Stage-specific cytosolic protein kinase C-like activity in human malarial parasite

*Plasmodium falciparum*

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Protein kinase C (PKC)-like activity was characterized in malarial parasite *Plasmodium falciparum* and its involvement in growth, maturation and differentiation functions, during the asexual stages (ring, trophozoite and schizont) of development was studied. PKC-like activity was found distributed in all the stages of the parasite maturation. The activity was predominantly cytosolic, however it was also present in the membrane fraction. The activation of cytosolic PKC required Ca$^{2+}$, phosphatidyl serine (PS), and either diacylglycerol or phorbol myristate acetate (PMA). The 9-fold increase in the activity was observed in the presence of the co-factors (Ca$^{2+}$, PS and PMA) in the late trophozoite stage, as compared to the ring stage. The activation of trophozoites with PMA resulted in redistribution of PKC-like activity from cytosol to membrane fractions. An antimalarial drug, chloroquine (CQ) inhibited directly the PKC-like activity in a dose-dependent manner (IC$_{50}$ of 45 nM) in trophozoites of chloroquine-sensitive CQ(S) strains, however, the activity remained unaltered in the chloroquine-resistant CQ(R) strains. Kinetic studies showed that the inhibition of cytosolic PKC-like activity by CQ was non-competitive with respect to ATP, histone and PS. The results suggest that the PKC-like activity is developmentally expressed during the parasitic survival and development.

**Keywords**: Malaria, *Plasmodium falciparum*, protein kinase C, cytosol, membrane, drug resistance, chloroquine, phorbol myristate acetate, phosphatidyl serine, schizont, trophozoite

**IPC Code**: A 61 P 33/06

*Plasmodium falciparum* malaria infects approximately 250 million people worldwide and is responsible for about 2 million deaths per year. The emergence of drug-resistant strains is responsible for the lethal complications of malaria, namely cerebral malaria, which represents one of the most important public health problems in many parts of the world. Malaria control methods are still inadequate and moreover, the prospect of a useful vaccine is uncertain. Therefore, design and development of new antimalarial drugs are needed for the identification of potential therapeutic targets unique to the parasite. Although the various stages of maturation of *P. falciparum* are well defined in morphological terms, little is known regarding the molecular and biochemical changes associated with the maturation and replication of the malarial parasite. Thus, there is a need to study the various cellular processes of a drug-resistant strain, which may prove helpful in identifying the susceptible targets.

Of the few reversible processes, phosphorylation and dephosphorylation reactions play an important role in the regulation of cellular events. Protein kinase C (PKC), a Ca$^{2+}$ and phospholipid-dependent protein kinase family of enzymes plays a key role in signal transduction and regulation of responses to diverse external agents, including growth factors and cytokines. PKC activity is associated with receptor-mediated response to hormones, neurotransmitters and growth factors, which require mobilization of Ca$^{2+}$ ions. Only limited studies have been reported on protein kinases and phosphoproteins of malarial parasites. A cAMP-dependent protein kinase was partially characterized from *P. falciparum*, and phosphorylated proteins were identified. A polyamine-stimulated type-I casein kinase activity and phosphorylation of endogenous substrates were...
reported in rodent *P. berghei*\textsuperscript{10}, *P. chabaudi*\textsuperscript{11} and *P. falciparum*\textsuperscript{12}. Proteins derived from the parasite were phosphorylated in the membrane of erythrocytes infected with *P. berghei*\textsuperscript{10}. A cdc 2-like protein kinase\textsuperscript{13} and GMP-dependent protein kinases\textsuperscript{14} were also found in malarial parasite, and genes with protein kinase-like consensus sequences\textsuperscript{15,16} were identified. Earlier, we demonstrated that the protein tyrosine kinase (PTK) activity in *P. falciparum* was inhibited by chloroquine and piceatannol, and provided a hypothesis to explain their antimalarial activity and efficacy\textsuperscript{17}. The activity was reported to be extremely labile\textsuperscript{18}.

