

Research Article

Uptake and kinetic properties of choline and ethanolamine in *Plasmodium falciparum*

H Ahiboh^{1,3}, AJ Djaman², FH Yapi², A Edjeme-Aké¹, ML Hauhouot-Attoungbré¹, ED Yayo¹, D Monnet¹

¹Département de Biochimie Biologie Moléculaire, UFR des Sciences Pharmaceutiques et Biologiques, Université de Cocody – Abidjan BP V34 ²Laboratoire de Pharmacodynamie Biochimique - UFR de Biosciences, Université de Cocody – Abidjan BP V34. ³Département de Biochimie Fondamentale et Clinique – Institut Pasteur de Côte d'Ivoire – 01 BP 490 Abidjan 01

Abstract

Purpose: The asexual proliferation of *Plasmodium*, inside the erythrocyte, is accompanied by the synthesis of huge quantities of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). These needful phospholipids for the cytoplasmic membrane of the merozoites are provided by the precursors choline and ethanolamine. PtdCho and PtdEtn are synthesized by the parasite because the erythrocyte is unable to do it. In order to assess the dynamism of the phospholipid pathways, we aimed to investigate the respective shape of the uptake of choline and ethanolamine by *Plasmodium falciparum*.

Method: Time-course experiments and kinetic assays were performed respectively with fixed and ranged concentrations of radioactively-labelled choline and ethanolamine. The labelled-precursors were added in the culture of *P. falciparum* infected-erythrocytes and the incorporated molecules in phospholipids were measured with a scintigraph counter.

Result: The results showed that the incorporation of precursors in the infected-erythrocyte occurred with a Michaelis-Menten's kinetic shape. According to the maximum rate (V_{max}), the pathway of ethanolamine incorporation was faster than that of choline. Similarly, affinity for ethanolamine was greater than that of choline.

Conclusion: Although PtdCho is the major phospholipid in the membrane, this study rules out that the influx of ethanolamine in the infected-erythrocyte, *in vivo* conditions, is more dynamic than choline.

Keywords: *Plasmodium*, phospholipids, kinetics, metabolism, choline, ethanolamine

*Corresponding Author: Email: hugues.ahiboh@ucocody.ci Tel : (+225) 07 22 41 11 Fax : (+225) 22 44 06 70

INTRODUCTION

Like many other animal cells, the cytoplasmic membrane of the *Plasmodium* is made of bulk phospholipid molecules organized in a double layer. The major ones are phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). After the invasion of the erythrocyte, the parasite starts a schizogonic cycle marked by the production of several merozoites. This asexual proliferation of the parasite inside the erythrocyte is accompanied by the synthesis of huge quantities of needful phospholipids for the membrane of the merozoites. It means a biosynthesis of PtdCho and PtdEtn by the parasite because the erythrocyte is unable to do it on its own. In order to assess the needs of phospholipids for the parasite, we evaluated the uptake of its precursors, choline and ethanolamine. Many studies reveal metabolic differences within the species of *Plasmodium*¹⁻³. The uptake of choline and ethanolamine by the *Plasmodium* seem to vary with the species, the stage of the life cycle and the experimental conditions⁴. In this study, we determined the uptake and apparent kinetic properties of choline and ethanolamine in *P. falciparum* infested-erythrocyte, the most widespread human malarial parasite in West Africa.

EXPERIMENTAL

In vitro culture of *P. falciparum*

The 3D7 strain of *P. falciparum* in infected erythrocytes was incubated in modified RPMI 1640 medium supplemented with 25 mM Hepes (pH 7.6). The RPMI 1640 was free of choline and ethanolamine. The parasitemia was 7 - 12 % with a hematocrit of 2.5 % in a total amount of 3.5×10^6 – 6×10^6 infected cells. The cell suspension was distributed in microwell plate and incubated at 37°C in a 5 % carbon dioxide atmosphere at time 0.

Labelling for time-course studies

To assess the uptake of the phospholipid precursors by the infected-erythrocyte, radioactive isotopes of [1-³H]ethan-1-ol-2-amine and [methyl-³H]choline, purchased with Amersham™ corp were used. They were added, in the respective microwells, at time

60, 120, 180 and 210 min (final concentration of 2.5 μM; specific activity of 2 Ci/mmol). The final volume of the suspension in each microwell was 200 μl. At time 240 min, reactions were stopped at 4°C. Radioactive incorporation in bulk molecules was measured after haemolysis, filtration and transfer onto glass-fiber filter plate using a cell harvester. Radioactivity was measured on a scintigraph counter from Beckman™. Incorporation of the labelled precursors into the infected-erythrocyte was expressed in nmol/10¹⁰ cells.

