# PfPDE1, a novel cGMP-specific phosphodiesterase from the human malaria parasite *Plasmodium falciparum*

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This is the first report of molecular characterization of a novel cyclic nucleotide PDE (phosphodiesterase), isolated from the human malaria parasite *Plasmodium falciparum* and designated PfPDE1. *PfPDE1* cDNA encodes an 884-amino-acid protein, including six putative transmembrane domains in the N-terminus followed by a catalytic domain. The *PfPDE1* gene is a single-copy gene consisting of two exons and a 170 bp intron. PfPDE1 transcripts were abundant in the ring form of the asexual blood stages of the parasite. The C-terminal catalytic domain of PfPDE1, produced in *Escherichia coli*, specifically hydrolysed cGMP with a $K_m$ value of 0.65 $\mu$M. Among the PDE inhibitors tested, a PDE5 inhibitor, zaprinast, was the most effective, having an $IC_{50}$ value of 3.8 $\mu$M. The non-specific PDE inhibitors IBMX (3-isobutyl-1-methylxanthine), theophylline and the antimalarial chloroquine had $IC_{50}$ values of over 100 $\mu$M. Membrane fractions prepared from *P. falciparum* at mixed asexual blood stages showed potent cGMP hydrolytic activity compared with cytosolic fractions. This hydrolytic activity was sensitive to zaprinast with an $IC_{50}$ value of 4.1 $\mu$M, but insensitive to IBMX and theophylline. Furthermore, an *in vitro* antimalarial activity assay demonstrated that zaprinast inhibited the growth of the asexual blood parasites, with an $ED_{50}$ value of 35 $\mu$M. The impact of cyclic nucleotide signalling on the cellular development of this parasite has previously been discussed. Thus this enzyme is suggested to be a novel potential target for the treatment of the disease malaria.

Key words: antimalarial drug, cGMP, malaria parasite, phosphodiesterase (PDE), *Plasmodium*, zaprinast.

## INTRODUCTION

Malaria is a parasitic disease caused by four species of the protozoan parasites of the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* in humans. *P. falciparum* is the most virulent form of the four. Several drugs, including chloroquine and quinine, are used for the treatment of malaria; however, *P. falciparum* has developed resistance to most antimalarial agents, such as chloroquine. Therefore in order to overcome malaria, the genome project of *P. falciparum* has been completed [1]. Potential drug target molecules are currently being discussed [2].

The life cycle of the malaria parasite is complex, with several stages [3]. Soon after a bite by an infected *Anopheles* mosquito, sporozoites invade hepatocytes. Intense asexual division, termed exoerythrocytic schizogony, occurs in the liver, and up to 40 000 merozoites are generated after schizont rupture. Merozoites invade erythrocytes and additional rounds of asexual replication, a pathogenic phase of malaria, termed erythrocytic schizogony, takes place. Some merozoites arrest their cell cycle to differentiate into male or female gametocytes (gametogenesis), which are infectious to the *Anopheles* mosquitoes. Thus cellular differentiation is an important process in the life cycle of the malaria parasite. Intracellular cyclic nucleotides cAMP and cGMP play a pivotal role in the growth and differentiation of this organism, acting as second-messenger molecules. For example, cGMP signalling has been shown to be implicated in *Plasmodium* gametogenesis [4,5]. Consistent with this, expression of PfGC (guanylate cyclase) protein is found in gametocytes [6,7]. The presence of PfPKG (cGMP-dependent protein kinase) in the ring form of asexual blood stages, but not gametocytes, suggests a role of cGMP signalling in this cell form [8].

This cyclic nucleotide signalling is regulated through cyclic nucleotide production by adenylate and guanylate cyclases and hydrolysis by cyclic nucleotide PDEs (phosphodiesterases) [9,10]. In mammals, PDEs have been categorized into 11 families (PDEs 1–11) according to their amino acid sequence similarity, biochemical properties and inhibitor sensitivity [11,12]. Inhibitors of mammalian PDEs are used, or have been developed, for the treatment of diseases such as erectile dysfunction, thrombosis, asthma and chronic obstructive pulmonary disease [13–15]. PDEs from lower single-cell organisms have been reported and revealed to be distinct from the 11 mammalian PDE families. PDEs from a soil amoeba, *Dictyostelium*, and a protozoan parasite, *Trypanosoma*, have been isolated using their genome sequences and their characteristics successfully demonstrated. In *Trypanosoma brucei,* two class I PDEs (TbPDE1 and TbPDE2), which are specific for cAMP, have been cloned [16–19]. Interestingly PDE inhibitors against TbPDE2 block proliferation of bloodstream form trypanosomes [17], and inactivation of TbPDE2 by RNA interference induces growth inhibition of bloodstream-form *T. brucei*. This suggests that specific PDE inhibitors

Abbreviations used: BLAST, basic local alignment search tool; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; Ht, hematocrit; IBMX, 3-isobutyl-1-methylxanthine; IPTG, isopropyl $\beta$-D-thiogalactoside; PDE(s), phosphodiesterase(s); PfPDE, *P. falciparum* PDE; PfGC, *P. falciparum* guanylate cyclase; PfPKG, *P. falciparum* cGMP-dependent protein kinase; RT, reverse transcriptase; Tb, *Trypanosoma brucei*.

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The nucleotide sequence of *PfPDE1* has been submitted to DDBJ, EMBL, GenBank®/EBI (European Bioinformatics Institute) and GSDB Nucleotide Sequence Databases under the accession number AB100091.

may be useful for anti-protozoal chemotherapy [19]. Thus cyclic nucleotide signalling in lower organisms is essential for their survival, and disruption of this signalling would be expected to cause cell stress, leading to cell death.

In *Plasmodium*, the presence, and some physiological roles, of PDEs have been predicted. Nevertheless, the molecular basis of *Plasmodium* PDEs has not yet been established, in spite of the genome project of *P. falciparum* having been completed. In the present paper, we report a novel PDE (PfPDE1) in *P. falciparum*. The *PfPDE1* cDNA was cloned and both the enzymatic characteristics and PDE inhibitor sensitivities of the gene product were investigated in detail. Endogenous PDE activities of this organism were also examined. The physiological effect of a PfPDE1 inhibitor on this organism was also demonstrated. The findings reported here provide us with fundamental knowledge of the cyclic nucleotide metabolism in the human parasite *P. falciparum*. *Plasmodium* PDE is suggested to be a novel therapeutic target for the disease malaria and discovery of inhibitors would lead to improved treatment of the disease.

## EXPERIMENTAL

### Materials

[$^3$H]cAMP, [$^3$H]cGMP and [$\alpha$-$^{32}$P]dCTP were purchased from Amersham Biosciences. IBMX (3-Isobutyl-1-methylxanthine) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Vinpocetine, EHNA [*erythro*-9-(2-hydroxy-3-nonyl)adenine], milrinone, rolipram, zaprinast, dipyridamole, and chloroquine were purchased from Sigma–Aldrich. Theophylline was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Papaverine, E4021 and sildenafil were synthesized at Tanabe Seiyaku Co. Ltd. (Osaka, Japan).

