Journal of Medical Sciences

ISSN 1682-4474

science alert

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Research Paper

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J. Med. Sci., 7 (4): 565-571
15th May, 2007

A Study of Calcium Dynamics in Infected Erythrocytes of Nigerian Children with *Plasmodium falciparum* Malaria

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This study was carried out to determine ghost membrane Ca^{2+}ATPase activity in the infected erythrocytes as a correlate of intraerythrocytic Ca^{2+} content, parasite maturation and age. In this study, 32 blood samples from children (mean age = 5.8 year) with *P. falciparum* parasitaemia were analyzed microscopically with erythrocytes submitted for ghost membrane preparation by hypotonic lysis and resealing, membrane Ca^{2+}ATPase activity (measured as micromole of inorganic phosphate liberated from ATP substrate per hour) and intracellular calcium content determination by spectrophotometric methods. The infected erythrocytes were observed to harbour parasites at the ring (56.3%), trophozoite+schizont (34.4%) and gametocyte (9.3%) stages of development ($\chi^2 = 11.2$; $p = 0.004$) with geometric mean parasite density of 9786.9, 14064.2 and 8450.5 parasites μL^{-1}, respectively ($p<0.05$). Compared to the control (n = 10, mean age = 6.3 year), a significant ($p<0.05$) 1.9-3.9 folds increase in intracellular calcium ion (0.615 vs. 1.17-2.37 $\mu g\ mL^{-1}$) and 1.4-2.0 folds decrease in erythrocyte ghost membrane Ca^{2+}ATPase (0.648 vs. 0. 317-0.475 U mg protein) were found in the infected erythrocytes. These alterations were further found to increase with parasite maturation independent of age with inverse calcium-Ca^{2+}ATPase relationship ($r = -0.93$; $p<0.05$). The results of this study have revealed lowered erythrocyte membrane calcium pump activity as a contributor to parasite maturation-dependent intracellular calcium elevation in children with *Plasmodium falciparum* malaria

Key words: *Plasmodium falciparum*, infected erythrocytes, erythrocyte membrane ghost, Ca^{2+}ATPase activity, intraerythrocytic Ca^{2+}

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INTRODUCTION

The asexual erythrocytic stage of *Plasmodium falciparum* development heralds the clinical phase of infection, culminating in the death of close to 1 million under five year-old African children who suffer from 1-2% cases of severe malaria annually (Greenwood *et al*., 1991; Luty *et al*., 2000). Unfortunately, severe malaria and associated prognostic markers of poor clinical outcomes have severally been found to emanate from uncomplicated infections that elicit persistence and down regulation of protective immune response due to inadequate or failed interventions (Mockenhaupt *et al*., 2000; Gupta *et al*., 1994). This persistence of parasitaemia has been described as the ability of the primary infection parasite to survive intraerythrocytically, display multiplicity and elicits secondary infections that co-opt new erythrocytes for life cycle propagation (Wasserman, 1990). The mechanisms underlying these pathogenic events are multifactorial.

However, studies have recognized and validated the centrality of calcium as an indispensable cation for invasion of erythrocyte by *P. falciparum* merozoite and subsequent formation of parasitophorous vacuole, growth, development and release after schizogony (Wasserman *et al*., 1999; Kirk *et al*., 1994). Kirk *et al.* (1994), Tanabe *et al*. (1982) and Kramer and Ginsburg (1991) in separate studies have reported continuous increase in calcium permeability of erythrocyte membrane following invasion resulting in 10-20 folds elevation in intraerythrocytic calcium ion (Ca^{2+}) content. This is contrary to the low intracellular calcium kept at or below 100 nM in uninfected erythrocyte, which lacks subcellular organelles, leaks calcium slowly but possesses high membrane Ca^{2+}ATPase activity for maintaining cytosolic calcium homeostasis (Zylinska and Soszynski, 2000).