Labelling for kinetic assay

To assess the kinetic features, different specific activity of radioactive choline and ethanolamine were added at indicated concentrations in the suspension of the infected-erythrocytes. The suspension was incubated for 2 hours at 37 °C in a 5 % CO₂ incubator. Reactions were stopped at 4 °C and radioactive incorporation was measured after filtration and transfer on fiber glass plate using a cell harvester. Radioactivity was measured as described above.

The blank test was performed with an uninfected-erythrocyte suspension provided by an uninfected human to assess non-specific uptake by the erythrocyte.

Data analysis

Experiments were performed twice. Blank test was deduced from each result and all of them were means of triplicate assays. Regression curve was drawn and the Michaelis constant (K_m) and V_{max} were calculated with the software GraphPad Prism® version 4.

RESULTS

Time course of choline and ethanolamine uptake

P. falciparum incorporates choline and ethanolamine in its bulk molecules retained by the cell harvester. For the two precursors, the uptake is constant and linear at least from 60 to 240 minutes. The rate of choline uptake is 0.91 ± 0.03 nmol.10⁻¹⁰ parasites.h⁻¹ and 5.01 ± 0.24 nmol.10⁻¹⁰ parasites.h⁻¹ for ethanolamine uptake (Figs.1 and.2). The *in vitro* conditions

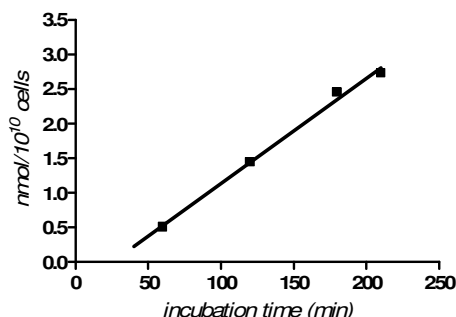


Figure 1: Choline incorporation rate in *P. falciparum*.

P. falciparum-infected erythrocytes (3D7 strain) (12 % parasitemia, 2.5 % hematocrit in 6.10^6 total infected cells) were incubated in a final volume of 200 μ l of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6). Suspensions were incubated at 37 °C in a 5 % CO₂ incubator at time 0. Radioactive choline (2.5 μ M, 2 Ci/mmol) was added at time 60, 120, 180 and 210 min. At time 240 min, reaction was stopped at 4 °C and radioactive incorporation in phosphatidylcholine was measured after filtration using a cell harvester. Incorporation of the labelled precursors into phosphatidylcholine is expressed in nmol/10¹⁰ cells and is mean of triplicate values \pm SEM.

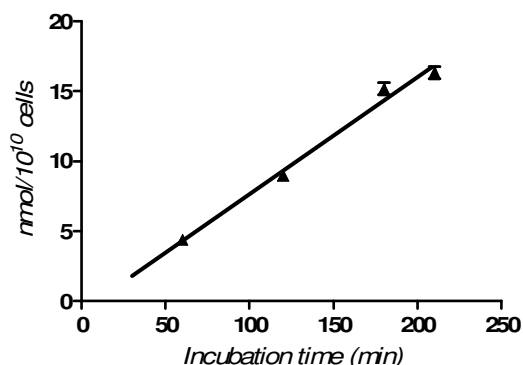


Figure 2: Ethanolamine incorporation rate in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (12 % parasitemia, 2.5 % hematocrit in 6.10^6 total infected cells) were incubated in a final volume of 200 μ l of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6). Suspensions were incubated at 37 °C in a 5 % CO₂ incubator at time 0. Radioactive ethanolamine (2.5 μ M, 2 Ci/mmol) was added at time 60, 120, 180 and 210 min. At time 240 min, reaction was stopped at 4 °C and radioactive incorporation in phospholipids was measured after filtration using a cell harvester. Incorporations of the labelled precursors into phospholipids are expressed nmol/10¹⁰ cells and are means of triplicate values \pm SEM.

show that the uptake of ethanolamine is greater than that of choline.

