Increased permeability to choline in simian erythrocytes after *Plasmodium knowlesi* infection

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The permeability of simian erythrocytes to choline was found to be considerably increased after infection by the malaria parasite, *Plasmodium knowlesi*. Choline entry occurs by a facilitated-diffusion system involving a carrier, which displays temperature-dependence, saturability with choline ($K_m = 8.5 \pm 0.7 \mu\text{M}$) and specificity. This carrier can also be inhibited by a thiol reagent, *N*-ethylmaleimide, at an inactivation rate which is, in the absence of choline, the same as in normal erythrocytes. Inactivation by *N*-ethylmaleimide can be accelerated by external choline and prevented by decamethonium, which acts as an inhibitor of choline entry in infected cells (as with dodecyltrimethylammonium). Both ethanolamine and imidazole act as inhibitors or activators of choline entry in infected erythrocytes, depending on the relative concentrations of choline and of the competing compound (i.e. ethanolamine or imidazole). After infection, the maximum velocity reached $2.84 \pm 0.5 \text{ nmol/min per } 10^{10}$ infected cells, which is more than 10 times the V_{max} of normal erythrocytes. Impairing the biosynthesis of phosphatidylcholine *de novo* in *Plasmodium*-infected erythrocytes by various methods (glucose or ATP depletion, high ethanolamine concentrations) did not result in any alteration of choline transport (K_m or V_{max}), indicating that the constant triggering and transformation of choline into phosphatidylcholine by the parasite is not directly responsible for the increase in the choline transport rate after infection. This high increase in choline transport activity is more likely related to modifications in choline carriers and/or in their environment after *Plasmodium* infection.

INTRODUCTION

A phospholipid (PL)-content increase of up to 500 % has been previously reported in erythrocytes after *Plasmodium* infection [1]. Particularly notable are the increases in phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which together account for as much as 85 % of the total PL content [2]. Numerous metabolic pathways provide the required PL molecules and make the parasite as complex as many multicellular organisms [3,4].

We have previously described the important role of PL metabolism, whose impairment is lethal to parasites [5–7]. Infected erythrocytes can take up small molecules which are analogous to ethanolamine, and incorporate them into false non-natural PLs that accumulate at the expense of PE and kill the parasite [5]. Moreover, larger molecules which, like choline, possess a quaternary ammonium group can also lead to the death of the parasite in a more efficient manner by impairing choline entry into infected cells [6,7]. We have also recently shown that choline entry into infected erythrocytes is a limiting step in PC biosynthesis *de novo* [8], corroborating the importance of this step in parasite development. We therefore decided to characterize entry mechanisms of the two main PL polar heads (choline and ethanolamine) into *Plasmodium knowlesi*-infected erythrocytes. For choline, our analysis is based on the model developed by Krupka and Devés, (described below) who have carried out a very thorough study of the choline carrier in normal erythrocytes.

In normal erythrocytes, choline entry occurs by a facilitated-diffusion system involving a membrane carrier characterized by its high specificity for choline, saturability, and temperature-dependence [9–11]. Krupka and Devés have demonstrated that

choline entry depends on the operation of a mobile element corresponding to a cyclic carrier model in which one substrate site is exposed on only one side at a time (in either an outward-facing or an inward-facing conformation) [12–15]. By contrast, ethanolamine, which is a primary amine, probably enters the erythrocyte by passive diffusion.

In the present paper, we show that ethanolamine enters normal and infected erythrocytes mainly by passive diffusion. On the other hand, choline entry, which involves a carrier-mediated process in normal erythrocytes, remains totally controlled after *Plasmodium knowlesi* infection, but the presence of the parasite greatly increases permeability to choline.

MATERIALS AND METHODS

Chemicals

[methyl- ^3H]Choline, [1- ^3H]ethan-1-ol-2-amine and (hydroxy-[^{14}C]methyl)inulin were purchased from Amersham International (Amersham, Bucks., U.K.). Choline chloride, ethanolamine, 2-dimethylaminoethanol, decamethonium (DM), dodecyltrimethylammonium (DoTMA), *N*-ethylmaleimide (NEM), phorbol 12-myristate 13-acetate (PMA) and dibutyl phthalate ($d = 1.047$) were supplied by Sigma (St. Louis, MO, U.S.A.). Special RPMI-1640 (i.e. RPMI 1640 [16] without choline, serine, inositol and methionine) was from Gibco (Cergy Pontoise, France). All reagents were of analytical grade.