### Parasite culture and isolation of nucleic acids

*P. falciparum 3D7* and Honduras-1 strains were cultivated in RPMI 1640 (Invitrogen) with 10 % (v/v) inactivated type A human plasma with 5 % (v/v) and 3 % (v/v) Ht (haematocrit) type A human erythrocytes respectively [20]. The plate was placed in a $CO_2$ incubator [$CO_2/O_2/N_2$ (5:5:90)] at 37 °C. Erythrocytes infected with *P. falciparum 3D7* were harvested and treated with 0.075 % (w/v) saponin in PBS to obtain the parasites. Chromosomal DNA was obtained from the parasites using QIAamp DNA Mini Kit (Qiagen). Total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan) and first-strand cDNA was prepared according to the instructions with GeneAmp RNA PCR Kit (Applied Biosystems).

### Cloning of chromosomal DNA and cDNA for *P. falciparum* PDE

The amino acid sequences of mammalian PDEs (PDEs 1–11) were used as queries to search the expressed sequence tag and genome databases. A BLAST (basic local alignment search tool) search [21] of the National Center for Biotechnology Information *P. falciparum* genome database identified putative *PfPDE* genes. PCR primers were designed based on the sequences retrieved from the database.

Chromosomal DNA fragments for the *PfPDE1* gene were generated by PCR amplification, using *P. falciparum* chromosomal DNA as a template. The 5′-region of the *PfPDE1* gene was amplified using the primer set (Pf1-F1: 5′-ATGGAATATTTTAATTGTGTTAATAATCTATGTTG-3′ and Pf1-R3: 5′-ATTTAATATATCTTCTATGGGCGATGTAGG-3′). To amplify the 3′-region of *PfPDE1* gene, the primer set (Pf1-F3: 5′-CCATCCTTTTATGAATATCTTATGTTTACGTTGATG-3′ and Pf1-R1: 5′-TTATTCAAATTTGATGAGCTCAAGTTTGCTTAG-3′) was used. PCR amplification was carried out through 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 3 min. PCR products were cloned into the TA-cloning vector pGEM-T Easy (Promega) and then sequenced.

The cDNA fragments encoding *PfPDE1* were obtained by PCR using *P. falciparum* cDNA as a template. N-terminal (nt 1–1329) and central to C-terminal (nt 367–2650) regions of *PfPDE1* were amplified using the above primer sets, Pf1-F1 plus Pf1-R3 and Pf1-F3 plus Pf1-R1 respectively. PCR amplification was carried out using the conditions described above and the amplified products were cloned into pGEM-T Easy. Five independent PCR clones encoding each region were sequenced to verify that a correct whole cDNA sequence had been cloned. One of the clones that included the C-terminal catalytic domain of PfPDE1, pGEM-PfPDE1c, was used for further experiments.

Nucleotide sequences were determined by an automated DNA sequencer ABI PRISM™ 310 using BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). Nucleotide and amino acid sequence data were analysed using the computer program GENETYX (Software Development Co., Tokyo, Japan). The deduced amino acid sequence was also analysed using the program SMART [22] for finding functional domains. Phylogenetic tree analysis was performed using CLUSTAL W and PHYLIP [23].

### Southern blot analysis

*P. falciparum* chromosomal DNA (2 $\mu$g/lane) was digested using the restriction endonucleases EcoRI, SpeI and XbaI, subjected to 0.8 % agarose gel electrophoresis and transferred on to Hybond-N+ nylon membrane (Amersham Biosciences). A 661-bp cDNA fragment of *PfPDE1* (nts 1147–1807) was $^{32}$P-radiolabelled using the Random Primer DNA Labelling Kit (TaKaRa Bio). Hybridization was performed in PerfectHyb™ (Toyobo, Osaka, Japan) containing the $^{32}$P-labelled probe at 60 °C for 4 h. The membrane was washed with 2 × SSC (1 × SSC: 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) and 0.1 % (w/v) SDS at 25 °C for 5 min, followed by two 10 min washes with 0.1 × SSC and 0.1 % (w/v) SDS at 60 °C. X-ray film was exposed to the membrane at − 80 °C for 12 h.

### Stage-specific expression of *PfPDE1* mRNA

To investigate stage-specific expression of *P. falciparum* cells at asexual stages, cultures were synchronized by sorbitol lysis and treatment with 66 % (v/v) Percoll™ (Amersham Biosciences) [24,25]. In brief, infected erythrocytes with late-stage asexual parasites, containing trophozoites and schizonts, were separated using Percoll™. These concentrated late-stage parasites were suspended with uninfected erythrocytes and cultured for 5–7 h. A proportion of the schizonts proceeded to the merozoite stage and invaded the fresh erythrocytes. These infected erythrocytes were treated with 66 % (v/v) Percoll™ to prevent minimal contamination with gametocytes and then with sorbitol to remove the remaining late-stage parasites (trophozoites and schizonts). The resultant ring forms were returned to culture and, 21, 32, 38, 43 and 50 h later, each sample was centrifuged at 830 ***g*** for 5 min at 4 °C. The parasites were obtained by treating with 0.075 % (w/v) saponin in PBS. Total RNA was obtained using Isogen and first-strand cDNA was prepared using the Advantage™ RT-for-PCR Kit (Clontech). Expression levels of *PfPDE1* transcripts were examined by PCR amplification using the 5′-primer 5′-AGCATGCTTTTCATGCTAGACATGAACCAC-3′ and the 3′-primer 5′-TTATTCAAATTTGATGAGCTCAAGTTTGCTTAG-3′, which generates a DNA fragment of 795 bp (nt 1870–2655). An 840 bp DNA fragment of the *P. falciparum* cysteine protease

falcipain-3 ([26]; GenBank™ accession number AF258791) was amplified by PCR using the 5′-primer 5′-AATAGTTTAT-ATAAAAGGGGTATG-3′ and the 3′-primer 5′-TAATGGTA-CATAAGCTTCTGTTCC-3′ (nt 686–1528 of the cDNA) as a control. PCR amplification was carried out through 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. PCR products were separated using 1.5 % agarose gel electrophoresis, and the DNA fragments were stained with ethidium bromide.

### Expression of PfPDE1 protein in *Escherichia coli*

To generate an expression plasmid for a catalytic domain of PfPDE1, the EcoT22I–SalI DNA fragment (approx. 1 kb) of pGEM-PfPDE1c was subcloned into the PstI and XhoI sites of the mammalian expression vector pcDNA4/HisMax A (Invitrogen) and the resultant plasmid named pHis-PfPDE1Δ. Transfection of this plasmid into COS-7 cells for recombinant PfPDE1 production was done according to the method described previously [12]. To express the PfPDE1 enzyme in *E. coli*, the BamHI–PstI DNA fragment (approx. 1 kb) of pHis-PfPDE1Δ was subcloned into the BamHI and PstI sites of the bacterial expression vector pQE-32 (Qiagen) and the resultant plasmid named pQE-PfPDE1Δ. Site-directed mutagenesis of the pQE-PfPDE1Δ plasmid was used to generate PfPDE1 mutants with alanine substitutions at $Asp^{762}$ and $Gly^{788}$; the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used following the manufacturer's protocol. To introduce the desired mutations, the following primers were used: 5′-ATTTTAAAGGCATCAGCTATTGGACACTCAACA-3′ with 5′-TGTTGAGTGTCCAATAGCTGATGCCTTTAAAAT-3′ for PfPDE1D762A, and 5′-GAATTCTATTTACAAGCTTTAC-TAGAAAAATCG-3′ with 5′-CGATTTTTCTAGTAAAGCTTG-TAAATAGAATTC-3′ for PfPDE1G788A. The mutations were confirmed by DNA sequencing analysis.