The elevated free cytosolic Ca^{2+} of infected erythrocyte paves way for calcium siphoning into the parasitophorous vacuole and parasite subcellular organelles: food vacuole, endoplasmic reticulum and acidocalcisomes, where calcium is stored at higher micromolar to millimolar concentrations (Kramer and Ginsburg, 1991; Rohrbach *et al*., 2005) for mobilization and use in regulating parasite-mediated signaling pathways when necessary (Garcia, 1999).

It is therefore not surprising to have agents such as calcium ionophores, calcium channel blockers and calmodulin antagonists eliciting antiplamodial activity via intraerythrocytic growth arrest and blockade of parasite-mediated survival and toxigenic pathways (Scheibel *et al*., 1987; Adovelande *et al*., 1993). While waiting for the exploitation of this understanding to improve malaria control, it is highly imperative to understand to the fullest all the available mechanisms responsible for the loss of calcium homeostasis in *P. falciparum*-infected erythrocytes. So far pathophysiological events such as the emergence of new permeation pathways (NPPs) (Kirk, 2001), disruption of phospholipid bilayer owing to lipid peroxidation (Hsiao *et al*., 1991) and complement mediated membrane perturbation (Pang and Horii, 1998) have been incriminated. However, the roles played by erythrocyte plasma membrane Ca^{2+} ATPase remains unclear.

We hypothesized that since the erythrocyte: a non-excitable terminally differentiated cell, suffers from ATP depletion following *P. falciparum* colonization (Sherman, 1988), membrane Ca^{2+}ATPase activity impairment is possible and could consequently compromise the effluxing of intracellular calcium.

In this regard and because of the enormous health burden of falciparum in Nigerian children (Iwalokun *et al*., 2001, 2004), we decided to examine the activity of the erythrocyte ghost membrane Ca^{2+}ATPase of infected-blood samples of children with *Plasmodium falciparum* malaria. Effect of parasite maturation on enzyme activity and relationships with intraerythrocytic calcium content were also evaluated.

MATERIALS AND METHODS

Blood samples: A total of 32 *Plasmodium falciparum*-infected blood samples were analyzed. They were obtained from children (mean age 5.8 year) presenting with *Plasmodium* falciparum parasitaemia at Massey Street Children Hospital, a major referral pediatric Hospital in Lagos Nigeria. This part of the country is holoendemic for falciparum malaria with perennial transmission mediated by *Anopheles gambiae* complex and high intensity during the raining season March-October (Iwalokun *et al*., 2000). Ten uninfected blood samples from apparently healthy children (mean age = 6.2 year) were used as controls. The children were enrolled based on absence of sickle cell disease and normal blood creatinine and urea levels to eliminate effects of abnormal hemoglobin and renal dysfunction on intraerythrocytic calcium level (Judd *et al*., 2003; Agroyannis *et al*., 2000).

Parasite erythrocytic stage detection and quantitation: Thick and thin smears of each of the malarial blood samples were made on grease free slides, stained with 4% Giemsa stain and viewed microscopically as oil immersion fields to speciate and examine the intraerythrocytic stage of development. The parasites were counted against 200-500 leukocytes in the thick film and Geometric Mean

Parasite Density (GMPD) was determined from parasite density based on 8000 leukocytes per microlitre of blood (Warhurst and Williams, 1996).

Erythrocyte ghost membrane preparation: A modified method of Braun and Fromherz (1997) was adopted for the preparation of ghost erythrocyte membranes of infected and uninfected cells. Citrated (0.39 g%) blood samples were centrifuged at 3500 rpm for 10 min at 25°C to remove plasma and buffy coat. The packed red blood cells were washed thrice with 5 volumes of 50 mM Tris-HCl buffer. (pH 7.4). The washed cells were then suspended in equal volume of the same buffer on ice for 4 h. Erythrocyte suspension (0.5 mL) was lysed by the addition of 15 mL of lysing solution (301 mg $MgSO_4$, 312 mg KCl in 500 mL of distilled water). This was immediately followed by the addition of 1 mL of resealing solution (53.7 g KCl+10.5 g NaCl in 400 mL of distilled water). The tubes were further kept on ice for 5 min and at 37°C for 30 min. The resulting white erythrocyte ghost was finally pelleted by centrifugation at 3500 rpm at 25°C for 10 min.