Biological material

Splenectomized *Macaca fascicularis* monkeys, weighing 3–6 kg (Sanofi, Montpellier, France), were infected with cryopreserved *P. knowlesi* Washington strain, variant 1 [17]. On days

Abbreviations used: DM, decamethonium; DoTMA, dodecyltrimethylammonium; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PMA, phorbol 12-myristate 13-acetate; IC₅₀, concn. giving 50 % inhibition; BSM, buffered saline medium.

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6–9, highly infected blood cells were collected as previously described [5]. When necessary, leucocytes were eliminated by passage through a cellulose-powder column (CF11; Whatman, Clifton, NJ, U.S.A.) [18], and then erythrocytes were washed with special RPMI 1640 supplemented with 35 mM-Hepes, pH 7.4 (modified RPMI). Control erythrocytes were obtained from non-infected monkeys and abundantly washed in choline-free medium for 2–3 h at 37 °C to allow cell depletion of choline.

Isolation of infected cells

Washed infected erythrocytes (10–30 % parasitaemia) were subjected to a Percoll/sorbitol fractionation as described by Kutner *et al.* [19]. Cells were suspended in phosphate-buffered saline ([19]; pH 7.4) containing 6 % sorbitol and layered on top of a Percoll/sorbitol discontinuous gradient (ranging from 60 to 90 % Percoll). Separation of infected from uninfected cells was achieved by centrifugation in a Sorvall RC-2B centrifuge for 20 min at 10000 rev./min in a fixed-angle rotor (SS-34). After centrifugation, the upper Percoll layer contained infected cell fractions, whereas the uninfected erythrocytes were in the lowest layer of the gradient. The layers were carefully removed from the gradient and cells were washed four times with modified RPMI at room temperature. After enrichment, parasitaemia was in the range of 95–100 %, as monitored by microscopic examination of Giemsa-stained smears.

Entry of radiolabelled choline into erythrocytes

Unless otherwise specified, choline influx was initiated by rapid mixing of 100 μ l of cells $[(0.65\text{--}3) \times 10^8$ erythrocytes] with 750 μ l of modified RPMI supplemented with 50 μ l of radio-labelled choline (sp. radioactivity 0.07–0.2 Ci/mmol) to give a final haematocrit of 0.8–3.4 %. Experiments were carried out at 37 °C for 6 min, in a final volume of 900 μ l. The flux was stopped at the end of the incubation period by adding 2.5 ml of ice-cold modified RPMI. Triplicate 1 ml portions of cell suspension were immediately overlaid on 400 μ l of ice-cold *n*-dibutyl phthalate in polyethylene tubes and centrifuged in a Beckman 11 Microfuge at 10000 *g* for 10 s at 4 °C. The cells sedimented below the dibutyl phthalate layer, which separated them from the overlying aqueous radioactive solution. Supernatants were discarded, and 2.5 ml of cold modified RPMI was carefully added on the dibutyl phthalate layer to wash the walls of the microtubes. After centrifugation, supernatants and dibutyl phthalate were discarded and cells were lysed and precipitated with 500 μ l of 10 % (w/v) trichloroacetic acid. After centrifugation, 400 μ l of the supernatant was counted for radioactivity in 10 ml of scintillation cocktail (Packard no. 299) in a Beckman 3801 scintillation spectrometer. In some experiments, [14 C]inulin was added to the incubation medium to determine (and deduce) the [3 H]choline present in the extracellular space [20]. But, in practice, [3 H]choline uptake was determined by subtracting values obtained by carrying out incubations in the presence of 1.2 mM-choline. [3 H]-Choline entry was expressed as nmol/min per 10^{10} cells. All experiments were done at least twice with triplicate independent incubations.

Effectors of the choline transport rate

Infected cells $[(0.7\text{--}3) \times 10^8$ cells/assay] were first preincubated for a short period (5–20 min) at 37 °C in the presence of various effectors (ethanolamine, imidazole, DoTMA, DM or PMA), and choline influx was then initiated by the rapid addition of radioactive choline (0.07–0.2 Ci/mmol). After 6 min at 37 °C, the reaction was stopped at 4 °C by adding an excess of cold modified RPMI as described above. Inhibition of choline influx was expressed as the concentration of the effector decreasing the entry of 10 μ M-[3 H]choline entry by 50 % (IC_{50}).

Glucose depletion was induced by preincubating cells at 20 °C for either 80 or 280 min in a medium constituted of 137 mM-NaCl, 4 mM-KCl, 1 mM-MgCl₂, 1 mM-CaCl₂ and 20 mM-Hepes (pH 7.4) (buffered saline medium, BSM). Control cells were incubated in BSM supplemented with 2 g of glucose/l.