Cyclin-dependent protein kinases (CDKs) have become attractive drug targets to identify effective inhibitors of *P. falciparum*\textsuperscript{19,20}, due to their crucial role in the control of cell division and/or development\textsuperscript{1}. Recently, modulation of PKC activity was reported in the *P. falciparum* infected erythrocytes\textsuperscript{21}, suggesting a role of host PKC in establishment of parasite in the RBCs. The various other kinases and their corresponding genes have been isolated from *P. falciparum*. One of these kinases belongs to a family of calcium-dependent protein kinases (CDPKs), which have been found only in the plants and protists\textsuperscript{16,23}. The stimulation of CDPKs by Ca\textsuperscript{2+} ions and lipids, their appearance as a multigenic and multi-functional family, and their possible involvement in signal transduction may indicate that they are the functional analogues of PKCs in the plants and protists\textsuperscript{23}. Since CDPKs are not found in vertebrates, they represent promising targets of antimalarial chemotherapy. Recent findings have confirmed altered activity of PTK and phosphorylation of proteins during cerebral malaria pathology and may seem to be an important cue in understanding the possible continuation of post-receptor signaling\textsuperscript{24,25}.

Although, the protein kinases have a critical role in parasite growth and maturation, the role of PKC (CDPKs) in asexual malarial parasite growth remains to be explored. Stage-specific expression of PKC and its inhibition in the intra-erythrocytic growth of the parasite postulate a key role of PKC\textsuperscript{26}. In the present study, we investigated the role of PKC activity during the development of asexual stages (ring, trophozoite and schizont) of *P. falciparum* and report the characterization of PKC-like activity. This is the first report on the presence of PKC-like activity in *P. falciparum* parasite.

### Materials and Methods

#### Reagents

RPMI 1640 was from Gibco Laboratories, Grand Island, NY; [\textsuperscript{32}P]-\gamma-ATP (sp. activity 2500 Ci/mM) was purchased from BRIT, Bombay, India. L-\alpha-Phosphatidyl serine (PS), histone IIIs, phorbol myristate acetate (PMA), dithiothreitol (DTT), ethylene glycol-bis tetraacetic acid (EGTA), phenylmethyl sulphonyl fluoride (PMSF), Triton X-100, chloroquine (CQ), percoll, polymyxin B and protease inhibitors were obtained from Sigma Chemical Co., St Louis, USA. Staurosporine was obtained from Boehringer Mannheim GmbH, Germany. All other chemicals were of analytical grade and were procured locally.

#### Parasite culture

CQ(S) (FJB-D9) and CQ(R) (FJB-D4) strains of *P. falciparum* were cultured in vitro in RPMI 1640 medium, supplemented with 25 mM HEPES/0.37 mM hypoxanthine/100 mg neomycin base/10% heat inactivated fetal calf serum. For culture, 10% hematocrit was adjusted with normal 0° red blood cells. Parasites were grown at 37°C in candle jar and parasitaemia was monitored at 24 hr\textsuperscript{27}. Parasitaemia and stage-specific parasitic development were examined microscopically in JSB stained blood smears.

#### Parasite isolation and cytosol preparation

Parasitized erythrocytes were isolated using different concentrations of percoll (30%, 45%, 50% and 65%) as previously described\textsuperscript{28}. Purity of each form was checked by microscopic examination after gradient centrifugation. Infected erythrocytes (at different stages) were harvested by centrifugation (700 g, 10 min, 4°C) and parasites were collected by saponin lysis (0.15%) and washed twice with phosphate buffer (5 mM, pH 7.0). Released parasites were concentrated by centrifugation (1500 g, 15 min, 4°C). Free parasites were found to contain minimal erythrocytic contamination (0.5-1.5% of total cells) upon microscopic examination.

