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Modulation of the Growth of *Plasmodium falciparum in Vitro* by Protein Serine/Threonine Phosphatase Inhibitors

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To elucidate the physiological roles of the protein serine/threonine phosphatases of *P. falciparum*, first we identified and characterized phosphatase activities of *Plasmodium falciparum* enzymologically and pharmacologically. We have demonstrated that *P. falciparum* possesses phosphatase-1-like activities predominantly over phosphatase-2A-like activities, while erythrocytes possess mainly phosphatase-2A-like activities. Then, we examined the effects of okadaic acid and calyculin A, potent inhibitors of protein phosphatase 1 and 2A, on the growth of *P. falciparum in vitro*. Both of the drugs inhibited parasite growth dose dependently. The manner of growth inhibition by calyculin A and okadaic acid suggested that these drugs inhibit parasite growth mainly by inhibiting parasite phosphatase-1-like activities. Both drugs were shown to inhibit the growth of three different developmental stages of parasites—ring forms, trophozoites, and schizonts—and inhibit trophozoites the most. This is the first report on *P. falciparum* protein serine/threonine phosphatase activities, which are essential to regulate the erythrocytic stage of parasite growth. © 1998 Academic Press

Plasmodium falciparum is an intraerythrocytic protozoan parasite which causes the most virulent form of human malaria. At present, falciparum malaria accounts for more than 2-3 million deaths per year and the worldwide spread of multidrug resistant *P. falciparum* is a serious public health concern. *Plasmodium* has a unique life cycle with various developmental events in the mosquito vector and in the vertebrate host. Sporozoites injected from an anopheline mosquito at feeding first invade host liver cells, multiply therein and produce thousands of daughter cells, merozoites, which appear in the circulation and invade red blood cells. Within the red blood cells, asexual development from ring forms through trophozoites and finally into schizonts takes place, forming new merozoites. Newly produced merozoites invade red blood cells and repeat the erythrocytic development process. Some parasites at the erythrocytic stage turn gametocytes, which occur sexual proliferation in the mosquito vector.

It has been proposed that phosphorylation and dephosphorylation play important roles in the developmental events of *Plasmodium*. Protein kinases in the erythrocyte membrane are involved in erythrocytic entry of merozoites (1). A large number of phosphoproteins, both of parasitic and host erythrocytic origin, have been identified in *Plasmodium*- infected erythrocytes (2-6), and phosphorylation status of these phosphoproteins changes during intraerythrocytic development (4-6). The major erythrocyte membrane phosphoproteins such as spectrin, band 3 and band 4.1 have been reported to be dephosphorylated during merozoite invasion (4). Recently, several kinases have been identified in *Plasmodium*, mainly by using molecular cloning techniques (7-14). It is likely that *Plasmodium* also contains protein phosphatases which play important roles in controlling cellular events, as is the case for many other organisms (15-17). However, few reports concerning protein phosphatases of *P. falciparum* have been published until now. In this study, in order to identify the roles of the phosphatases of *P. falciparum*, we measured phosphatase activities of parasite extracts in the presence or absence of calyculin A and okadaic acid, potent inhibitors of protein phosphatases 1 and 2A (18-20), and examined the effects of the inhibitors on the growth of *P. falciparum in vitro*. Results showed that *P. falciparum* predominantly possesses protein phosphatase 1 like activities, which appear to control the growth of the erythrocytic stage.

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Abbreviations: Tris, Tris (hydroxymethyl) aminomethane; IC50, 50% inhibitory concentration.

