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Selective internalization of choline-phospholipids in *Plasmodium falciparum* parasitized human erythrocytes

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We have incubated control and *Plasmodium falciparum* parasitized human erythrocytes with lipid vesicles containing radiolabeled long-chain phosphatidylcholine and sphingomyelin, in the presence of a nonspecific lipid transfer protein. Most of the radiolabeled phospholipids were, immediately thereafter, available for extracellular phospholipases, suggesting that uptake of vesicles as such did not occur. In time, the amount of phosphatidylcholine inserted in the outer leaflet of the host cell membrane of parasitized erythrocytes decreased, indicating that phosphatidylcholine was being internalized in parasitized erythrocytes. The exclusion of sphingomyelin from the internalization process suggests that the removal of phosphatidylcholine from the outer leaflet of the erythrocyte membrane is caused by transbilayer migration, rather than by endocytosis. The extent of phosphatidylcholine internalization indicates that part of it does not remain in the inner leaflet of the host cell membrane, but is taken up by the intraerythrocytic parasite. Individual phosphatidylcholine species, containing 16:0/18:1-, 16:0/18:2- and 16:0/20:4-fatty acids, showed similar extents of internalization, after being incorporated in parasitized erythrocytes by a phosphatidylcholine specific transfer protein.

Introduction

During its intraerythrocytic development, the malaria parasite, *Plasmodium sp*, induces several biochemical and biophysical changes in the host cell membrane [1–4]. These alterations are apparently needed for the traffic of large amounts of nutrients, e.g. lipids, required by the growing parasite, as well as for the passage of waste products resulting from its active metabolism. The parasite can synthesize phospholipids de novo [5], be it that fatty acids have to be imported [6]. In addition to de novo synthesis, direct import of intact phospholipids seems to occur [5,7]. The parasite itself can alter the phospholipid molecular species composition of the host cell membrane [4], but not its phospholipid polar head group composition and transbilayer distribution [8,9]. Therefore, an intensive phospholipid trafficking between host cell membrane and intraerythrocytic parasite is suspected, though its pathways are unknown.

In this work, we have incorporated radiolabeled sphingomyelin (SM) and phosphatidylcholine (PC) in the outer leaflet of the host cell membrane of *Plasmodium falciparum* parasitized human erythrocytes, and studied their internalization. The implications of our results for the phospholipid trafficking within *P. falciparum* parasitized human erythrocytes are discussed.

Materials and Methods

Materials. Human blood and AB^+ human serum were obtained from the local blood bank. RPMI medium 1640 was purchased from Gibco (Grand Island, NY, U.S.A.). Hepes and $NaHCO_3$ (analytical grade) were purchased from Merck (Darmstadt, F.R.G.). Percoll was obtained from Pharmacia (Uppsala, Sweden). L-3-Phosphatidylcholine,1,2-di[1-^{14}C]palmitoyl, L-3-phosphatidylcholine,1-palmitoyl-2-[1-^{14}C]oleoyl, L-3-phos-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; nsLTP, nonspecific lipid transfer protein; Pal-AMPA, palmitoyl-lysine^{116}-ε-amidinated porcine pancreatic phospholipase A_2; PC, phosphatidylcholine; PCTP, phosphatidylcholine specific transfer protein; PE, phosphatidylethanolamine; SM, sphingomyelin; TLC, thin-layer chromatography.

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phatidylcholine,1-palmitoyl-2-[1-^{14}C]linoleoyl, [*N-methyl*-^{14}C]sphingomyelin and [1α,2α(n)-^{3}H]cholesteryl ether were purchased from Amersham International (Buckinghamshire, U.K.). L-3-Phosphatidylcholine,1-palmitoyl-2-[1-^{14}C]arachidonyl was from New England Nuclear (Boston, MA, U.S.A.). Egg PC, egg phosphatidic acid and cholesterol were obtained from Sigma (St. Louis, MO, U.S.A.). Nonspecific lipid transfer protein was purified as described by Crain and Zilversmit [10], with some alterations [11]. Phosphatidylcholine specific transfer protein was isolated as previously described [12]. Sphingomyelinase C was purified from *Staphylococcus aureus* cultures [13]. Palmitoyl-lysine^{116}-ε-amidinated porcine pancreatic phospholipase A_2 (Pal-AMPA) was prepared as previously described [14].