Acetylcholine esterase (AchE) activity was determined as previously described\textsuperscript{29}. Briefly, 0.1-10 μg of enzyme protein (parasite and erythrocyte lysate) was incubated in a 200 μl reaction mixture containing 0.6 mM/L thioacetyl chloride, 0.5 mM/L 5,5′ dithiobis-2-nitrobenzoic acid and 100 mM/L NaH\textsubscript{2}PO\textsubscript{4}, pH 7.0 and absorbance at 415 nm was measured over a period of time. Based on AchE
activity (Table 1), 1% of erythrocytes co-purified with the parasites fractions on the percoll and the purity of the recovered parasites was determined by NADP-glutamate dehydrogenase assay (Table 1) as previously described. The PKC-like activity in infected and uninfected erythrocytes preparations in which the parasites were grown was assayed to determine any contribution from contaminating erythrocytes and leukocytes. The contaminating erythrocytes and leukocytes accounted for only about 0.02% of the total PKC-like activity in the free parasites (Table 2). Isolated parasites were washed twice and resuspended in lysis buffer (20 mM Tris, pH 7.5, 10 mM DTT, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 5 μg/ml leupeptin and 250 mM sucrose). Parasites were sonicated for 1 min with alternative cooling and centrifuged at 1,50,000 g for 30 min. The supernatant (cytosol) was separated and resuspended in 0.6% Triton X-100 in sonication buffer and allowed to dissolve for 1 hr at 4°C and stored for the assay of PKC-like activity. Erythrocyte lysates were prepared by homogenizing 100 μl washed packed RBCs in 10 ml PBS as above. Protein in cytosol and membrane fractions was determined by the method of Lowry et al. The PKC-like activity in infected and uninfected erythrocytes was assayed to determine any contribution from contaminating erythrocytes and leukocytes. The controls, representing non-specific kinase activity were run simultaneously in the absence of Ca²⁺ and PS and the values subtracted from total activity. PKC-like activity was expressed as pico moles [P³²]-γ-ATP. Reaction was started by adding 50 μl of enzyme (40-60 μg of parasite protein, erythrocyte lysate) and incubated for 3 min, and was stopped by adding 1.0 ml of 20% trichloroacetic acid. The contents were filtered on millipore membrane filters (HA type, 0.45 μm), washed with ethanol and counted in a liquid scintillation counter (Beckman LS 2800). The controls, representing non-specific kinase activity were run simultaneously in the absence of Ca²⁺ and PS and the values subtracted from total activity.

### Table 1 — Percentage of total recovered glutamate dehydrogenase, acetylcholine esterase and catalase in isolated P. falciparum parasites

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of Recovered enzymes (Parasite/parasite + red cells × 100)</th>
</tr>
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<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>84.30 ± 9.54</td>
</tr>
<tr>
<td>Acetylcholine esterase</td>
<td>1.42 ± 0.26</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.97 ± 0.32</td>
</tr>
</tbody>
</table>

### Table 2 — Specificity of AchE and PKC-like activity in RBCs and malarial parasite P. falciparum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AchE activity (μmoles/min/mg)</th>
<th>PKC-like activity (pmoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected RBCs</td>
<td>3.56 ± 0.12</td>
<td>12.93 ± 0.36</td>
</tr>
<tr>
<td>Infected RBCs</td>
<td>3.96 ± 0.38</td>
<td>13.54 ± 1.28</td>
</tr>
<tr>
<td>Parasite cytosol</td>
<td>0.08 ± 0.01</td>
<td>186.48 ± 15.20</td>
</tr>
<tr>
<td>Parasite membrane</td>
<td>0.04 ± 0.01</td>
<td>90.32 ± 6.27</td>
</tr>
</tbody>
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PKC-like activity was determined by histone IIIIs phosphorylation method. The reaction mixture (50 μl) consisted of 50 mM Tris HCl (pH 7.5), 5-100 μM ATP, 10 mM MgCl₂,6H₂O, 100 μM CaCl₂,2H₂O, 5 μg PS, 1 mM DTT, 50 μg histone IIIIs and 0.1 μCi[³²P]-γ-ATP. Reaction was started by adding 50 μl of enzyme (40-60 μg of parasite protein, erythrocyte lysate) and incubated for 3 min, and was stopped by adding 1.0 ml of 20% trichloroacetic acid. The contents were filtered on millipore membrane filters (HA type, 0.45 μm), washed with ethanol and counted in a liquid scintillation counter (Beckman LS 2800). The controls, representing non-specific kinase activity were run simultaneously in the absence of Ca²⁺ and PS and the values subtracted from total activity. PKC-like activity was expressed as pico moles [P³²]-γ-ATP transferred per mg protein per min under the experimental conditions. The effects of staurosporine, polymyxin B and CQ (dissolved in water and appropriate dilutions were used) on inhibition of the PKC-like activity were studied. Inhibition kinetics of CQ was examined by Line-weaver Burk double reciprocal plot, in respect to ATP, histone and PS, respectively.