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MATERIALS AND METHODS

Parasite culture. *P. falciparum* K1 strain originally provided by Dr. D. Walliker of Edinburgh University was maintained in asynchronous culture as described by Trager and Jensen (21), with a slight modification : Parasites were cultured in flasks containing a 1% erythrocyte suspension in RPMI 1640 medium (Gibco BRL, Life Technologies, Japan) supplemented with 25 mM 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, (HEPES; Boehringer Mannheim, Germany), 24 mM $NaHCO_3$, 10 mM D-glucose, 10 mg/L hypoxanthine (Kohjin Co., Ltd. Japan) and 30 mg/L gentamicin sulfate (Sigma Chemical Co., USA) (referred as *Pf*medium) with 10% (v/v) human serum (heat- inactivated) under an atmosphere of 5% O_2, 5% CO_2, 90% N_2 at 37 °C. *Pf*medium with serum (referred as *Pf*medium[+]) was exchanged twice a week. When parasites were cultured in a 5% erythrocyte suspension, a daily exchange of *Pf*medium[+] was performed. Erythrocytes obtained from CPD (citrate-phosphate-dextrose) supplemented blood were used for culture after removing plasma and buffy coats throughly by washing more than three times with *Pf*medium. Erythrocytes were taken from healthy volunteers. Human serum was supplied from Hyogo Red Cross Blood Center.

Preparation of parasite and erythrocyte extracts. Parasites of an asynchronous culture at 3-5% parasitemia in a 5% erythrocyte suspension were freed from the host erythrocytes by saponin lysis as described by Kimura et al. except that saponin treatment was carried out for 10 min on ice (22). Freed parasites pelleted by centrifugation were washed with HEPES- buffered saline (HBS: 140 mM NaCl, 10 mM KCl, and 1 mM MgCl2 in 10 mM HEPES-NaOH at pH 7.2) three times to remove erythrocyte ghosts on the top layer of the pellet, aliquoted and stored at −130 °C. To prepare parasite extracts, parasites were thawed, diluted in 10 folds of homogenate buffer (1% Triton X-100, 2 mM EDTA, 10 mM EGTA, 4 μg/ml leupeptin, 4 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride and 10 mM 2-mercaptoethanol, in 20 mM Tris-HCl at pH 7.4) and homogenated by sonication for 15 sec on ice, followed by centrifugation at 12,000 × g for 10 min. The supernatant was used as parasite extracts. Unparasitized erythrocytes and unparasitized erythrocyte ghosts obtained by saponin lysis were also homogenated in the same way and each supernatant was used as erythrocyte extracts and erythrocyte ghost extracts, respectively.

Preparation of ^{32}P-labeled substrates. [^{32}P]phosphorylase a and [^{32}P]casein were prepared according to the method described by MacKintosh (24). Briefly, 10 mg/ml phosphorylase b (Sigma Chemical Co., USA) was incubated with 200 μg/ml phosphorylase kinase (Sigma Chemical Co., USA) for 60 min at 30 °C in 100 mM sodium glycerol 1 phosphate at pH 8.0 containing 0.2 mM $CaCl_2$, 2 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP ($1 \sim 5 \times 10^6$ cpm/nmol). Ten mg/ml casein (Sigma Chemical Co., USA) was incubated with 50 μg/ml bovine cAMP- dependent protein kinase catalytic subunit (Nacalai Tesque, INC., Japan) for 16 h at 30 °C in 50 mM Tris-HCl at pH 7.4 containing 10 mM $MgCl_2$, 0.1 mM EGTA, 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol and 50 μM [γ-^{32}P]ATP ($1 \sim 5 \times 10^6$ cpm/nmol). After terminating phosphorylation by addition of a 1/10 volume of 100 mM EDTA, 500 mM NaF and 10 mM sodium pyrophosphate, each reaction mixture (500 μl) was subjected to a Sephadex G-25 column [Nap-5 column] (Pharmacia Biotech, Sweden) equilibrated in 50 mM Tris-HCl at pH 7.4 with 0.1% (v/v) 2-mercaptoethanol (buffer A) to remove [γ-^{32}P]ATP. Phosphorylated products were eluted in 1 ml of buffer A according to the manufacture's protocol.

