outset of the process.

Finally, PKG has also been shown to play a crucial role in the asexual cycle of *P. falciparum*. Schizont-stage infected erythrocytes treated with Compound 1 were unable to rupture and release the merozoites; when the experiment was repeated with the Compound 1-insensitive mutants, schizogony was completed as normal (Taylor et al., 2010). Similarly, hepatic merozoite release was blocked in liver-stage *P. berghei* PKG conditional knockouts (Falae et al., 2010), and liver-stage parasitaemia was also significantly reduced in mice treated with Compound 1 and challenged with *P. yoelii* sporozoites (Panchal & Bhanot, 2010). This demonstrates that cGMP signalling plays crucial roles in both asexual and sexual development of *Plasmodium* and that PKG is an excellent drug target for human malaria chemotherapy. Not only could a selective PKG inhibitor potentially cure an infected patient, but, because gametogenesis is also blocked by PKG inhibition, transmission to the mosquito vector would be reduced as well, limiting spread of the disease.

**Concluding remarks**

The cyclic nucleotide signalling systems of protozoan parasites share a number of conserved characteristics with their mammalian hosts, but also display some striking differences. The canonical building blocks of the cascades: cyclases, PDEs and nucleotide-specific protein kinases, are all, by and large, present and at least partially characterized. However, significant differences in the mode of activation and action raise a multitude of questions and highlight some unique aspects to the parasites’ signalling paradigm.

The apparent lack of G-protein-coupled receptors raises the issue of how ACs are regulated and, with only circumstantial evidence that the N-terminal region may act as a receptor to extracellular stimuli in kinetoplastids, the search for the identities of any stimulatory or inhibitory ligands remains of high importance. In apicomplexan ACs and GCs, the presence of putative ion channels in the transmembrane domains could allow a very direct method of communication between signalling systems, with the possibility that the activity of the cyclases could be directly regulated by ion concentration gradients.

Uniquely among Class I PDE enzymes, the apicomplexan PDEs have transmembrane domains, suggesting that they are integral membrane proteins. Kinetoplastid PDEs have a more standard structure and the localization of PDEB1 to the paraflagellar rod of *T. brucei* may allow compartmentalization of the cAMP signal. It is not inconceivable that the PFR could act as a scaffold for other cAMP effector proteins, such as PKA, in a manner similar to A-kinase anchoring proteins in mammalian systems, further aiding the specificity of action of the cAMP impulse. Indeed, we have barely even begun to scratch the surface of what downstream effector proteins and pathways are involved in transmitting the cyclic nucleotide signal.

One aspect of cyclic nucleotide signalling almost deafening by its silence in the academic literature is the cGMP signalling pathway in kinetoplastids: could it really be entirely absent from the organism? With only PDEC showing any affinity for cGMP and the surprising identification of an apparently cGMP-specific kinase in *T. brucei* being the most significant discoveries in this aspect of nucleotide signalling, much more work is clearly needed.
The above review demonstrates without doubt that cyclic nucleotide signalling in pathogenic protozoa performs many essential functions and offers promising drug targets – not only in human pathologies but also in veterinary medicine. The development of Compound 1 against coccidiosis in chickens is an excellent example of how veterinary and human medicine can overlap and stimulate drug discovery. However, these new insights have yet to translate into genuine preclinical candidates for therapy for the severe human diseases caused by these organisms. We feel that the field does not lack challenges or opportunities.

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