ATP depletion was achieved by preincubating the cells for 7 min with 1 mM-NaN₃ or for 2 h at 37 °C in BSM containing 0.2 mM-NaN₃ [21]. After one washing in BSM, the choline influx was measured as described above.

Entry of ethanolamine into infected erythrocytes

Experiments were carried out as for choline except that, owing to the rapidly diffusible nature of ethanolamine, the incubation medium containing cells and [3 H]ethanolamine (0.7 Ci/mmol) was overlaid directly on the 400 μ l dibutyl phthalate layer present in the microtubes. Under the dibutyl phthalate, 100 μ l of 10 % trichloroacetic acid was deposited at the bottom of each microtube. The flux was stopped by rapid centrifugation at 4 °C. The amount of [3 H]ethanolamine present in the extracellular space was determined by addition of [14 C]inulin [20].

Treatment of cells with NEM

An aqueous solution of 165 mM-NEM was freshly prepared. This solution was diluted with modified RPMI just before reaction with cells.

Cells (0.3–0.5 % final haematocrit) were preincubated for various times at 37 °C, with 1 mM-NEM without substrate. Controls were preincubated in the absence of NEM. The reaction was stopped by adding ice-cold modified RPMI containing 14 mM- β -mercaptoethanol as previously described [10]. Cells were then washed four times in modified RPMI, and transport was assayed for 10 min at 37 °C, in the presence of 3 μ M-[3 H]-choline; v_0 represents the transport rate of untreated cells (control) and v is the transport rate of NEM-treated cells. The rates of inactivation were determined by plotting the natural logarithm of the v/v_0 ratio against the time of treatment with NEM, in accordance with the method of Edwards [10]. The pseudo-first-order rate constant of inactivation by NEM (k_i) was obtained from the slope of the line by least-squares analysis.

Assay of PL metabolism

We have developed an original method to assess PL metabolism quickly (M. L. Ancelin, F. Vialettes & H. J. Vial, unpublished work) in which PL metabolism was monitored in microtitre plates by the incorporation of either 10 μ M-[3 H]choline (1.5 μ Ci/well) or 10 μ M-[3 H]ethanolamine (0.74 μ Ci/well) into PL. For comparison, incorporation of [3 H]hypoxanthine at trace concentrations (0.68 μ Ci/well) into nucleic acids was also monitored. Infected erythrocytes 4×10^6 cells/assay were incubated in a 96-multiwell plate for 3 h at 37 °C in presence of the radioactive precursor in a final volume of 250 μ l of modified RPMI. Incubations were carried out at 37 °C, by the candle-jar method [22]. After incubation, cells were collected on glass-fibre filters (Whatman GF/C), by using a cell harvester (Multimash 2000; Dynatech), and lysed with water. The insoluble materials (such as membrane lipids and nucleic acids) retained on the filter were abundantly washed with water for 50 s. Filters were then dried for 35 s and placed in scintillation vials containing 2 ml of scintillation cocktail. Final radioactivity counting was performed after 24 h at room temperature, owing to the time-dependent rise in the recovered radioactivity.

Determination of choline metabolite content

Incubations were carried out as described above for the choline entry assay, but at a higher specific radioactivity (1.3 Ci/mmol). After incubation, cells were lysed with 250 μ l of methanol instead

of 10% trichloroacetic acid. Cellular lipids were then extracted by the method of Folch *et al.* [23], modified as described previously [5]. Under our conditions of short-term incubation, radioactive choline was recovered exclusively in the aqueous phase, whereas no radioactive PC was present in the organic phase. The radioactive compounds of the aqueous phase (essentially choline and phosphocholine) were separated by silica-gel t.l.c. developed in methanol/0.5% NaCl/25% NH_3 solution (10:10:1, by vol.) as described previously [8].

RESULTS

Entry of choline and temperature-dependence

Erythrocytes are bathed in plasma whose choline level is in the range approx. 10–40 μM [24]. Preliminary experiments were carried out at choline trace doses (0.9 μM , 1 Ci/mmol) to characterize [^3H]choline uptake as a function of time in normal and *P. knowlesi*-infected erythrocytes at 0 and 37 °C (Fig. 1). Choline uptake was quite low in normal erythrocytes at 0 °C

(15 pmol/ 10^{10} cells after 6 min incubation) without any significant increase even after 15 min incubation. In contrast, at 37 °C, choline uptake increased linearly with time until 15 min, reaching 36 pmol/ 10^{10} cells after 6 min (see Fig. 1 insert). Adding 400 μM -*NN*-dimethylethanolamine at 37 °C drastically inhibited choline entry, whose rate fell to as low as at 0 °C (results not shown).