Phosphatase assay. [^{32}P]phosphorylase phosphatase activity and [^{32}P]casein phosphatase activity in parasite, erythrocyte and erythrocyte ghost extracts were examined according to the procedure described by MacKintosh (23). Briefly, 10 μl of each extract diluted in 5 folds of buffer A with 0.1% bovine serum albumin were premixed with 10 μl of buffer A containing various concentrations of calyculin A or okadaic acid (Research Biochemicals International, USA). Then, dephosphorylation reactions were initiated by adding 10 μl of [^{32}P]-phosphorylase a or 10 μl of [^{32}P]casein. Background reactions were performed with 20 μl of buffer A instead of 10 μl of diluted extract and 10 μl of each drug with buffer A. After 10 min of incubation at 30 °C, reactions were terminated by addition of 100 μl of 20% (w/v) trichloroacetic acid, and centrifuged at 12,000 × g for 5 min. The radioactivity in 100 μl of the supernatant was measured by scintillation counter (Beckman LS 3801). Phosphatase activity in respective extracts was expressed as pmol phosphate released per assay, subtracting background activity. Because the presence of hemoglobin or malaria pigment contained in erythrocytes or parasites disturbed accurate measurement of protein weight, pmol phosphate released per mg (protein) was not measured. The wet weight of parasites, erythrocytes and erythrocyte ghosts included in each assay was identical (200 μg of wet weight / assay). IC50, the drug concentration required to inhibit phosphatase activity by 50% as compared to drug-free controls was determined graphically by interpolation of log concentration- response curves. Calyculin A and okadaic acid were dissolved in dimethyl sulfoxide (DMSO) at 1 mM and used at less than 1% (v/v) final concentration of DMSO. Preliminarily, 1% DMSO was not found to have any effects in this assay.

In vitro growth assay in asynchronous culture. Parasitized erythrocytes (770 μl) at 0.1-0.3% parasitemia in a 5% erythrocyte suspension were placed in 24 well- microtiter plate wells and cultured in *Pf*medium[+] with various concentrations of calyculin A or okadaic acid and parasitemia was monitored every 24 h as the index of parasite growth. Both reagents were used at less than 0.1% (v/v) final concentration of DMSO in this assay. *Pf*medium[+] was exchanged daily in the presence of drugs. Parasitemia was determined by counting 10,000 Giemsa-stained erythrocytes smeared on a glass slide every 24 h for three consecutive days. $IC50_{72}$, the drug concentration required to inhibit parasitemia by 50% as compared to drug- free controls after 72 h, was determined graphically by interpolation of log concentration-response curves after 72 h. To examine the effects of short term- exposure to drugs, parasitized erythrocytes (0.1-0.3% parasitemia, 5% hematocrit) were exposed to various concentrations of drugs for 15 min, washed with *Pf*medium three times and then cultured in *Pf*medium[+] with daily exchange of *Pf*medium[+] containing no drugs.

In vitro growth assay in synchronous culture. To examine stage-specific effects of inhibitors, highly synchronized parasites at the ring form, trophozoite, and schizont stages were prepared by sorbitol treatment of culture as described by Lambros and Vanderberg (24). Then, parasites at each stage were treated by calyculin A or okadaic acid for short periods and parasitemia was examined 48 h- after drug treatment. Concretely, parasitized erythrocytes synchronized three times to ring form stage (0.5% parasitemia, 5% hematocrit) were divided into 3 groups and aliquots (770 μl) were placed in 24 well-microtiter plate wells. The first group of parasitized erythrocytes was treated with calyculin A or okadaic acid for 15 min immediately after the third sorbitol treatment (ring form stage). The second group was cultured for 20 h, and then treated for 15 min (trophozoite stage). The third group was cultured for 31.5 h, and then treated in the same manner (schizont stage). After exposure to each drug, parasitized erythrocytes of each stage were washed with *Pf*medium three times and cultured in the absence of drugs with a daily exchange of *Pf*medium[+] for 48 h more, and parasitemia was examined.