Production and isolation of P. falciparum parasitized human erythrocytes. The Nigerian strain of *P. falciparum* was cultivated using the Petri dish-candle jar method [15] without synchronization. Parasites were grown in erythrocytes, which were kept at a 7% hematocrit in RPMI medium 1640, supplemented with 25 mM Hepes, 24 mM $NaHCO_3$ and 10% (v/v) serum. When the parasitemia (% of the total erythrocytes which are parasitized) was between 10 and 15%, the cells were collected. Unparasitized (used as control) and parasitized erythrocytes were separated on a Percoll gradient [16], and washed four times with the adequate buffer. The Percoll gradient allows the isolation of a fraction of erythrocytes with 100% parasitemia.

Characterization of phospholipid classes composition. The lipids were extracted from both types of cells [17], separated by two-dimensional TLC [18], and the amount of phospholipid present in each spot determined [19].

Incorporation of trace amounts of radiolabeled phosphatidylcholine and sphingomyelin in the erythrocyte membrane. Radiolabeled PC and SM were incorporated by incubating donor vesicles with the cells at 7% hematocrit in 280 mM sucrose, 10 mM NaCl, 20 mM glucose, 1 mM EDTA, 10 mM Tris (pH 7.4) (sucrose buffer), in a shaking water bath at 37°C, for 15 min, in the absence and in the presence of the nonspecific lipid transfer protein (nsLTP, 0.147 mg/100 μl of packed cells). Donor vesicles were prepared by ultrasonic dispersion of PC/phosphatidic acid/cholesterol/SM (10:1:10:0.6, mol/mol) in the sucrose buffer. The PC fraction in these vesicles was egg PC, plus trace amounts of L-3-phosphatidylcholine,1-palmitoyl-2-[1-^{14}C]linoleoyl, (specific activity 1.2 mCi/mol); the specific activity of SM was 58 mCi/mmol. The ratio of erythrocyte membrane phospholipid to vesicle phospholipid was kept close to 10. Lipid transfer was stopped by washing the cells three times with 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM Tris (pH 7.4) (NaCl buffer).

Incorporation of trace amounts of radiolabeled 16:0/16:0-, 16:0/18:1-, 16:0/18:2- and 16:0/20:4-phosphatidylcholines in the erythrocyte membrane. Incorporation of the radiolabeled PC(s) was performed by incubating the cells (10–15% hematocrit in the NaCl buffer) for 2 h (unless otherwise indicated) in a shaking water bath at 37°C, in the absence and in the presence of 4 μM PC specific transfer protein (PCTP). The lipid donor system consisted of sonicated lipid vesicles [20] containing PC/phosphatidic acid/cholesterol/cholesteryl ether (10:1:10:0.01, mol/mol). The PC in these vesicles was egg PC to which trace amounts of various radiolabeled PC species were added to obtain a final specific activity of 0.25 mCi/mmol; the specific activity of radiolabeled cholesteryl ether was 58 mCi/mmol. The ratio of erythrocyte membrane PC to vesicle PC was kept close to 0.1. Lipid transfer was stopped by washing the cells three times with NaCl buffer.