Choline uptake was still very low at 0 °C after *P. knowlesi* infection (33 pmol/ 10^{10} infected cells after 6 min). However, at 37 °C there was a steep linear increase in choline entry: the entry rate was 470 pmol/ 10^{10} infected cells after 6 min, i.e. more than 10 times that in normal erythrocytes. Short-time incubations (from 30 s to 7 min) were also performed by the single-step centrifugation method in the presence of [^{14}C]inulin described in the Materials and methods section to determine choline entry into infected erythrocytes. Under these conditions also, a linear increase in radioactive choline entry was observed for up to 7 min (results not shown). As in normal erythrocytes, adding 400 μM -*NN*-dimethylethanolamine completely inhibited choline entry into infected cells at 37 °C (results not shown).

Fig. 1. Kinetics of [^3H]choline entry into normal and *P. knowlesi*-infected erythrocytes

Infected cells (—) (23% parasitaemia, trophozoite form) were centrifuged on a Percoll/sorbitol gradient and enriched to 95–100% parasitaemia. Uptake of [^3H]choline was performed in modified RPMI containing 0.9 μM -choline (1 Ci/mmol) at a final haematocrit of 0.8% (6.5×10^7 cells/assay) at 37 °C (●) and 0 °C (○). The flux was stopped at the indicated time by adding 2.5 ml of ice-cold modified RPMI, followed by centrifugation of a 1 ml portion through *n*-dibutyl phthalate at 4 °C as described in the Materials and methods section. Uptake by normal erythrocytes (----) (1.4×10^8 cells/assay) under the same conditions is also shown in the insert. Each point is the mean of triplicate determinations in one typical experiment. Vertical bars correspond to S.E.M.

Fig. 2. Entry of [^3H]choline into normal and *P. knowlesi*-infected erythrocytes as a function of external choline concentration

Experiments were carried out for 6 min at 37 °C as described in Fig. 1, except for choline concentration and specific radioactivity (0.07 Ci/mmol): ○, normal erythrocytes; ●, infected erythrocytes. (b) Double-reciprocal plot of the initial velocity of choline entry. Where not shown, error bars were within the symbols.

Fig. 3. Effect of alkyltrimethylammonium ions on choline entry into *P. knowlesi*-infected erythrocytes

Suspensions of infected erythrocytes (97.4% schizont) contained 1.5×10^8 cells/assay. Influx of $10 \mu\text{M}$ - $[\text{^3H}]$ choline (sp. radioactivity 0.07 Ci/mmol) was measured for 6 min at 37°C in the presence of DoTMA ($\square$) or DM ($\blacksquare$).

Fig. 4. Time course of the inactivation of choline transport by NEM into normal and *P. knowlesi*-infected erythrocytes

Cells (final haematocrit of 2.5%) were incubated for the indicated times with 1 mM -NEM in the absence of choline. Normal erythrocytes ($\circ$) were present at 1.7×10^8 cells/assay, and infected erythrocytes (schizont form; $\bullet$) were at 6.8×10^7 infected cells/assay. The reactions were stopped by adding 14 mM - β -mercaptoethanol. Cells were then extensively washed in modified RPMI before measuring the entry of $3 \mu\text{M}$ - $[\text{^3H}]$ choline for 10 min; v is the transport rate of NEM-treated cells and v_0 is the transport rate of untreated cells.

The effect of increasing external choline concentrations was investigated in both normal and infected erythrocytes after 6 min incubation (the time when initial velocities were measured) at 37°C (Fig. 2a). Under these conditions, choline entry followed Michaelis-Menten kinetics, allowing determination of kinetic characteristics (Fig. 2b). The parameters were $K_m = 7.2 \pm 1.2 \mu\text{M}$ and $V_{\text{max}} = 0.28 \pm 0.05 \text{ nmol/min per } 10^{10} \text{ cells}$ ($n = 5$) and $K_m = 8.5 \pm 0.7 \mu\text{M}$ and $V_{\text{max}} = 2.84 \pm 0.5 \text{ nmol/min per } 10^{10} \text{ infected cells}$ ($n = 12$) for normal and *P. knowlesi*-infected erythrocytes respectively. Thus the affinity for choline was not significantly modified after infection, whereas, as seen above, choline entry rate was increased more than 10-fold. Uninfected erythrocytes of infected monkeys exhibited the same kinetic characteristics as erythrocytes from healthy monkeys with regard to K_m and V_{max} . (results not shown). The kinetic parameters of choline entry as a function of parasite maturation were not investigated, but no significant differences were found between young and mature parasite stages among preparations in different experiments.