The expression plasmid pQE-PfPDE1Δ, pQE-PfPDE1-ΔD762A, pQE-PfPDE1ΔG788A or the control vector pQE-32 was introduced into *E. coli* JM109 cells. The recombinant *E. coli* cells were grown overnight at 37 °C in LB (Luria–Bertani) medium containing ampicillin (100 μg/ml). The culture was diluted 1:20 with fresh LB medium containing 2 % glucose and ampicillin (100 μg/ml), incubated at 37 °C in a shaking incubator for 2 h and then further incubated at 27 °C for 4 h, after addition of IPTG (isopropyl β-D-thiogalactoside) at a final concentration of 1 mM. The cells were then washed once with ice-cold PBS and resuspended in ice-cold lysis buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 5 μg/ml pepstatin A and 5 μg/ml leupeptin). After freeze–thaw treatment, suspended cells were disrupted by a sonicator (TOMY Seiko, Japan) for 15 s (5 times with 1 min intervals), and the lysates were centrifuged at 16000 ***g*** for 15 min at 4 °C. The supernatant was added to a plastic tube containing nickel nitrilotriacetate resin (Qiagen), equilibrated with the lysis buffer, and incubated with rotation at 4 °C for 3 h. The nitrilotriacetate resin was poured into a plastic column (0.8 cm × 5 cm) and allowed to drain. The packed resin was washed with wash buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 200 mM NaCl, 5 mM imidazole, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin), and the proteins were then eluted using elution buffer (40 mM Tris/HCl, pH 7.5, 15 mM benzamidine, 200 mM NaCl, 200 mM imidazole, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). The PfPDE1 fractions were dialysed against the lysis buffer and stored at −80 °C until use.

### SDS/PAGE, immunoblotting, and MS analysis

Proteins were subjected to SDS/PAGE (12.5 % gels) and stained using a Silver Staining Kit, Protein (Amersham Biosciences), omitting a step of fixation with glutardialdehyde. Immunoblot analysis was performed using PVDF membranes (Millipore) and anti-pentahistidine monoclonal antibody (Qiagen) as previously described [12]. Bound primary antibodies were detected using horseradish-peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) and visualized with ECL® Western Blotting Detection System (Amersham Biosciences). A gel slice containing a 41 kDa protein was treated with 10 mM DTT (dithiothreitol) at 56 °C for 45 min, and alkylation was performed in the dark with 50 mM iodoacetic acid at 25 °C for 30 min. The dried sample was immersed in 40 μl of 5 μg/ml trypsin solution in 25 mM $NH_4HCO_3$, and kept at 37 °C for 15 h. Tryptic peptides were extracted with 45 μl of 33 % $NH_4HCO_3$/66 % acetonitrile, 45 μl of 5 % formic acid/66 % acetonitrile and 75 μl of 5 % formic acid/80 % acetonitrile. The digests were subjected to automated LC (liquid chromatography)-MS/MS analysis HPLC using the Magic 2002 (Michrom BioResources, Auburn, CA, U.S.A.) connected online to a LCQ Deca ion-trap tandem mass spectrometer (Thermoquest, San Jose, CA, U.S.A.). Each chromatogram was subsequently analysed with the Mascot search algorithm (http://www.matrixscience.com).

### PDE and protein assays

The PDE assay was performed using the radiolabelled nucleotide method as described previously [12,27]. In brief, the assay buffer contained 50 mM Tris/HCl, pH 8.0, 5mM $MgCl_2$ or 1 mM $MnCl_2$, 4 mM 2-mercaptoethanol, 0.33 mg/ml BSA, 13 nM [$^3$H]cGMP or 5 nM [$^3$H]cAMP, and unlabelled cGMP or cAMP. Reactions were started by adding enzyme solution to 500 μl of assay buffer and the tubes were incubated at 37 °C for 30 min. After boiling for 2 min, the mixtures were added to 100 μl of 1 mg/ml rattlesnake (*Crotalus atrox*) venom and incubated at 37 °C for 30 min. Reactions were stopped by the addition of 500 μl of methanol and the resultant solutions were applied to Dowex (1 × 8 200–400; Dow Chemical Company, Midland, MI, U.S.A.) columns. Aqueous scintillation mixture was added to each eluate and the radioactivity was measured using a scintillation counter. The $K_m$ and $V_{max}$ values were calculated from Lineweaver–Burk plots [28]. Relative $V_{max}$ values were determined according to the method of McPhee et al. [29]. Relative concentrations of PfPDE1 proteins produced in *E. coli* were calculated by immunoblotting as described above. The membranes were incubated with ECL® reagents at 25 °C for 1 min and then exposed to X-ray film for 2–10 s, under conditions in which each X-ray film exposure did not reach saturation. The resultant films were scanned by ARCUS II (Agfa-Gevaert), and quantified using the Quantity One program (PDI, Inc., Huntington Station, NY, U.S.A.). The absorbances were plotted versus the amount of hexahistidine-tagged protein to measure the relative concentrations of PfPDE1 proteins. The protein concentration was determined using DC Protein Assay Kit (Bio-Rad), using the BSA as a standard.

### PDE assay of *P. falciparum* lysates

Erythrocytes infected with *P. falciparum* Honduras-1 were washed with AIM buffer (10 mM Pipes, pH 6.7, 120 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, and 5 mM glucose) and treated with 0.075 % (w/v) saponin in AIM buffer to obtain the parasite. The cells washed with AIM buffer were suspended in ice-cold lysis buffer and disrupted by freeze–thaw treatment and sonication as described above. To remove cell debris and haem polymer, the lysates were centrifuged at 10000 ***g*** for 15 min at 4 °C. Then the supernatants were centrifuged at 100000 ***g*** for 60 min at 4 °C and fractionated into pellets and soluble (cytosolic) fractions.

The pellets were dissolved with ice-cold lysis buffer containing 0.5 % Triton X-100, centrifuged at 10000 ***g*** for 15 min at 4 °C and the resultant supernatants were used as the membrane fractions. Prepared cytosolic and membrane fractions were used in a PDE assay.

### *In vitro* antimalarial activity assay

The following procedure is based on the antimalarial activity assay previously described [30]. Test compounds were dissolved in appropriate solvent and a serial dilution was prepared. In the 24-well plate, each well contained 1 ml of asynchronous *P. falciparum* Honduras-1 culture with a final Ht concentration of 3 % and 0.3 % parasitaemia, which contained 10 $\mu$l of test compound solution. Prepared culture was then incubated for 72 h using the gas conditions described above. To evaluate the antimalarial activity of the test compounds, more than 1000 erythrocytes, stained with Giemsa (Merck), were examined by microscopy. All of the test compounds were assayed in duplicate and, for zaprinast, the experiment was repeated three times. The $ED_{50}$ value, which is a dose giving 50 % reduction in the increase of infected erythrocytes, was determined by comparison with drug-free controls cultured under the same conditions.