Determination of the internalization of the radiolabeled phospholipids incorporated in the outer leaflet of the erythrocyte membrane. In those experiments where radiolabeled PC and SM had been incorporated in the presence of nsLTP, the cell suspension was kept in the NaCl buffer (2% hematocrit) to allow the newly introduced lipids to equilibrate over both membrane leaflets. Samples were removed at timed intervals and exposed to the action of phospholipases for 5 min in a shaking water bath at 37°C. Two phospholipases were independently used, namely a modified phospholipase A_2 (Pal-AMPA, 0.5 IU/10 μl packed cells), and sphingomyelinase C (0.5 IU/10 μl packed cells). When different molecular species of radiolabeled PC had been incorporated, the cells were immediately incubated with Pal-AMPA, after the removal of PCTP and donor vesicles. After inhibition of phospholipase action by addition of an excess of EDTA, cells were pelleted and the lipids extracted [21], separated by TLC [18] and the radioactivity present in the different lipid spots quantified in a Packard Tri-Carb 1500 Liquid Scintillation Analyzer. In those experiments where a combination of different molecular species of radiolabeled PC had been used, the PC fraction was extracted from the TLC plates, treated with phospholipase C and, after converting the diacylglycerols into their benzoyl-derivatives, separated by HPLC as previously described [4]. Radioactivity was determined in the corresponding fractions.

Presentation of data. All results are the means of three or four experiments, ± standard deviation (in the figures indicated by vertical bars).

Results

Table I shows the phospholipid classes composition of control and parasitized human erythrocytes. It is apparent that the relative amounts of PC and phosphatidylethanolamine (PE) are augmented in parasitized erythrocytes, whereas the relative content of SM is reduced to approximately 1/3 of its original value.

TABLE I

Phospholipid classes composition of control and P. falciparum parasitized human erythrocytes

Phospholipid	Control erythrocytes (%)	*P. falciparum* parasitized erythrocytes (%)
PC	30.6 ± 1.2	45.1 ± 1.2
SM	26.4 ± 0.7	8.3 ± 1.6
PE	26.1 ± 1.0	34.7 ± 2.0
PS + PI	11.4 ± 2.6	8.5 ± 0.6
PA	3.9 ± 0.6	1.7 ± 0.3
LPC	1.6 ± 0.6	1.6 ± 0.6

Since the absolute phospholipid content of the erythrocytes increases 2–5-times upon development of the parasite in their cytosol [1], the data shown in Table I probably indicate that, similar to what has been observed in the case of *P. knowlesi* [8], *P. falciparum* is enriched in PC and PE, and has little or no SM.

The major goal of our study was to compare the internalization of exogenously supplied long-chain PC and SM. As shown in Table II, some spontaneous movement of radiolabeled lipids from the vesicles to the cells occurred when the cells were incubated in the absence of the transfer protein. Since, in control cells, the transfer of PC was higher than that of SM, this spontaneous lipid transfer cannot be explained by sticking of the vesicles to the erythrocytes only; a possible alternative explanation would be a collision-mediated monomer transfer [22]. Parasitized erythrocytes spontaneously incorporated more radiolabeled PC and SM than control cells do. Since the PC/SM ratio of the incorporated material is similar to that in the vesicles, this may result from sticking of entire vesicles to parasitized erythrocytes, or, alternatively, from an easier insertion of SM (and PC) into this membrane, which is known to exhibit a loosened lipid packing [23]. After washing away the vesicles and the nsLTP, most (90% in the case of sphingomyelin) of the incorporated radiolabeled probes was available for extracellular phospholipases in both types of erythrocytes, indicating that no uptake of entire vesicles as such occurred.

TABLE II

Spontaneous and nonspecific lipid transfer protein-mediated incorporation of phosphatidylcholine and sphingomyelin in control and P. falciparum parasitized human erythrocytes

Erythrocytes/ type of incorporation	Phospholipid incorporated (nmol/45 μl packed cells)	
	phosphatidylcholine	sphingomyelin
Control/spontaneous	0.13 ± 0.02	0.004 ± 0.003
Parasitized/spontaneous	0.25 ± 0.13	0.02 ± 0.01
Control/protein-mediated	1.87 ± 0.55	0.04 ± 0.01
Parasitized/protein-mediated	1.97 ± 0.83	0.04 ± 0.01