Ethanolamine entry

Since normal erythrocytes can be very permeable to ethanolamine, we measured ethanolamine entry into normal and infected erythrocytes after a rapid single-step centrifugation as

described in the Materials and methods section. In normal erythrocytes and infected cells, there was a linear increase in ethanolamine entry with extracellular ethanolamine concentrations (from 2 to $100 \mu\text{M}$) and no saturation plateau was reached (results not shown).

Inhibition of choline transport in *P. knowlesi*-infected erythrocytes by alkyltrimethylammonium compounds

We have previously shown that alkyltrimethylammonium ions, such as DoTMA or DM, inhibit PC biosynthesis in *P. knowlesi*-infected erythrocytes by impairing choline transport into infected erythrocytes [6,7]. Consequently, we studied the effect of these compounds on the entry of $10 \mu\text{M}$ -choline, a concentration that corresponds to the K_m of choline transport.

Fig. 3 shows that DoTMA inhibited choline transport in a dose-dependent manner, with IC_{50} of $8 \pm 3 \mu\text{M}$ ($n = 4$). The same inhibition pattern was obtained for DM, with an IC_{50} of $20 \pm 8 \mu\text{M}$ ($n = 2$).

Inactivation of choline transport by NEM

In normal human erythrocytes, Edwards [10] has shown that freely diffusible NEM could irreversibly inactivate the choline transporter in a pseudo-first-order reaction with respect to the transport system. The inactivation of choline transport by NEM was shown to proceed faster when there is a substrate of the transport system outside the cell. On the other hand, in the presence of the substrate within the cell, or of a competitive inhibitor (such as DM) outside the cell, inactivation proceeds more slowly [10,15,25]. This has been explained by the asymmetry of the carrier, associated with a preferential and rapid attack by NEM on the inward-facing carrier. Adding choline outside provokes an accumulation of the carrier in the inward-facing state (owing to the rate-limiting step, i.e. the re-orientation of the free carrier) and therefore accelerates carrier inactivation by NEM. By contrast, DM binds to the outward facing site of the carrier, preventing its re-orientation toward the inward-facing state, hence preventing the reaction of NEM with the carrier.

Compared with normal human erythrocytes [10], choline transport in normal simian erythrocytes exhibited a similar time-course of inactivation by NEM with a rate constant of inactivation, k_0 , of 0.17 min^{-1} (Fig. 4). After *Plasmodium* infection, the inactivation reaction by NEM was still pseudo-first-order, with $k_0 = 0.16 \text{ min}^{-1}$, i.e. quite similar to that in normal erythrocytes.

We then investigated the effect of external choline on the rate

Fig. 5. Effects of external choline and DM on the rate of inactivation of the choline carrier by NEM

Infected cells were preincubated with 1 mM -NEM at 37°C and with the indicated concentrations of choline (a) or DM (b), for 4 or 8 min respectively. Reactions were stopped by adding β -mercaptoethanol, and cells were then extensively washed and incubated in RPMI for 2 h at 37°C . Then $[\text{^3H}]$ choline (0.09 Ci/mmol) entry was monitored for 10 min at 37°C . The rate constant of choline-transport inactivation by NEM, k (expressed as min^{-1}), was determined as explained in the Materials and methods section.

of NEM inactivation. Cells were incubated with 1 mM-NEM at various choline concentrations (12.5–100 μM) for 4 min at 37 °C. After addition of β -mercaptoethanol and abundant washing, the entry of 3 μM -[^3H]choline was assayed for 10 min at 37 °C. Fig. 5(a) shows that external choline progressively increased the rate constant of inactivation by NEM. At saturating concentrations of choline outside the cell, the rate of inactivation by NEM increased by 3.5-fold, demonstrating that choline outside the cell progressively accelerates the inactivation of choline transport by NEM. Devés and Krupka have shown that in normal erythrocytes the ratio (Q_{To}/Q) of the rates of inactivation in the presence of saturating (50 μM) choline concentration (Q_{To}) and in the absence of choline (Q) is equal to the ratio of the rates of exit of radioactive choline in the presence ($\bar{v}^{\text{T}}$) and absence ($\bar{v}$) of choline in the external solution [$(\bar{v}^{\text{T}}/\bar{v})S_{1/2}$] [15]. These ratios are higher than 1 for accelerated exchange [15]. In our study, at 50 μM -choline this ratio was 3.2, which means that accelerated choline exchange also occurs in *Plasmodium*-infected erythrocytes.