## RESULTS

### Cloning of chromosomal DNA and cDNA for a novel *P. falciparum* PDE

A search of draft genome sequence databases using the amino acid sequences of 11 mammalian PDEs (PDE 1–11) resulted in four putative *PfPDE* (*P. falciparum* PDE) genes. During the course of this study, whole genome sequencing of this organism was completed [1] and the protein-coding regions have been predicted by computer analysis (http://www.plasmodb.org/). On that web site, the above four putative *PfPDE* genes have been registered as PFL0475w, MAL13P1.118, MAL13P1.119 and PF14_0672. All these gene products contained a sequence consistent with the class I PDE signature sequence $HDX_2HX_4N$ [31]. Based on the alignment of PDE catalytic domain sequences, a phylogenetic tree was inferred by the NJ method (Figure 1). The four putative *PfPDEs* did not belong to any PDE family previously described, but showed a low degree of evolutionary relatedness with PDE9A and the *Dictyostelium* PDE, RegA. Thus the *PfPDE*s constitute a new family of PDE. We focused on one of these *PfPDE*s, PFL0475w (here designated *PfPDE1*). The *PfPDE1* gene was found on chromosome 12 in the genome database. Based on this sequence, we designed PCR primers and performed PCR amplification using *P. falciparum* chromosomal DNA as a template to obtain chromosomal DNA for *PfPDE1*. PCR products of the appropriate size were detected, subcloned into the TA-cloning vector pGEM-T Easy and sequenced. The nucleotide sequences of the isolated clones carrying a chromosomal DNA for *PfPDE1* were in full agreement with the sequences found in the genome database (Figure 2A). Southern blot analysis of *P. falciparum* chromosomal DNA, digested with restriction endonucleases, EcoRI, SpeI and XbaI, indicated that *PfPDE1* is a single-copy gene (Figure 2B).

*PfPDE1* cDNA was also obtained in the same way by PCR amplification using *P. falciparum* cDNA as a template. An open reading frame of 2655 bp was identified and predicted to encode an 884-amino-acid protein with a predicted molecular mass of 107 kDa. The cDNA sequence was A/T-rich [75.6 % (2112 bp/2655 bp)] and showed a characteristic codon usage similar to those of the *P. falciparum* genes reported to date. Two

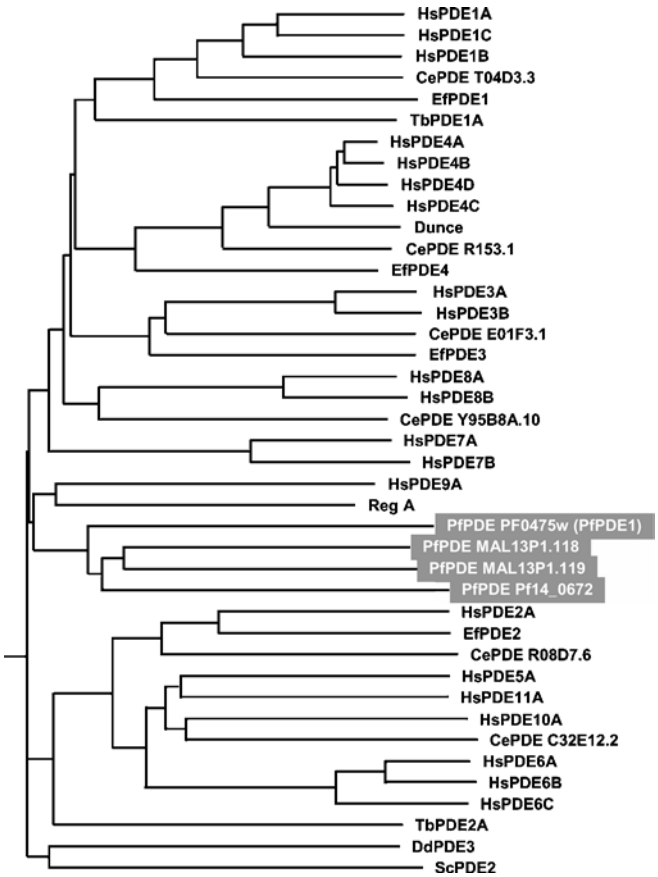


**Figure 1 Phylogenetic tree of the PDE families inferred from their catalytic domain sequences**

The phylogenetic tree was generated using the NJ algorithm of PHYLIP on the basis of a multiple alignment of the catalytic domain sequences of PDEs analysed with CLUSTALW. The following PDEs were included in the analysis: human PDE1A (HsPDE1A) (GenBank accession number, U40370), HsPDE1B (U56976), HsPDE1C (U40371), HsPDE2A (U67733), HsPDE3A (U36798), HsPDE3B (U38178), HsPDE4A (U68532), HsPDE4B (U85048), HsPDE4C (Z46632), HsPDE4D (L20969), HsPDE5A (D89094), HsPDE6A (M26061), HsPDE6B (X66142), HsPDE6C (X94354), HsPDE7A (U67932), HsPDE7B (AB038040), HsPDE8A (AF056490), HsPDE8B (AB085824), HsPDE9A (AF048837), HsPDE10A (AB020593), HsPDE11A (AB036704), *Ephydatia fluviatilis* PDE1 (EfPDE1) (AB017021), EfPDE2 (AB017022), EfPDE3 (AB017023), EfPDE4 (AB017024), *Caenorhabditis elegans* PDE (CePDE) T04D3.3 (Z81114), CePDE R153.1 (U28729), CePDE E01F3.1 (Z93376), CePDE Y95B8A.10 (AC024877), CePDE R08D7.6 (Z12017), CePDE C32E12.2 (U80032), *Trypanosoma brucei* PDE1A (TbPDE1A) (AF253418), TbPDE2A (AF263280), *Saccharomyces cerevisiae* (yeast) PDE2 (ScPDE2) (M14563), *Drosophila melanogaster* Dunce (AH006406), *Dictyostelium discoideum* RegA (AJ005398), *D. discoideum* PDE3 (DdPDE3) (AY162269).

motifs of a bivalent-cation-binding domain ($YHNX_2HG/AX_{23}E$ and $HDX_2HX_{24-26}E$) conserved in class I PDEs [32] are also found in PfPDE1 ($Y^{609}HtX_2HAX_{23}d^{639}$ and $H^{650}DX_2HX_{25}E^{680}$, conserved and irregular amino acids are shown in upper- and lower-case letters respectively). [32]. The catalytic domain sequence of PfPDE1 (amino acid residues 607–776) was compared with those of PDEs from human, yeast, *Dictyostelium* and trypanosome. The catalytic domain sequence of PfPDE1 exhibited the highest identity (36 %) to PDE9A among these PDE sequences. Comparison of the catalytic domain sequence of PfPDE1 with those of the three other predicted PfPDEs revealed the highest identity (40 %) and similarity (60 %) to MAL13P1.118. The amino acid sequence of PfPDE1 outside the catalytic domain was searched using SMART and BLAST. The SMART program revealed that N-terminal region of PfPDE1 has six putative transmembrane regions (Figure 3), suggesting that PfPDE1 is