Ghost membrane protein determination: The ghost membranes of Plasmodium-infected and uninfected erythrocytes were analyzed spectrophotometrically for total protein content according to Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard (50-2500 μg).

Sorbitol lysing assay and intracellular calcium determination: Methods adopted by Wagner *et al.* (2003) and Krasilnikov *et al.* (1997) with little modifications were used for erythrocyte suspension preparation and lysis. Briefly, fresh blood sample was mixed with 20 volumes of isotonic standard physiological solution (SPS, 150 mM NaCl, 5 mM Tris citrate, 1% EDTA, pH 7.4) and centrifuged at 1500 g for 5 min. The supernatant was removed and the erythrocyte sediment was washed three times with the same buffer, centrifuged and re-suspended in EDTA-free SPS to a final hematocrit of 10% using a capillary hematocrit centrifuge. An aliquot of each erythrocyte suspensions (30 μL) was then subjected to lysis by adding 1.5 mL of pre-warmed isotonic sorbitol buffer (280 mM sorbitol, SPS, pH 7.4) at 37°C in a 1 cm cuvette. Since uninfected erythrocyte is non-susceptible to osmotic swelling and lysis (Bowman and Lwitt, 1977) induced by sorbitol in plasmodium infected erythrocytes, we monitored hemolysis spectrophotometrically as decrease in absorbance compared to the controls (uninfected erythrocytes) at 660 nm over 20 min at 4 min intervals. Fifty percent lysis time T_{50} was defined as time to 50% decrease in absorbance of infected erythrocyte suspension subjected to sorbitol lysis.

In another experiment, both infected and uninfected erythrocyte suspension were subjected to hypotonic lysis by adding 10 volumes of buffered ammonium chloride solution (0.83% NH_4Cl, 5 mM Tris citrate, pH 7.4) at 37°C for 20 min. The resulting haemolysate was centrifuged at 1500 g for 5 min and the supernatant collected into separate sterile bottles for colorimetric determination of calcium according to Lorentz (1982).

Calcium ATPase assay: The enzyme assay was a 1 mL reaction volume containing 500 μg protein of the ghost membrane preparation and enzyme reagent containing 1.0 mM $CaCl_2$, 2.5 mM $MgCl_2$ with or without trisodium vanadate (0.15 mM) in 25 mM Tris-HCl (pH, 7.4). The resulting mixture was equilibrated at 37°C for 10 min. The reaction was started by addition of freshly prepared disodium-ATP to a final concentration of 1.0 mM followed by incubation at 37°C for 1 h. The reaction was stopped by adding 500 μL of 10% SDS.

The concentration of inorganic phosphate released was determined according to Fiske and Subbarow (1925). Enzyme activity was measured as the micromoles of Pi released per minute per mg protein. The true Ca^{2+}ATPase activity was calculated from the difference in activity in the absence and presence of Vanadate (Carafoli and Cromption, 1978).

Statistical analysis: Data were expressed as Mean+standard Error of Mean (SEM) and analyzed by one-way analysis of variance (ANOVA). Investigation of parasite density and maturation as predictors of altered membrane Ca^{2+}-ATPase activity was evaluated by regression analysis at 95% confidence limits. Probability p-value <0.05 was regarded as significant.

RESULTS

The results presented in Table 1 show that 18 (56.3%), 11 (34.4%) and 3 (9.7%) of the infected blood samples examined harbored *P. falciparum* parasites at the ring, trophozoites/schizonts and gametocyte stages of development. Geometric Mean Parasite Density (GMPD) determination further revealed highest (17400.4±2215.7 parasites μL^{-1}) and lowest (8450.5±543.2 parasites μL^{-1}) parasitaemia in the parasite trophozoites/schizont and gametocyte stages, respectively. While the ring stage parasites produced intermediate parasitaemia of 9786.9±717.6 parasites μL^{-1}. The observed disparity in GMPDs between the three stratified stages of parasite development was found to be significant ($p<0.05$).