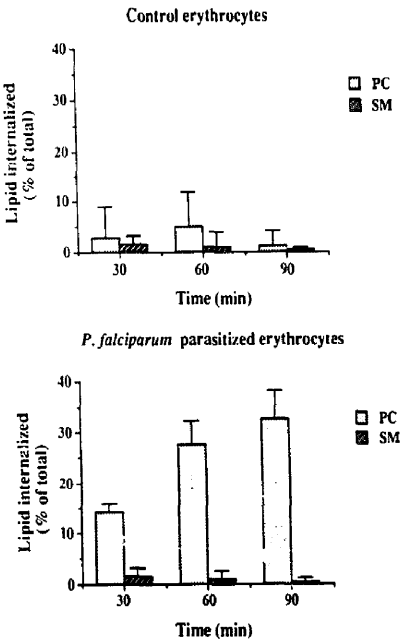

Fig. 1. Internalization of phosphatidylcholine and sphingomyelin (% of total) inserted in the outer leaflet of the erythrocytic membrane of control and of *P. falciparum* parasitized human erythrocytes. Percentage internalized lipid represents that fraction of radiolabeled PC (or SM) initially available to extracellular Pal-AMPA (or sphingomyelinase) that could not be degraded any more at 30, 60 and 90 min after insertion of the probe molecules in the outer membrane leaflet of control or parasitized cells. Initially, 60% of the incorporated radiolabeled PC and 90% of the incorporated radiolabeled SM were available for extracellular hydrolysis, in both types of cells. Lysis was below 15% in all cases and comparable during the incubations with Pal-AMPA and sphingomyelinase.

Fig. 1 shows that, once inserted in the outer membrane leaflet of parasitized human erythrocytes, PC, but not SM, moves inwards in a time-dependent way. This is evidenced by a time-dependent decrease in the pool of radiolabeled PC that is accessible to the exogenous Pal-AMPA, under non-lytic conditions. Essentially all the newly inserted radiolabeled SM remained accessible to sphingomyelinase, even after incubation of the cells for up to 90 min (see Fig. 1). It was not possible to study the internalization of the phospholipids during a period longer than 90 min, because the parasitized cells showed excessive lysis when exposed to phospholipases beyond that time-point. Alternatively, in some experiments the cells were kept under the conditions used for the transbilayer equilibration of the phospholipids for up to 7 h. Degradation of the incorporated radiolabeled SM by an endogenous sphingomyelinase, that might eventually be present, could not be observed. Since the parasite has little or no SM, this suggests that the SM that had been incorporated in the host cell membrane had not been transferred to the parasite and, conse-

quently, must have stayed behind in that membrane. It is important to note that, during the experiments, the radiolabeled PC and SM remained fully intact; neither their degradation, nor their conversion in any other phospholipid could be detected.

PCTP was used to incorporate four different species of radiolabeled PC into the outer leaflet of the erythrocyte membrane, because this protein promotes a one for one exchange of PC molecules, leaving the other characteristics of this membrane unaltered [24]. Fig. 2 shows the time-course for spontaneous and PCTP-mediated incorporation of radiolabeled 16:0/16:0-, 16:0/18:1-, 16:0/18:2- and 16:0/20:4-PC in control and in parasitized human erythrocytes. Radiolabeled cholesteryl ether was included in these lipid vesicles (see Methods), as a non-exchangeable marker, in order to monitor the extent of contamination of erythrocytes by vesicles which could not be removed during the washing procedure [25]. The contamination that could be calculated this way, coincided with the spontaneous uptake of radiolabeled PC by both control and parasitized erythrocytes, and did not increase with time. This suggests that the spontaneous uptake is probably due to sticking of vesicles to the cells, rather than to some kind of endocytosis of the whole vesicles. In both types of cells, the PCTP-mediated radiolabeled PC incorporation increased with the degree of unsaturation of the PC species used, in agreement with previous data obtained with normal human erythrocytes [25]. The high amounts