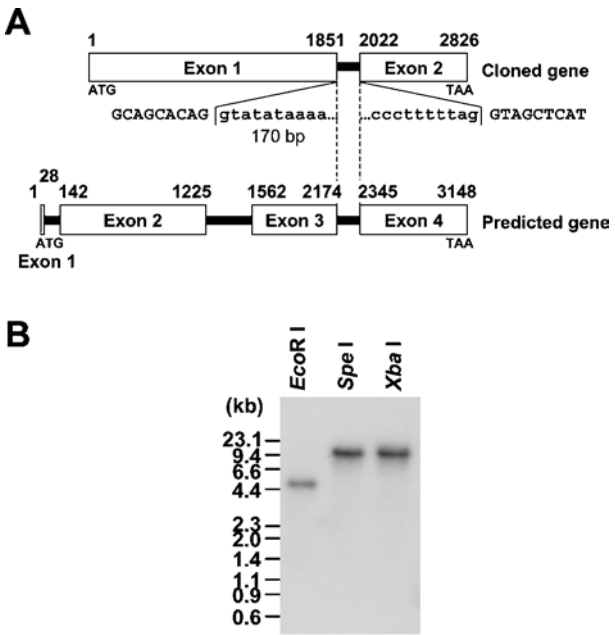


**Figure 2 Structure of the *PfPDE1* gene**

(**A**) The *PfPDE1* gene structure is illustrated. Open boxes with numbers represent exons. The computationally predicted organization is indicated above. Exon–intron organization, determined by comparison of the cDNA and genomic sequences, is shown below. Exon sequences are indicated by *upper*-case letters and intron sequences by lower-case letters. Exon–intron boundaries in common to both organizations are indicated by a broken line. A chromosomal region, which is computationally predicted as a second intron but actually is a coding region, is shown between thin lines. The initiation ATG codon of the cloned cDNA was indicated by a thick line. (**B**) Southern-blot analysis. *P. falciparum* chromosomal DNA, digested with the indicated (above the gel) restriction endonucleases, was fractionated and transferred on to a nylon membrane. The blot was hybridized with a $^{32}$P-labelled *PfPDE1* cDNA fragment.

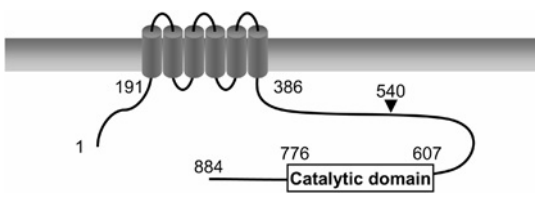


**Figure 3 Schematic structure of PfPDE1**

The cylinders represent hydrophobic segments that are thought to form transmembrane $\alpha$-helices. An arrowhead indicates the start site of PfPDE1$\Delta$.

a membrane protein. The BLAST search revealed no significant similarity to other reported sequences.

The *PfPDE1* cDNA sequence was compared with a computationally predicted coding sequence for PFL0475w and the PfPDE1 chromosomal DNA sequence. As shown in Figure 2(A), the *PfPDE1* gene was revealed to consist of two exons and a 170 bp intron, although the presence of three introns (nt 29–141, 1226–1561, and 2175–2344 in the predicted organization) has been predicted in the coding region of the genome sequence (accession no. NC004316). The nucleotide sequence, observed at the 5′ donor and 3′ acceptor splice sites, demonstrated that the sites are consistent with the canonical GT-AG rule (5′ donor; GTATATAAAAAA . . . and 3′ acceptor; . . . ATCCCTTTTTAG) [33]. The second intron in the predicted organization (nt 1226–1561; see accession no. AE014845) was revealed to be a coding region of PfPDE1, composed of 112 amino acid residues

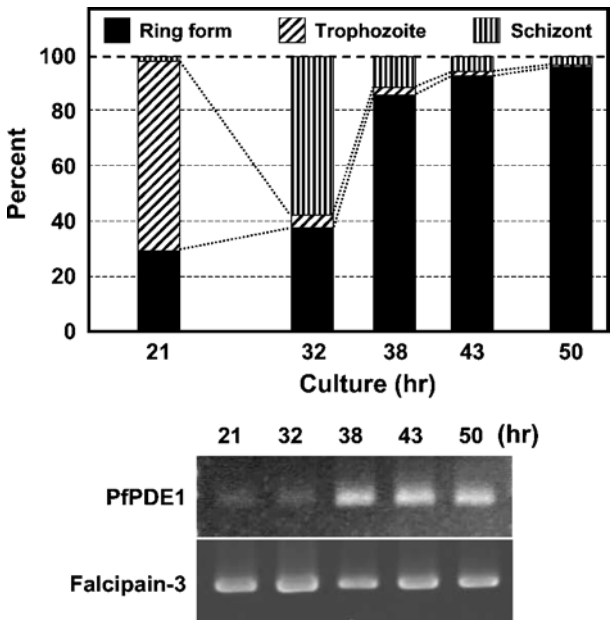


**Figure 4 Stage-specific expression of *PfPDE1* transcripts**

In order to obtain a synchronous culture, ring form-rich parasites, prepared as described in the Experimental section, were cultured. After 21, 32, 38, 43 and 50 h, each sample was collected and the stage-specific cDNAs were prepared. The percentage of ring form, trophozoite, and schizont was calculated by counting the numbers of each cell form (upper panel). Expression levels of *PfPDE1* transcripts (nt 1870–2655) and falcipain-3 (GenBank® accession number AF258791, nt 686–1528) were examined by RT-PCR. After separation on a 1.5% agarose gel, PCR products were detected with ethidium bromide staining (lower panel).

(Figure 2A). Finally, PfPDE1 contains six potential transmembrane regions (PFL0475w has been predicted as a membrane protein with three potential transmembrane regions).

### Stage-specificity of *PfPDE1* expression in *P. falciparum*

*Plasmodium* has a complex life cycle that differs at morphological and biochemical levels. To investigate changes in *PfPDE1* expression during blood stage development, RT (reverse transcriptase)-PCR analysis was performed using total RNA isolated from synchronously cultured parasites as shown in Figure 4. Cell populations were determined by counting the numbers of the three cell forms. To eliminate PCR products amplified from contaminating chromosomal DNA, sense and antisense PCR primers were designed from coding sequences of exons 1 and 2 respectively. PCR products amplified from chromosomal DNA were not detected. The levels of *PfPDE1* transcripts were in accordance with the proportion of ring form. Amounts of mRNA loaded in each lane were confirmed by RT-PCR analysis of falcipain-3, which is expressed in all stages of asexual blood stages [26]. Thus stage-specific transcriptional regulation of *PfPDE1* was observed.

### Production of recombinant PfPDE1 in *E. coli*

Production of the recombinant PfPDE1 protein as a hexahistidine-tagged N-terminally truncated form (PfPDE1$\Delta$) was first attempted with COS cells, which we have previously employed successfully as described in [12]. However, no significant plasmid-directed PDE activity was detected in transfected COS cells, probably owing to an A/T-rich coding sequence of *PfPDE1*. Therefore an *E. coli* expression plasmid encoding a hexahistidine-tagged PfPDE1$\Delta$ (pQE-PfPDE1$\Delta$) was generated and introduced into *E. coli*. After induction with IPTG, the cell extracts prepared