Treatment of the erythrocytes with sorbitol under iso-osmotic condition revealed a reduction in T_{50} sorbitol lysis time in infected erythrocytes (3.6-5.6±0.04-0.1 min)

Table 1: Levels of parasitaemia and lysis time of infected erythrocytes at various developmental stages of *Plasmodium falciparum*

Parameter	Control	Parasite developmental stages: Ring	T+S	Gametocytes
N (%)	10.0 (100)	18 (56.3)	11 (34.4)	3 (9.3)
†GMPD	-	9786.9±717.6^a	17400.4±2215.7^b	8450.5±543.2^c
‡T_{50} (min)	7.8±0.3^a	5.6±0.1^b	4.1±0.1^c	3.6±0.04^d

N (%) represents number and percentage of uninfected and *P. falciparum*-infected blood samples harbouring ring, trophozoites/schizont (T+S) and gametocytes stages of parasite development. †Parasitaemia was measured as geometric mean parasite density±SEM of N samples in duplicate determinations. ‡Time to 50% transmittance value of erythrocytes (N≥3) lysed with sorbitol in duplicate determinations. Different superscripted letters along the same horizontal line indicate significance ($p<0.05$) (ANOVA)

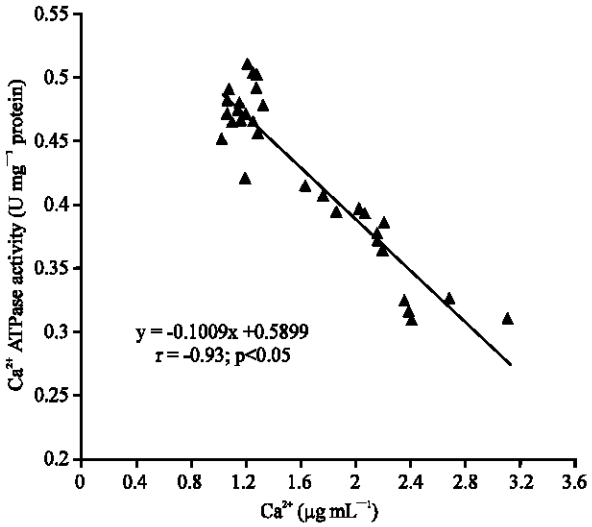

Fig. 1: Relationship between intraerythrocytic free calacium ion level and erythrocyte ghost membrane Ca^{2+} ATPase activity. Each determination was done in duplicates and association was expressed by an equation and correlation coefficient (r)

compared to the control (7.8±0.3 min). The increasing order of fragility: gametocytes (3.6±0.04 min)> trophozoite/schizont (4.1±0.1 min) > ring (5.6±0.1 min) was further found to be significant ($p<0.05$).

The level of intracellular free calcium ion was found to correlate inversely with Ca^{2+}-ATPase activity ($r = -0.93$; $p<0.05$) (Fig. 1). Further stratification of intracellular calcium by stage of erythrocytic development showed that Ca^{2+} concentration increased significantly ($p<0.05$) with maturation from the ring stage (1.17±0.02 µg mL^{-1}) through the trophozoite and schizont stages (2.16±0.09 µg mL^{-1}) to gametocyte (2.37±0.01 µg mL^{-1}) stage of development. The corresponding values for Ca^{2+}ATPase activity were 0.475±0.01, 0.377±0.07 and 0.317±0.01 U mg^{-1} protein, respectively (Table 2). On whole, significantly ($p<0.05$) lower calcium content

Table 2: Comparison of intracellular calcium concentration and calcium ATPase activity in infected erythrocytes at various developmental stages of *Plasmodium falciparum*

Blood sample@	Calcium (µg mL^{-1})	Change* (fold)	Calcium ATPase (U mg^{-1} protein)	Change* (Fold)
Control	0.615±0.01	1	0.648±0.03	1
Ring	1.17±0.02^a	+1.9	0.475±0.01^a	-1.4
T+S	2.16±0.09^a	+3.5	0.377±0.007^a	-1.7
Gametocyte	2.37±0.01^a	+3.9	0.317±0.01^a	-2.0