We also studied the effect of DM, which does not penetrate the erythrocytes [26] and which has been reported to inhibit choline transport by reacting with the outward-facing site of the choline carrier [10]. Infected cells were first preincubated for 8 min with 1 mM-NEM in the presence of DM. The entry of 3 μM -[^3H]choline was then monitored for 10 min. Fig. 5(b) shows that increasing the DM concentration, from 20 to 160 μM , resulted in a progressive decrease in the rate constant of inactivation by NEM (k). At 160 μM , DM totally prevented the inactivation of choline transport by NEM ($k = 0.004 \text{ min}^{-1}$)

Activation of choline transport

Several small diffusible molecules (or 'competing substrates') have been shown to accelerate choline transport of normal erythrocytes [27]. This was related to the existence of the rate-limiting step in the transport, i.e. re-orientation of the free carrier (without choline). Indeed, small molecules which are structurally similar to choline can be loaded by the choline carrier, which can re-orientate more rapidly than when it is free.

We thus studied the effect of ethanolamine, which differs from choline in terms of the volume of the polar head, on the rate of

zero-*trans* entry of choline (Fig. 6a). Lineweaver–Burk plots were drawn from experiments performed without or with four different ethanolamine concentrations, in the range 50 μM –1.5 mM. The straight line in the control experiment, when no ethanolamine was present, intersects with every other straight line when ethanolamine was present. Thus ethanolamine either inhibited or activated choline entry. At the intersection point, ethanolamine had no apparent effect on choline influx. When the external ethanolamine concentration was increased, the no-effect point was displaced to higher choline concentrations. Addition of ethanolamine led to a modification of the maximum rate of choline entry. As shown in the insert, V_{max} first progressively increased in the presence of external ethanolamine, reaching a maximal 2.4-fold augmentation at 0.25 mM, then decreased and returned to a level close to that of the control.

Imidazole, another quickly diffusible compound, was shown to have a similar biphasic effect on normal erythrocytes [27], and was consequently also investigated (Fig. 6b). The straight lines in the Lineweaver–Burk plots (with and without imidazole) once more intersect each other; at choline concentrations lower than those at which the lines intersect, transport was inhibited by imidazole, whereas at higher concentrations choline entry was accelerated. As for ethanolamine, it is worth noting that the intersection point (where imidazole has no apparent effect) was dependent on the imidazole concentration, and increasing the imidazole concentration led to a shift of the intersection point to higher choline concentrations. Imidazole could increase the V_{max} , with a maximum effect at 2.5 mM (see the insert), with V_{max} being increased by 2.5-fold. It should be noted that, compared with the results obtained in normal erythrocytes [27], the maximum increase in V_{max} also occurred at imidazole concentrations of about 2.5 mM.

The above experiments demonstrated that choline transport could be accelerated by diffusible compounds. Thus we investigated this possibility with some catabolites released during infection from infected cells into the external medium (notably the products of haemoglobin degradation, or parasite catabolites [28]) to activate choline transport. Indeed, small catabolites could also act as 'competing substrates' and be responsible for the high increase in V_{max} after infection. With this objective,

Fig. 6. Effect of ethanolamine and imidazole on choline influx into infected erythrocytes

Experiments were carried out as in Fig. 2, except that the infected erythrocytes were preincubated for 10 min in the presence of the indicated ethanolamine concentration (ETH; a) or for 15 min with imidazole (IMDZ; b), before addition of [^3H]choline (0.17–0.19 Ci/mmol) at the indicated concentration. The *P. knowlesi*-infected erythrocyte suspension was 98 % parasitized (trophozoite form) with $(1.3\text{--}1.7) \times 10^8$ cells/assay. Inserts show the V_{max} (in nmol/min per 10^{10} cells; derived from a least-squares analysis) as a function of the external ethanolamine (a) or imidazole (b) concentration.

Table 1. Kinetic parameters of choline influx and choline metabolite content in *P. knowlesi*-infected erythrocytes under various depletion conditions

Pure infected erythrocytes (10^8 cells, haematocrit) were incubated in three different media (modified RPMI, BSM, or BSM + 2 g of glucose/l) at room temperature for 80 min (with gentle agitation). Cells were then centrifuged for 5 min at 1100 rev./min to replace the initial medium by the corresponding fresh medium. Then either choline influx or choline metabolite content was determined as described in the Materials and methods section. Results are means of one typical experiment carried out in triplicate.