Kinetics of uptake

This assay was performed assuming an enzymatic process for the uptake of precursor

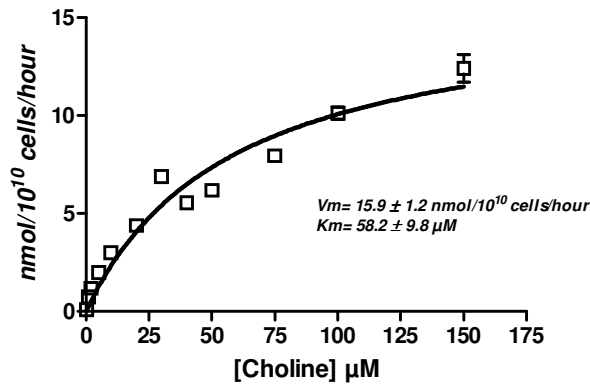


Figure 3: Kinetics of choline incorporation in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (7 % parasitemia, 2.5 % hematocrit in 3.5×10^6 total infected cells) were incubated in a final volume of 200 μ l of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6) and choline at indicated concentration (0,066 - 84 Ci/mmol). Suspension was incubated for 2 hours at 37 °C in a 5 % CO₂ incubator. Reaction was stopped at 4 °C and radioactive incorporation was measured after filtration using a cell harvester. Results are means of triplicate values \pm SEM.

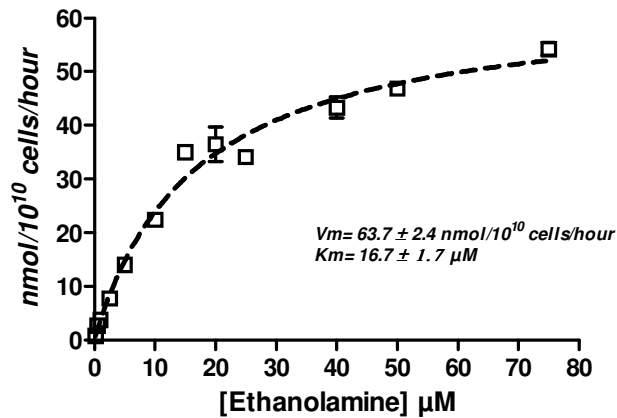


Figure 4: Kinetics of ethanolamine incorporation in *P. falciparum*

P. falciparum-infected erythrocytes (3D7 strain) (7 % parasitemia, 2.5 % hematocrit in 3.5×10^6 total infected cells) were incubated in a final volume of 200 μ l of modified RPMI 1640 supplemented with 25 mM Hepes (pH 7.6) and ethanolamine at indicated concentration (0,066 - 28 Ci/mmol). Suspension was incubated for 2 hours at 37 °C in a 5 % CO₂ incubator. Reaction was stopped at 4 °C and radioactive incorporation was measured after filtration using a cell harvester. Results are means of triplicate values \pm SEM.

and their incorporation in bulk molecules of the parasite. The uptake of choline and ethanolamine in *P. falciparum* showed an apparent *Michaelis-Menten* kinetics with the following features: choline uptake had a V_{max}

of 15.9 ± 1.2 nmol. 10^{-10} cells.h⁻¹ with an apparent K_m of 58.2 ± 9.8 μ M and ethanolamine uptake had a V_{max} of 63.7 ± 2.4 nmol. 10^{-10} cells.h⁻¹ with an apparent K_m of 16.7 ± 1.7 μ M (Figs 3 and 4). The metabolic

pathway of precursor incorporation in the phospholipids of *P. falciparum* seems to have a greater affinity for ethanolamine than for choline. Beside this, the incorporation of ethanolamine was faster than that of choline.

DISCUSSION

In human infection, signs of clinical morbidity, associated with malaria may occur at a parasitaemia range of about 100 – 720,000 per μl (i.e., 0.02 to 15%)^{5, 6}. Our experimental conditions were chosen to get an optimal growth of the parasite. It means not too low parasitaemia to get a sufficient measurable metabolic reaction through the radioactivity signal. But not too high parasitaemia to get a dynamic and optimal growth of the parasite and avoid a high amount of lactic acid or other biological wastes that a high parasitaemia can release. It is the same caution that led to a relatively short incubation time (less than 240 min).

In human blood, the choline concentration is about 10 - 12 μM and ethanolamine is about 2.5 μM ⁷. In order to assess the match of choline and the ethanolamine pathways in *in vitro* conditions, we chose an identical final concentration of precursors (2.5 μM for each). The uninfected-erythrocyte is not expected to incorporate choline or ethanolamine in any metabolic pathways. However, Staines and Kirk showed that in an infected mammalian, the choline enters in uninfected erythrocyte with the same rate than in infected erythrocyte⁴. Therefore we performed a blank test with uninfected-erythrocytes from healthy humans to deduce the non-specific adsorption of the precursors on any bulk molecules of the erythrocytes.