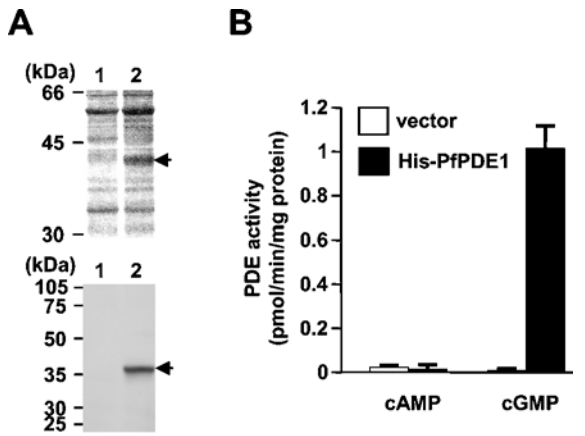


**Figure 5 Production of the recombinant PfPDE1 protein**

(**A**) Expression of the catalytic domain of PfPDE1 was examined by SDS/PAGE and immunoblotting. The control vector pQE-32 (lane 1) and pQE-PfPDE1Δ (lane 2) were used to transform the *E. coli* JM109 cells. The hexahistidine-tagged PfPDE1Δ was partially purified using a nickel affinity column. Partially purified PfPDE1 protein was separated by SDS/PAGE (12.5 % gels), and visualized with silver staining (upper panel) and with immunoblotting using anti-pentahistidine antibody (lower panel). (**B**) Cyclic nucleotide hydrolytic activities of partially purified proteins from the cells carrying a control vector (open bars) and pQE-PfPDE1Δ (filled bars) were measured using 5 nM cAMP or 13 nM cGMP as a substrate. Bivalent cation used was 5 mM $MgCl_2$.

were subjected to nickel affinity column purification, as described in the Experimental section. SDS/PAGE analysis visualized by silver staining and immunoblot analysis using anti-pentahistidine antibody demonstrated the production of a 41 kDa protein in the lysates of the *E. coli* cells carrying pQE-PfPDE1Δ (Figure 5A). The value was in reasonable agreement with the molecular mass predicted for the hexahistidine-tagged PfPDE1Δ. The masses of tryptic peptides of the 41 kDa protein, determined by LC-MS/MS, corresponded to those of predicted tryptic fragments derived from the PfPDE1Δ sequence, such as 'ANTFISIGYK', 'LLYPLGVLEANFDKEK', 'AILSTDMK', 'LELIKFE', etc. (results not shown). The eluates were employed in the PDE assay using either 5 nM cAMP or 13 nM cGMP as a substrate. Partially purified proteins from PfPDE1Δ-transformed cells exhibited approx.135-fold higher levels of cGMP PDE activity than those from cells transformed with the control vector (Figure 5B). No significant cAMP hydrolytic activity of the PfPDE1Δ enzyme was observed under these conditions. cGMP hydrolysis was neither activated nor inhibited by cAMP at concentrations of up to 100 $\mu$M (results not shown). These results indicated that PfPDE1 is a cGMP-specific PDE.

### Characterization of PfPDE1 activity

PDEs require bivalent cations for their activities [32,34]. For example, cGMP hydrolytic activity of human PDE9A is supported by $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$, and this enzyme exhibits the highest activity with $Mn^{2+}$ [34]. The catalytic domain sequence of PfPDE1 shows the highest similarity with that of PDE9A among mammalian PDEs, and both PfPDE1 and PDE9A are cGMP-specific PDEs, suggesting that PfPDE1 activity also may be supported by $Mn^{2+}$. The effect of several bivalent cations ($Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$) on PfPDE1 enzyme activity was examined, and as expected, $Mn^{2+}$ was revealed to be a more potent activator of PfPDE1 than $Mg^{2+}$ and $Ca^{2+}$ at concentrations of 0.01–10 mM. Maximum activity was observed at 1 mM $MnCl_2$ (Figure 6). $Ca^{2+}$ was less potent at concentrations of 0.01–1 mM.

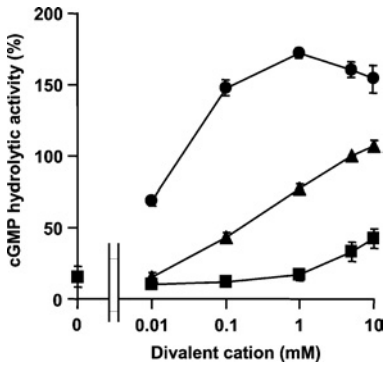


**Figure 6 Effect of bivalent cation on PfPDE1**

PDE activity of the catalytic domain of PfPDE1 expressed in *E. coli* was measured with 13 nM cGMP in the presence of different concentrations (0–10 mM) of $MgCl_2$ (▲), $MnCl_2$ (●), and $CaCl_2$ (■). The results are presented relative to the activity with 5 mM $MgCl_2$.

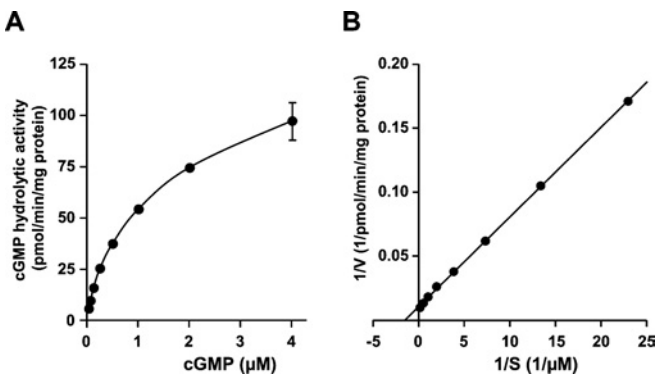


**Figure 7 Kinetic analysis of partially purified PfPDE1**

(**A**) Michaelis–Menten kinetics of PfPDE1. Partially purified PfPDE1 protein was prepared as described in the Experimental section. PfPDE1 was assayed at various cGMP concentrations (0.03125–4 $\mu$M). (**B**) Lineweaver–Burk plots of the same sets of data are shown. The data are expressed as the means ± S.E.M. for three independent experiments. $K_m$ and $V_{max}$ values are expressed as the means ± S.E.M. for triplicate assays. The bivalent cation used was 1 mM $MnCl_2$.

The $K_m$ and $V_{max}$ values of PfPDE1 in the presence of $Mn^{2+}$ were calculated from Lineweaver–Burk plots of activities using cGMP (0.03125–4 $\mu$M) as a substrate. As shown in Figure 7, Lineweaver–Burk plots showed apparent linearity over the substrate concentration range examined. The $K_m$ value of PfPDE1 for cGMP was $0.65 \pm 0.033$ $\mu$M, indicating that PfPDE1 is categorized into high-affinity cGMP PDEs. The $V_{max}$ value was $91 \pm 4.1$ pmol/min per mg of protein with the partially purified recombinant protein. Consistent with the $HDX_2HX_4N$ motif in the PfPDE1 catalytic domain, high affinity for cGMP also indicated that PfPDE1 is a class I, not a class II, PDE.