Data are presented as mean±SEM and analysed by one-way analysis of variance (ANOVA). @Data were obtained for uninfected blood samples (control) and infected samples at various stages of parasite development. T+S = Trophozoite/Schizont stage. Change is indicated by fold increase (+) or decrease (-) in measured parameters relative to the control. One unit (1U) = 1 micromole of inorganic phosphate (Pi) liberated per 1h under the assay conditions. $p<0.05$ indicates significance at 95% confidence limits. *$p<0.05$ compared to the control

Table 3: Regression analysis of age and parasite development as determinants of reduced erythrocyte ghost Ca^{2+}ATPase activity in children with *Plasmodium falciparum* malaria

Parameter	Mean@	Constant	r	SEM	p-value
Age (year)	6.1	15410	-0.22	0.5	0.09
Infected erythrocyte (GMPD) Ring	9786.9	0.6165	-0.85	1037.5	0.003
T+S	14064.2	0.6258	-0.88	2135.2	0.002
Gametocyte	8450.5	0.3560	-0.38	1325.1	0.07

@Mean value of parameters with *P. falciparum* parasitaemia (n≥3), n = number of samples

(0.615±0.01 µg mL^{-1}) but higher membrane pump activity (0.648±0.03 U mg^{-1} protein) were observed in uninfected erythrocyte control. While the observed 1.7-2.0 fold reduction in Ca-pump activity corresponded to 1.9-3.9 fold increment in intracellular calcium as the parasite developed to gametocytes (Table 2).

Multivariate regression analysis revealed age as a non-significant correlate of reduced calcium pump activity in the infected erythrocyte but showed that Ca^{2+} ATPase reduction improves with the stage of parasite intraerythrocytic development (Table 3).

DISCUSSION

In the present study we found a consistently higher intraerythrocytic free calcium ion level in the infected erythrocytes of children with *Plasmodium falciparum* malaria compared to level in apparently healthy controls. Our observation supports previous works that *Plasmodium falciparum* induces increased calcium permeability and subsequently intracellular free calcium ion following invasion of the erythrocytes (Wasserman, 1990; Garcia *et al*., 1996). However, disparity exists regarding the magnitude of intraerythrocytic Ca^{2+} elevation observed in this study. For instance, our observed 1.9-3.9 folds are greater than 0.3 folds increase observed by Wasserman *et al.* (1999), falls within the 2 fold elevation observed *in vitro* at schizont stage of

development by Adovelande *et al.* (1993) but lower than 10-20 folds increase observed independently by Tanabe *et al.* (1982) in *P. chabaudi* schizonts and gametocytes in mice and Kramer and Ginsburg (1991) in *P. falciparum* infected erythrocytes. While parasite and experimental types are most likely causes for this disparity, the method for calcium ion estimation may also be a factor. In most of the previous studies, intraerythrocytic free calcium ion was estimated using atomic absorption spectroscopy, fluorometry and laser scanning microscopy with calcium indicators (Wasserman, 1990; Tanabe *et al.*, 1982; Rohrbach *et al.*, 2005) contrary to the spectrophotometric methods used in this study. In addition, analysis has also been based on parasite culture system at regulated cell density as against 10% infected erythrocyte suspension used in this work. Aside malaria, 2 and 4 fold increases in intraerythrocytic calcium was observed by Corry *et al.* (1996) using fluorometry and Agroyannis *et al.* (2002) using AAS for calcium ion estimation in uraemic patients. That the host erythrocytes exhibit increase membrane permeability is further supported by the results of our sorbitol lysis experiment where lower T_{50} values of 3.6-5.6 min in infected erythrocyte suspension compared to 7.8 min in uninfected erythrocytes were observed. The latter has been reported to exhibit sorbitol impermeability (Bowman and Lwitt, 1977; Kirk *et al.*, 1993), while in the former complete lysis within 15 min and between 10-12 min have been reported (Wagner *et al.*, 2003; Go *et al.*, 2004). Wagner *et al.* (2003) further attributed this increased erythrocyte permeability to the presence of an anion channel called plasmodial erythrocyte anion channel (PESAC) within 18 h of infection and with a density of 1000-2000 further copies per cell reported by Desai *et al.* (2000).