Several pathways from choline or ethanolamine are known to lead to phosphatidyl choline and phosphatidyl ethanolamine. The one known as the Kennedy pathway, consists in the phosphorylation of choline in phosphocholine (PC), catalyzed by a choline kinase (EC 2.7.1.32). Then a cytidylmonophosphate (CMP) is transferred on the PC leading to CDP-Choline. This reaction is catalyzed by a cholinephosphate cytidyltransferase (EC

2.7.7.15). And finally, the CDP-Choline is transferred on a diacylglycerol (DAG) to get the PtdCho. This reaction is catalyzed by CDP-Choline:1,2 diacylglycerol choline phosphotransferase (EC 2.7.8.2). Another pathway goes through the tri-methylation of the phosphoethanolamine (PE) into the PC and then leads to PtdCho via CDP-Cho⁸. The last one, known as occurring in mammalian cells and yeast, is the tri-methylation of PtdEtn into PtdCho⁹. The involvement of each pathway depends on the type of cell.

The respective biosynthetic and catabolic pathways of phosphatidylcholine and phosphatidylethanolamine involve many kinds of molecules : choline is known to be mainly taken up and incorporated in phosphatidylcholine (PtdCho) without betaine or acetylcholine biosynthesis¹⁰. Ethanolamine is retrieved from PtdCho and Phosphatidylethanolamine (PtdEtn). But nothing is known about the kinetics of their catabolism.

Our experiments show that *P. falciparum* incorporates choline in a constant way within 60 – 240 minutes while *P. knowlesi* optimal incorporation of choline into PtdCho is less than 180 min. But the rate of choline uptake, supposed to be mainly incorporated in PtdCho, is about 100 fold lesser for *P. falciparum* than for *P. knowlesi*¹¹. In the same way, Staines and Kirk showed that the rate of choline uptake by *P. vinckei* is greater than that of *P. falciparum* one⁴. This correlates with the length of life cycle of *P. knowlesi* or *P. vinckei* which is 24 hours while *P. falciparum* is much longer, 48 hours.

Infestation of the erythrocyte by *Plasmodium* entails a huge change of phospholipid composition in the erythrocyte membrane and in the whole complex parasite-erythrocyte relationship. At the schizonte stage phospholipid composition increases to about 500 – 700% and is mainly composed of PtdCho and PtdEtn (75 – 85%) with predominantly PtdCho¹²⁻¹⁴. However in our condition of experiments, we noticed that the rate of ethanolamine uptake was superior to the rate of choline uptake. The normal

mammalian plasma choline concentration is in the range 10 – 40 μM ¹⁵. Therefore, we expected the rate of choline uptake to increase and be superior to that of ethanolamine. But on the one hand V_{max} of choline uptake was inferior to V_{max} of ethanolamine uptake. On the other hand, the apparent K_m of ethanolamine uptake showed a higher affinity of the parasite for ethanolamine rather than choline. These observations contribute to the hypothesis that PtdCho is mainly issued from ethanolamine according the three steps of N-methylation of phosphoethanolamine to phosphocholine and catalyzed by phosphoethanolamine N-methyltransferase (EC 2.1.1.103)^{8, 16}. Otherwise, in *in vivo* conditions, the ethanolamine pathway, is inhibited by a transcriptional repression of the phosphoethanolamine methyltransferase as recently shown by Witola and Ben Mamoun¹⁷. This apparent feature of Michaelis-Menten kinetics for the choline and ethanolamine uptakes hide the possibility that these pathways possess any key-steps regulated by a possible allosteric enzyme.

CONCLUSION

Choline and ethanolamine are incorporated in bulk molecules of *P. falciparum*. The uptake of these precursors by infected-erythrocyte increased when compared with uninfected erythrocyte. Such differences in the apparent kinetic properties of the choline and ethanolamine uptake are easily understandable if we are aware that ethanolamine has to provide both PtdCho and PtdEtn metabolic pathways. On the contrary, choline provides only the PtdCho pathway. These results are in favor for a most dynamic influx of ethanolamine in the *P. falciparum* phospholipids but may not be the case in *in vivo* conditions.