### Data analysis

Results are expressed as mean ± SD of 6 independent assays. The differential responses of cytosolic and membrane preparations with or without PMA were determined by the method of one-way ANOVA.

### Results

#### Developmental expression of PKC

Of the total PKC-like activity, about 90% was of parasitic origin, and only about 10% could be attributed to the host enzyme activity (Table 2). PKC-like activity was found distributed in membrane and cytosolic fractions [CQ(S) and CQ(R) strains] of...
Fig. 1—PKC-like activity in the developmental stages of *Plasmodium falciparum* [Parasites were homogenized, sonicated and samples were centrifuged at 150000 g for 15 min. The supernatant was then separated and PKC-like activity was determined in the membrane (solid bar) and cytosol (open bar) as described in ‘Materials and Methods’. Values in parentheses indicate the percent of the total PKC-like activity in the membrane fraction of each parasite stage. Data represent mean ± SEM of 5 separate independent experiments]

*P. falciparum*. As the activity was predominantly cytosolic, cytosol was used as a source of the enzyme. The study on the translocation of PKC-like activity was carried out on cytosol and parasite membrane.

Total PKC-like activity was found increased as the parasite matured from ring to schizont stage (Fig. 1). The distribution of the enzyme activity between cytosolic and membrane fractions was compared between different developmental stages. The 82% of the activity was found in the cytosol, while only 18% in the membrane fraction in the ring stages. In contrast, in the schizont stage, 72% and 28% of the activity was found in cytosolic and membrane fractions. Interestingly, in trophozoites, the activity was distributed equally at 50% in each fraction (Fig. 1).

**Redistribution of PKC-like activity**

To determine whether PKC was required for parasite growth and development, the parasites were incubated overnight with 2 μmole/L PMA, and cytosolic and membrane enzyme activities were measured. The dose was sufficient to cause complete down-regulation of PKC. Upon stimulation with PMA, cytosolic PKC-like activity (P<0.001) was translocated to the membrane fraction (Fig. 2). The redistribution of PKC-like activity in the cytosolic and membrane fractions at trophozoite stage was also found to be statistically significant (P< 0.01) and is shown in Fig. 2.

**Characterization of PKC-like activity**

PS-stimulated PKC-like activity was about 40% above the background (Table 3). The activity was independent of Ca²⁺, because the enzyme was stimulated to the same degree with phospholipid...
alone. Addition of PMA to PS, however, increased the activity 2.5-times over the background. Moreover, when PMA was added to extracts (parasite enzyme) containing Ca\(^{2+}\) and PS, the activity was stimulated to about 9-folds. The replacement of Ca\(^{2+}\) with 1 mM EGTA resulted in a marked decrease in the PKC-like activity. The dependence of enzyme activity on Ca\(^{2+}\), PS and phorbol esters strongly suggest that the enzyme activity is of a PKC\(^{22}\).