### Inhibitory profile of PfPDE1

The effects of various PDE inhibitors on PfPDE1 were examined (Table 1). $IC_{50}$ values for the non-specific PDE inhibitors IBMX, papaverine, theophylline and pentoxifylline were more than 100 $\mu$M. Furthermore, vinpocetine, EHNA, milrinone and rolipram, which are inhibitors for mammalian PDE1, PDE2, PDE3 and PDE4 respectively, were also inactive up to 100 $\mu$M. Compounds that inhibit PDE5 showed inhibitory effects on PfPDE1. Dipyridamole, E4021 and sildenafil demonstrated a moderate inhibitory effect ($IC_{50} = 22 \pm 0.58$, $46 \pm 1.8$, and $56 \pm 11$ $\mu$M, respectively). Among the PDE inhibitors tested, zaprinast

**Table 1 Inhibitory effect of the various PDE inhibitors and chloroquine on PfPDE1**

Partially purified PfPDE1 proteins produced in *E. coli* were used for the assay. The concentrations of cGMP used were 0.6 $\mu$M. $IC_{50}$ values were calculated by linear regression. Data are expressed as the means $\pm$ S.E.M. for three independent determinations. All assays were performed in duplicate. The bivalent cation used was 1 mM $MnCl_2$.

| Inhibitor | $IC_{50}$ for PfPDE1 ($\mu$M) |
|---|---|
| Chloroquine | > 100 |
| IBMX | > 100 |
| Papaverine | > 100 |
| Theophylline | > 100 |
| Pentoxifylline | > 100 |
| Vinpocetine | > 100 |
| EHNA | > 100 |
| Milrinone | > 100 |
| Rolipram | > 100 |
| Sildenafil | 56 $\pm$ 11 |
| E4021 | 46 $\pm$ 1.8 |
| Dipyridamole | 22 $\pm$ 0.58 |
| Zaprinast | 3.8 $\pm$ 0.23 |

**Table 2 Comparison of $IC_{50}$ for zaprinast of PfPDE1 mutants**

Wild-type PfPDE1 enzyme and its mutants, PfPDE1D762A and PfPDE1G788A, were produced in *E. coli* and partially purified by using a nickel affinity column. The $K_m$ values of PfPDE1 were calculated from Lineweaver–Burk plots of activities using cGMP. $IC_{50}$ values for zaprinast were measured with 0.6 $\mu$M cGMP by linear regression. Data are expressed as the means $\pm$ S.E.M. for three independent determinations All assays were performed in duplicate. ND, not detected; NT, not tested.

| Protein | $K_m$ ($\mu$M) | $IC_{50}$ for zaprinast ($\mu$M) |
|---|---|---|
| PfPDE1 (wild-type) | 0.65 $\pm$ 0.033 | 3.8 $\pm$ 0.23 |
| PfPDE1D762A | ND | NT |
| PfPDE1G788A | 0.77 $\pm$ 0.054 | 5.6 $\pm$ 0.67 |

was the most effective antagonist for PfPDE1 with an $IC_{50}$ value of 3.8 $\pm$ 0.23 $\mu$M. This value is approximately 10 times higher than that obtained for human PDE5 ($IC_{50}$ = 0.5 $\mu$M; [35]). A potent antimalarial, chloroquine, showed 35 % inhibition at 100 $\mu$M.

Previous reports revealed that $Asp^{754}$ and $Gly^{780}$ of bovine PDE5A are critical for interaction with zaprinast [36,37]. The Asp residue is conserved among PDEs and the involvement of this residue in binding to the substrate cGMP has been reported [38]. Since PfPDE1 also contained $Asp^{762}$ and $Gly^{788}$ in the conserved positions, we examined a role of these residues for cGMP hydrolytic activity and zaprinast-sensitivity of PfPDE1. Two PfPDE1 mutants, PfPDE1D762A and PfPDE1G788A, with alanine substitutions at $Asp^{762}$ and $Gly^{788}$ respectively (see the Experimental section) were used. PfPDE1D762A showed no cGMP hydrolytic activity (Table 2), although the protein was produced at almost the same level as PfPDE1$\Delta$ in immunoblot analysis (results not shown), indicating that $Asp^{762}$ is indispensable for the PDE activity. On the other hand, another PfPDE1 mutant, PfPDE1G788A, retained the cGMP-PDE activity with a $K_m$ value of 0.77 $\pm$ 0.054 $\mu$M, which was about the same as that of the wild-type (0.65 $\pm$ 0.033 $\mu$M). The $IC_{50}$ value of the mutant for zaprinast (PfPDE1G788A) was 5.6 $\pm$ 0.67 $\mu$M, and introduction of this amino acid substitution did not alter the zaprinast-sensitivity drastically.

### cGMP hydrolytic activity in *P. falciparum*

Endogenous cGMP PDE activity in *P. falciparum* was examined. The cytosolic and membrane fractions were prepared from mixed asexual blood-stage malaria parasites and cGMP PDE activities and their subcellular localization were tested. Consistent with the predicted protein feature of PfPDE1 as a membrane protein, most cGMP hydrolytic activity was detected in membrane fractions prepared from infected erythrocytes (Figure 8A). Uninfected erythrocytes showed no significant cGMP hydrolytic activities in either cytosolic or membrane fractions. Interestingly, zaprinast inhibited *P. falciparum* cGMP PDE activity of membrane fractions with an $IC_{50}$ value of 4.1 $\pm$ 0.32 $\mu$M, which was in good agreement with the value obtained with recombinant PfPDE1 (Figure 8B). Furthermore, E4021 inhibited 85 % of cGMP hydrolytic activity at 100 $\mu$M. On the other hand, IBMX and theophylline were also weak inhibitors at a concentration of 100 $\mu$M. At this concentration, chloroquine did not inhibit the cGMP-hydrolytic activity at all.

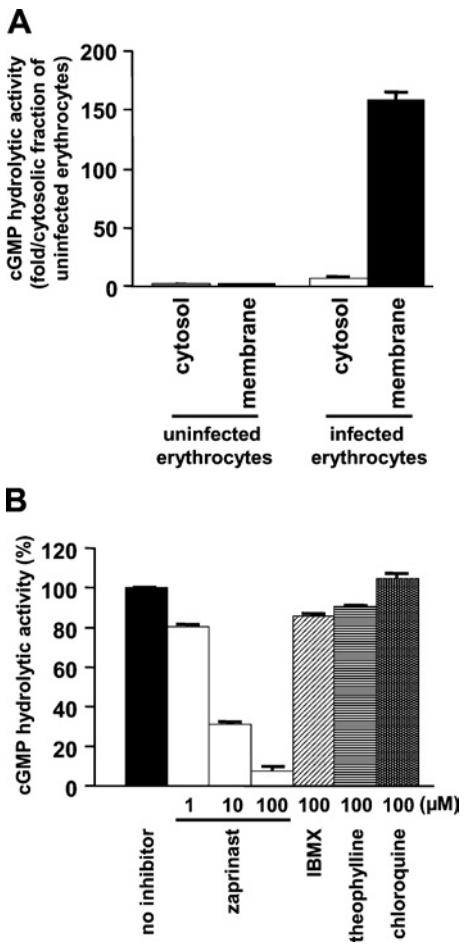


**Figure 8 cGMP hydrolytic activity in *P. falciparum***

(**A**) Cytosolic and membrane fractions of *P. falciparum* were prepared as described in the Experimental section. PDE activity was measured using 13 nM cGMP as a substrate. The data are expressed as the means $\pm$ S.E.M. for three independent experiments. (**B**) Membrane fractions of *P. falciparum* were used for the PDE assay using 13 nM cGMP. Data are expressed as the means $\pm$ S.E.M. for three independent experiments. All assays were performed in duplicate. The bivalent cation used was 1 mM $MnCl_2$.