RESULTS

To identify and characterize phosphatase activities of *P. falciparum*, phosphatase assays were performed on parasite extracts. In many organisms whose phosphatases are well characterized, [^{32}P]phosphorylase phosphatase activity and [^{32}P]casein phosphatase activity are known to represent the activities of phospha-

tase 1 and 2A and the activity of phosphatase 2A, respectively (23). The activities of [^{32}P]phosphorylase phosphatase and [^{32}P]casein phosphatase were detected in both parasite and erythrocyte extracts. Freed parasites obtained by saponin lysis were microscopically confirmed to be completely free from intact red blood cells. However, due to possible contamination by erythrocyte ghosts of freed parasites, the phosphatase activities of erythrocyte ghost extracts were examined. The same weight of erythrocyte ghosts as that of parasites showed little, if any, activities of [^{32}P]phosphorylase phosphatase or [^{32}P]casein phosphatase. These results clearly indicated that the phosphatase activities of parasite extracts were of parasite origin. [^{32}P]phosphorylase phosphatase activity in parasite extracts (9.7 pmol phosphate released / assay) were at similar levels in erythrocyte extracts (12.4 pmol phosphate released / assay). On the other hand, [^{32}P]casein phosphatase activity in parasite extracts (0.67 pmol phosphate released / assay) were considerably lower than that in erythrocyte extracts (3.6 pmol phosphate released / assay). These results suggested that phosphatase 1 like activities and phosphatase 2A like activities exist in the parasites, and that phosphatase 1 like activities are predominant.

Calyculin A and okadaic acid are potent inhibitors of protein phosphatase 1 and 2A, and the concentration of okadaic acid to inhibit phosphatase 2A is similar to the concentration of calyculin A (IC50; 0.5 ~ 1 nM) (16, 19), while the concentration of okadaic acid to inhibit phosphatase 1 is about 100 times higher than that of calyculin A (IC50; 60 ~ 500 nM for okadaic acid and 2 nM for calyculin A). [^{32}P]phosphorylase phosphatase activity and [^{32}P]casein phosphatase activity in parasite extracts and erythrocyte extracts were inhibited by calyculin A and okadaic acid in a dose- dependent manner (Fig. 1A and B). Calyculin A inhibited [^{32}P]-phosphorylase phosphatase activity in parasite extracts by 50% at 3 nM, while okadaic acid inhibited parasite [^{32}P]phosphorylase phosphatase activity by 50% at 300 nM, 100 fold higher concentration. Calyculin A and okadaic acid inhibited [^{32}P]phosphorylase phosphatase activity in erythrocyte extracts by 50% at 2 ~ 3 nM (Fig. 1A). These results suggested that phosphatase 1 like activities exist predominantly over phosphatase 2A like activities in *P. falciparum*. They also indicated the almost exclusive presence of phosphatase 2A in the host erythrocytes. On the other hand, [^{32}P]casein phosphatase activities in erythrocyte and in parasite extracts were inhibited by calyculin A and okadaic acid at similar IC50 levels (<2 nM), demonstrating that both parasites and erythrocytes have similar phosphatase 2A like activities (Fig 1B).

To investigate the roles of the phosphatases of *P. falciparum* on the parasite growth, *in vitro* growth assay was carried out. In this *in vitro* growth assay using asynchronous culture, control parasites (cultured