Furthermore, we assayed for Ca^{2+}ATPase activity in the ghost membrane of the infected erythrocyte and found a marked reduction in pump activity compared to the control coupled with inverse relationship with the elevated free calcium ion level. The present result thus provides a support for reduced calcium pump activity as a contributing factor to the elevated calcium ion level and loss of intracellular calcium homeostasis in *Plasmodium falciparum* infected erythrocytes. However, it disagrees with the finding of Tiffert *et al.* (2000) who reported non-impairment of calcium pump activity in terms of vmax. But aligns with the work of Uyemura *et al.* (2000) who reported a 20-30% reduction in pump activity in *Plasmodium berghei*-infected mice erythrocytes. Meanwhile, in pathologies where elevated erythrocytic calcium level has been observed, reduced calcium pump activity has consistently been found as the underlying mechanism (Agroyannis *et al.*, 2000).

However, the observation made by Tiffert *et al.* (2000) may also be a possibility considering the fact that erythrocyte Ca^{2+}ATPase elicits improvement in Ca^{2+} affinity in the presence of calmodulin (Downes and Mitchell, 1982), which has been found to increase with parasite maturation (Scheibel *et al.*, 1987) and which is similar to our observed pattern of intraerythrocytic calcium. Unfortunately, biological processes such as raised erythrocyte osmotic fragility and elevated nitric oxide levels (Iwalokun, 2004, 2005), membrane lipid peroxidation and oxidative stress (Hsiao *et al.*, 1991) observed as crucial events in the pathogenesis of malaria have been found as factors responsible for decline Ca^{2+}ATPase activity in pathologies where intraerythrocytic calcium is elevated significantly beyond the physiological limit of < 100 nM (Agroyannis *et al.*, 2000, 2002).

We observed the level of parasitaemia, to increase with parasite development except in the schizont stage to suggest that the observed elevated intraerythrocytic calcium and associated Ca pump depletion may be influenced by parasite density. However, disparity observed at the gametocyte stage may be due to the small size of the gametocytes harbouring samples analyzed and the disproportionately higher GMPD in the T+S group may be due to higher parasitaemia (≥40, 000 parasites μL^{-1} observed in 3 of the positive samples (results not shown). Meanwhile, in support of our present observations, Prabha *et al.* (1998) and Davis *et al.* (1998) had previously reported hypocalcaemia in acute malaria and inverse relationship between calcium level and parasite load.

Furthermore, the observed non-significant correlation with respect to age of children from which infected erythrocytes were collected suggests that the altered calcium-Ca^{2+}ATPase level found in this study is age independent. This is contrary to the reported influence of erythrocyte age on choline transport (Kirk *et al.*, 1992).

While our observation that Ca^{2+}ATPase activity declines 1.4-2.0 folds with parasite maturation with infected erythrocyte may provide a reason for the observed elevated intraerythrocytic calcium, it also indicates the possibility of other contributing factors because of fold disparity. The parasite mediated anion selective new permeation pathways (NPPs) (Kirk, 2001) have consistently been reported contribute to intraerythrocytic elevation of calcium, a signaling molecule, which not only mediates parasite invasion, growth and development but also evoke shape abnormality, morphological derangement and commit the infected erythrocyte to lysis and death eventualities.

In line with the results of this study, it can be concluded that the asexual development of *Plasmodium falciparum* in the erythrocyte in children with acute uncomplicated malaria is associated with reduced membrane calcium-pump activity in relation to raised intracellular calcium according to the parasite stage of development. This does not exclude the contribution of other membrane transport pathways evoked by the parasite.

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