ACKNOWLEDGEMENT

The authors acknowledge Wein S, Maynadier M, Boudou F for their technical assistance. We thank Herbute S, Wengelnick K and Vial H at the Laboratory of "Dynamique des Interactions Membranaires Normales et

Pathologiques" (CNRS UMR 5235) Université Montpellier II for their technical assistance and helpful advice concerning the protocol of experiments. This work was funded via an SCAC grant from the Cooperation Department of the French Ministry of Foreign Affairs.

REFERENCES

1. Biagini GA, Pasini EM, Hughes R, De Koning HP, Vial HJ, O'Neill PM, Ward SA, Bray PG. Characterization of the choline carrier of *Plasmodium falciparum*: a route for the selective delivery of novel antimalarial drugs. *Blood* 2004;104:3372-3377.
2. Lehane AM, Saliba KJ, Allen RJ, Kirk K. Choline uptake into the malaria parasite is energized by the membrane potential. *Biochem Biophys Res Commun* 2004;320:311-317.
3. Ancelin ML, Vial HJ. Several lines of evidence demonstrating that *Plasmodium falciparum*, a parasitic organism, has distinct enzymes for the phosphorylation of choline and ethanolamine. *FEBS Letters* 1986;202:217-223.
4. Staines HM, Kirk K. Increased choline transport in erythrocytes from mice infected with the malaria parasite *Plasmodium vinckei vinckei*. *Biochem J* 1998;334 (Pt 3):525-530.
5. McGuinness D, Koram K, Bennett S, Wagner G, Nkrumah F, Riley E. Clinical case definitions for malaria: clinical malaria associated with very low parasite densities in African infants. *Trans R Soc Trop Med Hyg* 1998;92:527-531.
6. Gazin PP, Goncalves K, Kone B, Lochouart L. [Effects of malaria outbreak in a quarter of the city of Bobo-Dioulasso (Burkina Faso)]. *Bull Soc Pathol Exot* 1996;89:200-203.
7. Fossati T, Colombo M, Castiglioni C, Abbiati G. Determination of plasma choline by high-performance liquid chromatography with a postcolumn enzyme reactor and electrochemical detection. *J Chromatogr B Biomed Appl* 1994;656:59-64.
8. Pessi G, Kociubinski G, Mamoun CB. A pathway for phosphatidylcholine biosynthesis in *Plasmodium falciparum* involving phosphoethanolamine methylation. *PNAS* 2004;101:6206-6211.
9. Kent C, Carman GM. Interactions among pathways for phosphatidylcholine metabolism, CTP synthesis and secretion through the Golgi apparatus. *Trends Biochem Sci* 1999;24:146-150.
10. Ancelin ML, Vial HJ. Regulation of phosphatidylcholine biosynthesis in *Plasmodium*-infected erythrocytes. *Biochim Biophys Acta* 1989;1001:82-89.
11. Vial HJ, Ancelin ML, Thuet MJ, Philippot JR. Phospholipid metabolism in *Plasmodium*-infected erythrocytes: guidelines for further studies using radioactive precursor

- incorporation. *Parasitology* 1989;98 Pt 3:351-357.
12. Vial HJ, Ancelin ML, Philippet JR, Thuet MJ. Biosynthesis and dynamics of lipids in *Plasmodium*-infected mature mammalian erythrocytes. *Blood Cells* 1990;16:531-555; discussion 556-561.
 13. Vial HJ, Ancelin ML. Malarial lipids. An overview. *Subcell Biochem* 1992;18:259-306.
 14. Van der Schaft PH, Beaumelle B, Vial H, Roelofsen B, Op den Kamp JA, Van Deenen LL. Phospholipid organization in monkey erythrocytes upon *Plasmodium knowlesi* infection. *Biochimica Biophysica Acta* 1987;901:1-14.
 15. Das I, de Belleruche J, Moore CJ, Rose FC. Determination of free choline in plasma and erythrocyte samples and choline derived from membrane phosphatidylcholine by a chemiluminescence method. *Anal Biochem* 1986;152:178-182.
 16. Pessi G, Choi J-Y, Reynolds JM, Voelker DR, Mamoun CB. In Vivo Evidence for the Specificity of *Plasmodium falciparum* Phosphoethanolamine Methyltransferase and Its Coupling to the Kennedy Pathway. *J Biol Chem* 2005;280:12461-12466.
 17. Witola WH, Ben Mamoun C. Choline induces transcriptional repression and proteasomal degradation of the malarial phosphoethanolamine methyltransferase. *Eukaryot Cell* 2007;6:1618-1624.