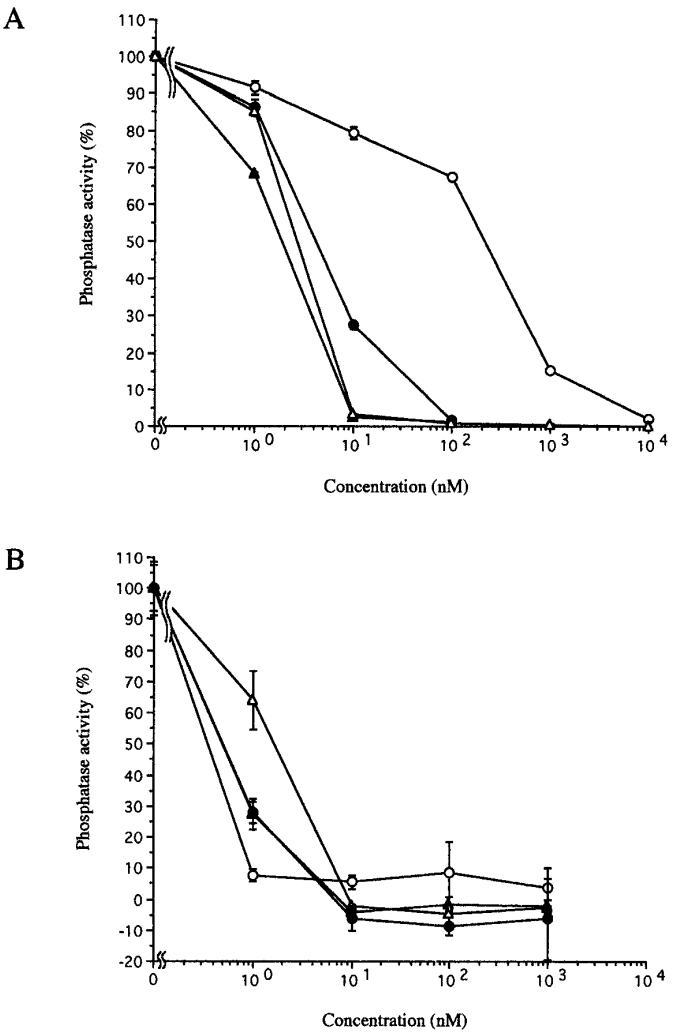

FIG. 1. Log concentration–response curves of phosphatase inhibitors for phosphatase activities. [^{32}P]phosphorylase phosphatase activity (A) and [^{32}P]casein phosphatase activity (B) of parasite extract (●, ○) and erythrocyte extract (▲, △) at indicated concentrations of calyculin A (●, ▲) and okadaic acid (○, dT) were examined as described under Materials and Methods. Data are expressed as % activity of drug-free controls. Each point is shown as the mean ± SEM of two determinations.

in *Pf*medium[+] without drugs) exhibited exponential growth for three days with a multiplication rate of 2-3 times per day. Calyculin A (≥5 nM) and okadaic acid (≥50 nM) inhibited parasite growth after 24 h. Inhibition progressively increased after 48 h and was the most evident after 72 h (data not shown). The log concentration- response curves of calyculin A and okadaic acid after 72 h demonstrated that both calyculin A and okadaic acid caused dose- dependent inhibition of parasite growth (Fig. 2), with the $IC50_{72}$ of calyculin A and okadaic acid being 9.9 nM and 38.5 nM, respectively. As phosphatase inhibitory effects of drugs have frequently been studied using short term (15 min) exposure (25, 26), parasites were exposed to the drugs briefly (15

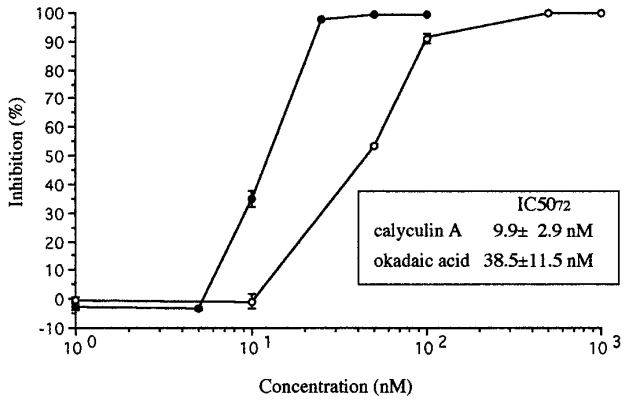

FIG. 2. Log concentration–response curves of phosphatase inhibitors for the growth of *P. falciparum in vitro.* Asynchronous parasitized erythrocytes at 0.1–0.3% parasitemia were continuously treated with indicated concentrations of calyculin A (●) and okadaic acid (○). Data are expressed as % inhibition of parasitemia 72 h after drug treatment as compared to drug-free controls. The curves are representative of two separate experiments for each drug with two determinations: each point is the mean ± SEM. $IC50_{72}$ values were determined graphically by interpolation of the curves. $IC50_{72}$ values indicated in the insert are shown as the mean ± SEM of two experiments.

min) and growth was examined. As shown in Fig. 3, both drugs inhibited parasite growth dose- dependently with $IC50_{72}$ of calyculin A and okadaic acid being 0.49 μM and 1.7 μM, respectively.