Incubation medium	K_m (μM)	$V_{\max}$ (mmol/min per 10^{10} cells)	Radioactive intracellular content (nmol/ 10^{10} infected cells) in:	
			Choline	Phosphocholine
RPMI	17	0.9	0.07 ± 0.01	0.56 ± 0.02
BSM with glucose (2 g/l)	14	0.7	0.10 ± 0.01	0.43 ± 0.03
BSM without glucose	18	0.7	0.29 ± 0.01	0.27 ± 0.01

Fig. 7. Equilibrium between the pathway of PC biosynthesis *de novo* and the methylation pathway

Pure infected erythrocytes (4×10^6 parasites, ring-trophozoite) were incubated for 3 h at 37°C at a final haematocrit of 0.2% in 96-multiwell plates containing modified 1640 RPMI medium in the presence of the indicated ethanolamine concentrations, as described in the Materials and methods section. The incorporation of [^3H]ethanolamine (0.75–300 Ci/mol) into both PE and PC (●), or of $10 \mu\text{M}$ -[^3H]choline (0.6 Ci/mmol) into PC (□), was measured.

P. falciparum-infected erythrocytes were cultivated, by the candle-jar method [22], at 7% haematocrit in modified RPMI 1640 buffered with 25 mM-Hepes (pH 7.4) and supplemented with 10% (v/v) human serum (blood group AB⁺), with daily change of medium. The supernatants from a 10–15%-parasitized suspension (schizont form) were harvested and then adjusted to pH 7.4. Control supernatants obtained from non-infected blood suspension were treated under the same conditions. Supernatants were then applied to normal erythrocytes (1.5×10^8 cells/assay) for 12 min at 37°C . Then various concentrations of [^3H]choline (0.12 Ci/mmol) were added for 6 min to determine choline influx. The $V_{\max}$ of choline transport, assayed with the supernatant of the infected preparation, was not significantly changed compared with the normal supernatant (results not shown).

PC metabolism and choline transport

The considerable increase in $V_{\max}$ could be due to the appearance of a high PC-biosynthetic activity in the erythrocyte after infection, resulting in a constant removal of intracellular choline from the erythrocyte cytoplasm. To test this hypothesis, we altered the PC biosynthesis *de novo* and investigated the impact of this alteration on choline transport.

First, experiments such as glucose depletion were carried out in order to inhibit PC metabolism. Infected erythrocytes were incubated for 80 min at room temperature in a buffered saline medium (BSM) (as defined in the Materials and methods section), devoid of glucose (Table 1). As expected, glucose depletion provoked a significant decrease in the phosphocholine content of infected cells (by 50%) with a concomitant increase (4-fold) in intracellular choline, indicating that the kinase activity was highly altered, owing to ATP depletion induced in the absence of glucose. However, no significant modification in the kinetic characteristics of choline transport was found compared with cells incubated in BSM supplemented with 2 g of glucose/l. When preincubation was carried out for 280 min, a slight decrease in $V_{\max}$ occurred, probably owing to the long-term incubation in a medium that is not quite adapted to sustain good parasite growth (results not shown). But no significant difference was observed between cells incubated in BSM supplemented or not with glucose.

We also pretreated 100%-trophozoite-infected cells at 37°C with NaN_3 either at 1 mM for 7 min or at 0.2 mM for 2 h. This last condition was previously reported to deplete ATP greatly (by about 40%) of erythrocytes infected by mature parasite stages [21]. We also failed to detect any change in choline transport activity under these conditions (results not shown).

Finally, PC biosynthesis *de novo* by the parasite was altered by high non-toxic concentrations of ethanolamine. This resulted in an increase in radioactive ethanolamine incorporation into PE and PC, its methylated derivative. Moreover, PC biosynthesis *de novo* progressively decreased, by 23% at $500 \mu\text{M}$ -ethanolamine and by 42% at 1 mM-ethanolamine (Fig. 7). On the other hand, ethanolamine (up to 4 mM) had no effect on hypoxanthine incorporation into nucleic acids, showing that parasite viability was not affected (results not shown). The parasite therefore regulated the total amount of PC biosynthesized by decreasing PC biosynthesis *de novo* in response to an over-production of PC synthesized via the methylation pathway.

P. knowlesi-infected erythrocytes were preincubated for 3 h in the presence of high ethanolamine concentrations under the same conditions, and then washed extensively to eliminate external ethanolamine. No significant modifications in either the K_m or the $V_{\max}$ of choline transport occurred, even at 4 mM-ethanolamine (results not shown).