### *In vitro* antimalarial activity of zaprinast

Finally, we examined the effect of PDE inhibitors in the cell proliferation of asexual blood parasites using an *in vitro* antimalarial activity assay. A solvent for PDE inhibitors, DMSO, did not affect the increase in the *P. falciparum* cell number (results not shown). Intriguingly, treatment with zaprinast, a potent inhibitor

**Table 3 *In vitro* antimalarial activity of zaprinast**

Asynchronous *P. falciparum* Honduras-1 were cultured with final Ht 3 % and 0.3 % parasitaemia in the absence or presence of PDE inhibitors. After 72 h, more than 1000 erythrocytes stained with Giemsa were examined under microscopy. The $ED_{50}$ value, which is a dose giving 50 % reduction of the increase of infected erythrocytes, was determined by comparison to drug-free controls cultured under the same conditions. Data are expressed as the means ± S.E.M. for three independent determinations.

| Inhibitors | $ED_{50}$ ($\mu$M) |
|---|---|
| IBMX | > 100 |
| Theophylline | > 100 |
| Zaprinast | 35 ± 4.2 |

of both recombinant PfPDE1 enzyme and endogenous cGMP PDE activity in *P. falciparum*, resulted in growth inhibition of the parasites with $ED_{50} = 35 \pm 4.2$ $\mu$M (Table 3). In contrast, neither IBMX nor theophylline inhibited parasite growth at the concentration of 100 $\mu$M. These data reaffirm the importance of the involvement of zaprinast-sensitive PDE activity, including PfPDE1, in the asexual stage development.

## DISCUSSION

Currently, PDEs comprise a superfamily of enzymes that are divided into three major classes (class I, II and III) on the basis of sequence similarity. Class I PDEs contain a signature sequence, $HDX_2HX_4N$, show high affinity for cAMP and cGMP and have been identified in organisms ranging from lower eukaryotes to mammals. *P. falciparum* was predicted to contain four class I PDE genes in its genome, based on sequence analysis. One of the *P. falciparum* PDEs, PfPDE1, showed high affinity for cGMP and was sensitive to some of the generally used PDE inhibitors, typical enzymatic characteristics of class I PDEs. Moreover, the PDE activity was lost by mutagenesis of the conserved $Asp^{762}$, which is predicted to be involved in the formation of a metal-binding pocket essential for class I PDEs [39]. Thus intracellular cyclic nucleotide levels in this lower organism would be multiply controlled by the four class I PDEs including PfPDE1.

The presence of six putative transmembrane domains in the N-terminal to central region of PfPDE1 suggested that PfPDE1 is a membrane protein. Interestingly, we found that the inhibitor sensitivity of cGMP hydrolytic activity in membrane fractions of *P. falciparum* at mixed asexual blood stages is similar to that of the recombinant PfPDE1 enzyme, suggesting the presence of PfPDE1 in these fractions. However, as well as PfPDE1, the other three putative PfPDEs are also predicted to contain transmembrane regions in their N-termini and it has been reported that theophylline-sensitive cGMP PDE activity, not a characteristic of PfPDE1, may exist in gametocytes of *P. falciparum* [6]. Thus, although inhibitor sensitivity of endogenous PDE activity is analogous to that of PfPDE1, it is unclear whether PfPDE1 is a major cGMP-PDE in asexual blood stages of *P. falciparum* or not. Therefore analysis of the three other putative PfPDEs is needed to clarify the regulation of cyclic nucleotide signalling through hydrolysis by the PDEs in *P. falciparum*.

Previous reports have discussed the cyclic nucleotide signalling in *P. falciparum*. cAMP is involved in the asexual stage of development and erythrocyte invasion in *P. falciparum* and *Plasmodium berghei* [40,41]. In contrast, cGMP has been shown to play an important role in gametogenesis of both parasites [4,5]. Guanylate cyclase (PfGC) activity has been demonstrated in gametocytes. A gametocyte-activating factor, xanthurenic acid, is known to increase PfGC activity [6], suggesting that exflagellation, a requirement for fertilization, may be mediated by cGMP signalling. However, Deng and Baker [8] have reported predominant expression of *P. falciparum* cGMP-dependent protein kinase (PfPKG) in the ring stage. In mammals, cGMP has three major receptor proteins, namely cGMP-dependent protein kinase, cyclic nucleotide-gated ion channel and cGMP-regulated PDE. Since PfPDE1 and the other three putative PfPDEs do not have a cGMP-binding domain, and a cyclic nucleotide-gated ion channel has not yet been identified in *Plasmodium*, PfPKG is the only effector of cGMP. Stage-specific expression of PfPDE1 transcripts was consistent with PfPKG, but not PfGC, and the lysates prepared from the parasite in asexual blood stages actually contained cGMP hydrolytic activity. The presence of cGMP-PDE and PKG activities suggests a role for cGMP signalling in the development of the parasite at the asexual blood stages.

The *in vitro* antimalarial activity assay demonstrated that treatment of *P. falciparum* with zaprinast results in significant growth inhibition of the parasites in the asexual blood stages. Interestingly, a cell-permeable cGMP analogue, 8-bromo-cGMP, inhibited growth of the parasites in the asexual blood stages with $ED_{50}$ values less than 10 $\mu$M (results not shown), indicating the significance of cGMP signalling in the survival of this organism. Zaprinast is a potent mammalian PDE5 inhibitor, and therefore it is important to know whether synthesis of a specific inhibitor for PfPDE1 is promising or not. Mutagenesis of $Gly^{788}$ in PfPDE1, which is implicated in the zaprinast binding of bovine PDE5 [36,37], did not alter the inhibitory effects of this compound on PfPDE1. This indicates that the amino acid residues concerned with the binding of zaprinast differ between mammalian PDE5 and PfPDE1; that is, the discovery of a PfPDE1 specific inhibitor is achievable.

In addition to zaprinast ($IC_{50} = 3.8$ $\mu$M), PfPDE1 was also inhibited moderately by dipyridamole ($IC_{50} = 22$ $\mu$M). The antimalarial activity of dipyridamole has been reported previously [42,43]. A 50 % inhibition of *P. falciparum* has been observed at 30 nM dipyridamole after 24 h treatment [43]. However, the reported antimalarial activity of dipyridamole [42] is strong compared with that of zaprinast observed here. Dipyridamole is known to have various effects such as nucleoside transport inhibition [44], anti-tumour-drug-effluxing inhibition [45], blockage of chloride transport [46], band-3-mediated anion-exchange inhibition [47] and antioxidant properties [48]. It has been assumed that the antimalarial effects of dipyridamole are associated with the inhibition of some transport system [42]. On the other hand, dipyridamole is also established as a potent PDE inhibitor [49]; nevertheless, the involvement of its inhibitory effects on PDEs in the organism was not discussed in [42]. Dipyridamole-sensitive PDE may be included in the three other *Plasmodium* PDEs and dipyridamole might exhibit more potent antimalarial effects through inhibition of the enzyme than zaprinast.

In summary, we have described the first *P. falciparum* PDE, PfPDE1, and the effect of a PfPDE1 inhibitor, zaprinast, on antimalarial activity. PfPDEs in the asexual blood stages are not a target molecule of chloroquine, and therefore, considering the need for the development of a novel treatment of chloroquine-resistant malaria, PfPDEs must be a unique target for chemotherapy. Further investigation of the PfPDEs of *P. falciparum* will elucidate the involvement of cyclic nucleotide signalling in the physiological responses of this parasite and will provide us with a new treatment of the most lethal malaria.

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