To examine the stage- specific effects of both reagents, the ring form, trophozoite and schizont stages were each exposed to inhibitors for short periods. Our method yielded highly synchronized parasites, 100% of ring forms, 89% of trophozoites and 89% of schizonts. The level of susceptibility of each stage to 1 μM of calyculin A was evaluated by % inhibition of parasitemia 48 h- after drug treatment. One μM of calyculin A was required to cause submaximal inhibition (approximately 70% inhibition as compared to drug- free control) of the asynchronous parasite growth 48 h- after 15 min- exposure to the drug (data not shown). Calyculin A (1 μM) inhibited parasites at all stages with the highest inhibition at the trophozoite stage (62.1% inhibition at the ring form stage, 99.0 % at the trophozoite stage and 71.2 % at the schizont stage). Statistical analysis (ANOVA and Fisher's PLSD as post hoc test) revealed that only trophozoites were significantly susceptible to calyculin A as compared to the two other stages, as measured by % inhibition of parasite growth. The similar investigation with okadaic acid (3 μM, the concentration required to inhibit the parasite growth approximately by 60 % 48 h- after 15 min- exposure to the drug) also showed all stages were inhibited, with the greatest inhibition at the trophozoite stage (data not shown).

DISCUSSIONS

Protein phosphatases can be divided into two families, serine/threonine and tyrosine phosphatases, based on the amino acid residues dephosphorylated. Protein serine/threonine phosphatases are classified into protein phosphatase 1, 2A, 2B, and 2C according to substrate specificity, sensitivity to endogenous inhibitors and regulation by divalent cations (27, 28). Calyculin A and okadaic acid are specific inhibitors of protein serine/threonine phosphatases and known to inhibit protein phosphatase 1 and 2A at nanomolar and protein phosphatase 2B at micromolar concentrations (16, 18-20). In contrast, protein phosphatase 2C is not affected by either calyculin A or okadaic acid.

There are only a few reports concerning plasmodial phosphatases. Evidence that okadaic acid reversed the inhibitory effect of staurosporine, a kinase inhibitor, on erythrocyte invasion suggests the existence of phosphatases in a different species of *Plasmodium* (*P. knowlesi*) (29). Bell et al. have informally reported (30) that the activities of protein phosphatase 2B, a calcium- dependent phosphatase, can not be detected in *P. falciparum*, while the activities of other types of phosphatases have not been well characterized. On the other hand, erythrocytes have been shown to possess phosphatase 2A and phosphatase 1 (31, 32).

To elucidate the physiological roles of the protein serine/threonine phosphatases of *P. falciparum* on the parasite growth, first we identified phosphatase activities in the parasites as well as in the erythrocytes. We also characterized their sensitivities to the inhibitors. The results demonstrated that phosphatase 1 and 2A like activities, which are inhibited by calyculin A and okadaic acid, exist in the parasites and that phosphatase 1 like activities are predominant over phosphatase 2A like activities. In *in vitro* growth assay, both calyculin A and okadaic acid dose- dependently inhibited