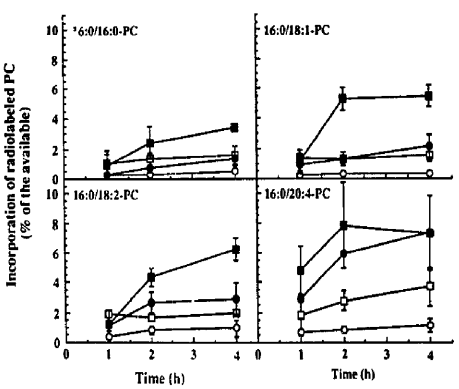

Fig. 2. Spontaneous and phosphatidylcholine specific transfer protein-mediated incorporation of several radiolabeled phosphatidylcholine molecular species (% of the amount available in the medium) in control and *P. falciparum* parasitized human erythrocytes. (○) spontaneous transfer towards control; (●) PCTP-mediated transfer towards control; (□) spontaneous transfer towards parasitized erythrocytes; (■) PCTP-mediated transfer towards parasitized erythrocytes. Taking 16:0/18:1-PC as representative for bulk PC in the vesicles [26], the amount of the PC transferred towards the erythrocytes, after 4 h incubation in the presence of PCTP, corresponded approximately to 20 and 50% of the PC present in the erythrocyte membrane of control and parasitized erythrocytes, respectively. Lysis was below 10% in all cases.

TABLE III

Internalization of 16:0/18:1-, 16:0/18:2- and 16:0/20:4-phosphatidylcholine inserted in the outer leaflet of the erythrocytic membrane of control and P. falciparum parasitized human erythrocytes

The various radiolabeled PC molecular species were incorporated in the outer leaflet of the erythrocyte membrane, as described under Methods. The data are presented as % of the amount of each species that was incorporated and that could not be degraded by extracellular Pal-AMPA. Cell lysis was in all cases below 15%.

Radiolabeled PC species	Radiolabeled PC inaccessible for hydrolysis in	
	P. falciparum parasitized erythrocytes (%)	control erythrocytes (%)
16:0/18:1	60±8	23±6
16:0/18:2	49±3	16±8
16:0/20:4	55±7	26±6

of PC that could be transferred to the host cell membrane of parasitized cells by PCTP, might be an indication of the weakened intermolecular interactions in the erythrocyte membrane of parasitized cells when compared to those in normal erythrocytes.

Because parasitized cells turned out to become very fragile during the long term incubations that are required for the PCTP mediated insertion of the PC species, they could not be subjected to subsequent incubations to study the time-dependence of the transbilayer reequilibration of those species. Alternatively, we incubated the erythrocytes with Pal-AMPA, immediately after a 2 h incubation with vesicles (containing the four types of radiolabeled species) plus PCTP, and subsequent washes. It appeared that under our experimental conditions, the incorporated 16:0/16:0-PC is not easily accessible for Pal-AMPA, similarly to what has been reported in the case of *Naja naja* phospholipase A_2 [26] and the results with this particular species are not therefore shown here. In control erythrocytes Pal-AMPA was able to hydrolyze approximately 80% of the other (saturated/unsaturated) species of radiolabeled PC (Table III). It has been previously shown, under experimental conditions close to those that we have used in our present study, that the radiolabeled PC newly inserted in the normal human erythrocyte is located in its outer leaflet, immediately after the incubation with PCTP [27]. The fact that we could not hydrolyze all the radiolabeled PC inserted in control erythrocytes, immediately after the incubation with PCTP and subsequent washes, is probably related with the differences in the enzymes used to assess the sidedness of PC localization in both studies. In the previous studies, a combination of *Naja naja* phospholipase A_2 and sphingomyelinase C was used, which provides an essentially complete hydrolysis of all the PC present in the outer leaflet of the erythrocyte, under nonlytic conditions [27]. The above mentioned fragile character of the parasitized erythrocytes precluded the

use of such a drastic method. Therefore, we have used Pal-AMPA because its superior membrane penetrating properties allow an extremely rapid, though not complete, hydrolysis of its substrates. In parasitized cells, these species of radiolabeled PC showed much higher degrees of protection from hydrolysis (Table III). The three species seemed to be equally protected, a small but statistically significant difference ($p < 0.05$) being detected only between 16:0/18:1- and 16:0/18:2-PC. Our data suggest that in parasitized erythrocytes radiolabeled 16:0/18:1-, 16:0/18:2- and 16:0/20:4-PC had already been partially internalized by the time cells were incubated with Pal-AMPA.