**Inhibition and kinetic studies**

Polymyxin B and staurosporine inhibited the enzyme activity in a dose-dependent manner (Fig. 3), which further confirmed the presence of PKC-like activity in *P. falciparum* in CQ(S) strain\(^{32,33}\). CQ also inhibited PKC-like activity in a dose-dependent manner (IC\(_{50}\) 45 nM) in CQ(S) strains (Fig. 4), however, no inhibition was found in CQ(R) strains, suggesting a possible role of the enzyme in mechanism of resistance (Fig. 4). Kinetic studies with Lineweaver-Burk plot showed that inhibition of PKC-like activity by CQ was non-competitive with respect to ATP (Fig. 5A), histone (Fig. 5B) and PS (Fig. 5C), implying that CQ did not directly interact with the corresponding binding sites on PKC.

**Discussion**

**PKC-like activity is developmentally regulated**

PKC (a calcium-dependent protein kinase) plays a key role in the regulation of cellular events, including cell growth and proliferation\(^{6,34,35}\). In the present study, the PKC-like activity associated with the cytosol of the *P. falciparum* trophozoites has been characterized. We examined the changes in the cytosolic PKC-like activity during the various stages of the maturation. Earlier\(^ {22}\), the presence of PKC activity was reported in the erythrocytes lysates of *P. falciparum* infected red cells, and it was postulated to be important for the establishment of parasite in the host RBCs. Our results on the presence of PKC-like activity in the parasite are based on the co-factor requirements for PKC activation, namely Ca\(^{2+}\), PS and phorbol esters\(^ {36}\). PKC-like activity was inhibited by the known inhibitors of mammalian PKC, such as staurosporine and polymyxin B\(^ {32,33}\). In addition, the activity was also stimulated with phorbol esters in *P. falciparum*, like in the other cells\(^ {37}\). A decrease of PMA-induced PKC in the cytosol (Fig. 2) suggests that the enzyme is important for the early stages of the parasite development, and may be of functional significance in parasite survival.

The PKC-like activity belonged to CDPKs family, which have been found in plants and protozoa\(^ {23,38}\). Total PKC-like activity was found increased significantly as the parasite matures from the ring to the schizont stage, suggesting it is developmentally-regulated. The results are in agreement with the earlier findings, where lower kinase activity was reported in ring stage, as compared to schizont stage\(^ {18}\). An increased PKC-like activity during maturation may reflect its increased expression during development. Thus, we may speculate that our observation of a lower activity in ring stage may be having a correlation with the proteolytic activity\(^ {18}\). It is, therefore, suggested that the proteolytic processing of kinase protein may be a physiological regulator of the protein kinases, PTK\(^ {17}\) and PKC during the maturation of malarial parasite.
Fig. 5 — Kinetic analysis of CQ inhibition of PKC-like activity in cytosol of *P. falciparum* trophozoites with respect to ATP (A), histone (B) and PS (C). [The final concentrations of CQ in the assay mixture were 0 (---), 25 (■ ■ ■), 50 (○ ○ ○) and 100 (λ — λ) nM, respectively. Each point is average of triplicate determination of 5 independent experiments]

CQ inhibits PKC-like activity

It is noteworthy that CQ inhibited the PKC-like activity only in the CQ(S) strains in a dose-dependent manner, and only very marginally in the CQ(R) strains (Fig. 4). The reason for this observation is not clear. It is possible that PKC may be a site for the action of CQ. It is also quite plausible to speculate the possibility of P-glycoprotein, which is expressed approximately 3-times more in the resistant strains and may be the target of CQ. Such factor(s) may modulate CQ concentrations and affect the metabolic processes of the CQ(S) strains.

Kinetic studies showed that the inhibition of PKC-like activity by CQ was non-competitive with respect to ATP, histone and PS, implying that the drug does not interact directly with their corresponding binding sites. Previous studies have shown that the phorbol esters binding sites are on the regulatory domain of PKC, and specific PKC inhibitors, such as sphingosine and calphostin-C also bind to this domain. In contrast, other PKC inhibitors, such as H-7 do not show a high specificity for PKC. Some oncogenes and oncoproteins have been shown to selectively inhibit PKC. However, whether the inhibition of PKC by CQ plays a role in the pharmacological action, remains to be elucidated. The purification and further characterization of the PKC-like activities in *P. falciparum* may throw the light on the functional aspects of this important set of protein kinases.

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References