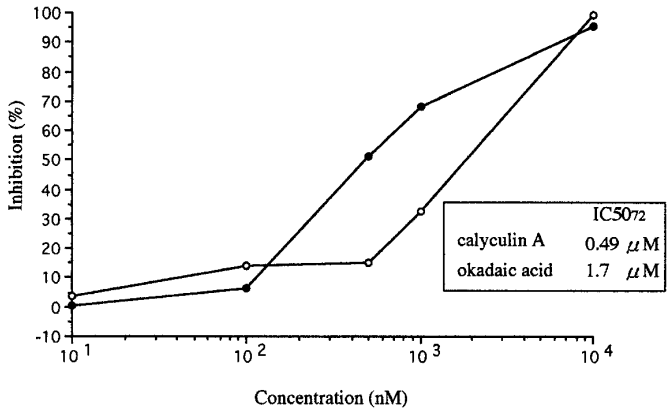

FIG. 3. Log concentration–response curves of short term exposure to phosphatase inhibitors for the growth of *P. falciparum in vitro.* Asynchronous parasitized erythrocytes at 0.1–0.3% parasitemia were exposed to indicated concentrations of calyculin A (●) and okadaic acid (○) for 15 min. Data are expressed as % inhibition of parasitemia 72 h after drug treatment as compared to drug-free controls. $IC50_{72}$ values indicated in the insert were determined graphically by interpolation of the curves.

the growth of *P. falciparum* at ≥5 nM and ≥50 nM, respectively. Since the structures of these two drugs are completely different, growth inhibition is probably due to specific inhibition of phosphatases by calyculin A and okadaic acid rather than by nonspecific cellular toxicity. $IC50_{72}$ values of calyculin A and okadaic acid were very small (9.9 nM and 38.5 nM, respectively), representing drug concentrations where only phosphatase 1 and 2A are inhibited. Short term- exposure to the drugs also caused dose-dependent inhibition, with the $IC50_{72}$ of calyculin A and okadaic acid being 0.49 μM and 1.7 μM, respectively. These values are comparable to those reported to affect cellular function by inhibition of phosphatase 1 and/or 2A in other cell types (25, 26). Taken together, our results suggest that calyculin A and okadaic acid affected parasite growth by inhibiting protein phosphatase 1 and/or 2A. Further, in *in vitro* growth assays, the IC50 of okadaic acid was considerably higher than the IC50 of calyculin A. Thus, it is likely that these phosphatase inhibitors affect parasite growth by mainly inhibiting parasite phosphatase 1 like activities rather than erythrocyte and/or parasite phosphatase 2A like activities. During preparation of this manuscript, protein phosphatase 2A like gene of *P. falciparum* was cloned (33) and mRNA of the gene was expressed mainly at the sexual stage (gametocytes) but not at the asexual stage. Enzymological characterization has not been performed in this report. The findings in this report do not contradict our present findings, considering that gametocytes are not produced in the culture of *P. falciparum* K1 strain used in this study. However, we cannot exclude the possibility that the phosphatase 1 like activities of *P. falciparum* we detected originated from an unknown type of plasmodial phosphatase whose enzymological and pharmacological features are similar to phosphatase 1. In fact, serine/threonine protein phosphatases which cannot be classified into protein phosphatase 1, 2A, 2B or 2C have recently been isolated (16, 34). However, it is not presently possible to enzymologically characterize the phosphatases in detail, due to difficulties in obtaining enough crude parasite extracts for this purpose.

Most of the erythrocytes membrane proteins, including cytoskeletal proteins and integral proteins, can be phosphorylated. Phosphorylation of membrane proteins is reported to alter their affinity to various cytoskeletal components and, therefore, the shape and rigidity of the erythrocytes (35-37). Interestingly, some erythrocyte membrane proteins have been observed to be dephosphorylated after merozoite invasion (4), suggesting that dephosphorylation of erythrocyte membrane proteins may be required for invasion or parasite development in the host erythrocyte. It is possible that the parasite phosphatases identified in this study play a role in this dephosphorylation step. A large number of parasite phosphoproteins are identified and the extent of phosphorylation of these proteins change during intraerythrocytic development (4-6, 38, 39). These parasite phosphoproteins are also target candidates for *P. falciparum* protein phosphatases and seem to minutely regulate intraerythrocytic development by regulating the activities of parasite protein phosphatases and protein kinases.

Our results on the stage- specific effects of these drugs suggest that protein phosphatases play trophozoite stage- specific roles in addition to house keeping roles at all stages of parasite development. Further studies are needed, including identification of stage-specific substrates of parasite phosphatases in order to clarify stage- specific roles of protein phosphatases of *P. falciparum*.

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