Discussion

Several possibilities can be envisaged for the transport of phospholipids from the host cell membrane towards the intraerythrocytic Plasmodium and vice-versa, namely a (more or less) permanent membrane connection, endocytosis, and/or protein- or vesicle-mediated transfer. We have studied the internalization of choline-containing phospholipids in those cells. The choline-containing phospholipids were chosen because they are the most abundant in the outer leaflet of the erythrocyte membrane of both control and parasitized erythrocytes [8,9,28], and because of the fact that – during parasite development – the relative amount of PC in the parasitized cell increases while that of SM decreases. Our results show that PC is internalized in parasitized cells, under conditions where it remains in the outer membrane leaflet in control erythrocytes. Since uptake of entire vesicles is highly unlikely (see above) and SM is apparently excluded from the internalization process, we have to postulate a transfer of PC (and SM) from the vesicles towards the outer leaflet of the host cell membrane, followed by a fast transbilayer movement specific for PC. An enhanced flip-flop of all phospholipid classes, including SM, in the host cell membrane of parasitized erythrocytes has already been reported in a study on *P. knowlesi* parasitized monkey erythrocytes, involving spin-labeled phospholipids [29]. However, this type of probes possess physico-chemical properties which differ markedly from those of natural (long chain) phospholipids, and that might account for the disparity between their results and ours. In another study [30], a fluorescent analog of PC was used, which showed that its transbilayer movement was higher in *P. falciparum* parasitized cells than in controls. It also appeared that this process might be protein-mediated and energy-dependent. A protein, synthesized by the parasite and inserted in the host cell membrane, that is capable of promoting PC transbilayer movement, could well account for our results. A similar function has already been attributed to a protein present in endoplasmatic reticulum [31].

Once present in the inner leaflet of the erythrocyte membrane, the PC molecules might eventually become accessible for cytosolic lipid transfer proteins (of parasitic origin) that would transport them towards the parasite. Alternatively, the physico-chemical properties of the inner leaflet phospholipids, which can easily form hexagonal phases [32], might favour the exchange of lipids between cytosolic vesicles and the inner membrane leaflet. The technique we have used does not allow us to directly determine whether the internalized phospholipid ends up in the inner leaflet of the host cell membrane or in the intraerythrocytic parasite. However, it is known that PC is distributed asymmetrically over the two leaflets of the erythrocyte membrane in both control and parasitized cells, only 25% of it in the inner leaflet [28,9]. Therefore, the fact that in our experiments more than 25% of the incorporated radiolabeled PC was found to be internalized, can be taken to indicate that an appreciable fraction of it ended up in the parasite. *P. knowlesi* is able to alter the PC and PE molecular species composition of its host erythrocyte, and an equilibrium seems to exist between the PC and PE pools in the host cell membrane and those in the parasite [4]. Our results, which reveal an increase in the internalization of PC, corroborate this suggestion. Since marked differences in flip-flop rates among different molecular species of PC are observed in the membrane of the healthy erythrocyte [27], it is of particular interest to note that the considerably enhanced internalization of this phospholipid in the parasitized cells is no longer species selective (Table III). The lack of SM flip-flop is consistent with the particularly stable transbilayer localization of this phospholipid in the human erythrocyte. Its highly asymmetric distribution is already found in pro-erythroblasts [33], and appeared to be maintained in sickled [34] and chemically modified erythrocytes [35]. Furthermore, the SM pools present in the outer and inner leaflets of the erythrocyte membrane show distinct molecular species compositions [36].

In summary, our results show that in *P. falciparum* parasitized human erythrocytes, the behaviour of the two choline-containing phospholipids, of which the majority is present in the outer membrane leaflet, is sharply distinct: while SM does not show any transbilayer migration, PC does so at a much higher rate when compared to that in normal human erythrocytes. Furthermore, the extent of transbilayer movement of PC seems, *grossomodo*, to be identical for different molecular species of PC and compatible with PC trafficking between host cell membrane and intraerythrocytic parasite.

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