We otherwise tried to modify choline incorporation into PC using phorbol esters. Infected cells were pretreated for 20 min at 37°C with PMA, ranging from 1 nM to $0.1 \mu\text{M}$, which was reported to stimulate the incorporation of choline into PLs in various cells [29]. We then assayed [^3H]choline influx at two choline concentrations (9 μM and 18 μM). Under these conditions, phorbol esters did not affect rates of choline influx even at $0.1 \mu\text{M}$ (results not shown).

DISCUSSION

Choline entry into normal erythrocytes has been well documented [9,30,31]. Our analysis is based on the model developed by Krupka and Devés, who have carried out a very thorough study of the choline carrier in normal erythrocytes (for review see [30]). Only entry rates, but not exit, could be determined in infected erythrocytes. Indeed, it is impossible to load infected cells with saturating choline concentrations, owing to the possible occurrence of haemolysis and mainly to the intense choline metabolism in infected cells. In normal erythrocytes, Krupka and Devés have established that choline entry occurs via a mobile carrier corresponding to a cyclic model, in which one substrate site is exposed on only one side at a time (in either outward-facing or inward-facing conformation) [30]. Re-orientation and dissociation of the carrier-substrate complex are far more rapid than re-orientation of the free carrier, which is the limiting step of choline transport [13]. This asymmetric choline carrier has different possible inhibition sites, depending on its facing conformation [30]. NEM specifically binds to a thiol group on the inner conformation, irreversibly inhibiting the choline carrier. In contrast, monoalkyl-substituted choline analogues with long alkyl chains [such as *NNN*-(dimethyldodecyl)-2-hydroxyethylammonium ion] do not enter the erythrocyte, and form an attachment with the outward-facing conformation [30].

We show here that, after *Plasmodium* infection of erythrocytes, choline entry still involves a carrier-mediated mechanism. No significant passive diffusion occurs, as revealed by the absence of entry at 4 °C (Fig. 1) or by the complete inhibition of choline entry at 37 °C by dimethylethanolamine, even in the late stage of the maturing parasite, when membrane permeability to certain usually impermeant substances (such as carbohydrates, polyols, amino acids, nucleosides and anions) is seriously altered [19,32]. This increase in permeability has been attributed to 'new permeability' pathways, which would display selectivity according to a model based on hydrocarbon partitioning of solute across the membrane [33]. The present study shows that this does not apply to the entry of choline, a cationic compound which remains entirely under carrier control throughout parasite development.

Characteristics of the choline carrier after infection appear to be close to those of the carrier in normal uninfected erythrocytes, in terms of temperature-dependence, affinity for choline ($K_m = 7.2 \pm 1.2 \mu\text{M}$ in normal erythrocytes and $K_m = 8.5 \pm 0.7 \mu\text{M}$ in infected erythrocytes) or rate constant of inactivation by NEM ($k_0 = 0.17 \text{ min}^{-1}$ and 0.16 min^{-1} for normal and infected erythrocytes respectively). Compounds such as DoTMA or DM are capable of inhibiting the choline carrier, suggesting that this carrier possesses a hydrophobic region adjacent to the active site which can accommodate the alkyltrimethylammonium ions, as is the case for normal erythrocytes [12,26].

The major difference involves the large increase (more than 10-fold in the choline transport rate after infection ($0.28 \pm 0.05 \text{ nmol/min per } 10^{10} \text{ cells}$ in normal erythrocytes, compared with 2.84 ± 0.50 in infected erythrocytes). It is worth noting that uninfected erythrocytes of malaria-infected monkeys have the same kinetic parameters as normal erythrocytes, showing that the large increase in V_{max} in infected cells is only due to the parasite itself, not to the other events occurring in the host during malarial infection.

Before investigating the reasons for the increase in V_{max} after infection, we will detail carrier function. Many studies have dealt with choline function in erythrocytes [30,31], and multiple approaches have been developed to characterize the consecutive steps that together bring about substrate translocation. First, the substrate is bound to the carrier site, exposed on one side of the

membrane, followed by re-orientation of the carrier complex. Then the substrate is released on the other side, and the free carrier reverses its orientation to return to its original position. In normal cells, binding of the substrate to the carrier on one side, and release of the substrate from the complex on the other side, are the quickest steps. In contrast, re-orientation within the membrane is the rate-limiting step, and the free carrier is slower than the carrier-substrate complex to translocate across the membrane [13]. In infected erythrocytes, once choline is released from the complex, it is quickly removed from the erythrocyte cytoplasm, directed to the intracellular parasite and metabolized [8]. We checked that the 10-fold increase in the V_{max} of choline transport after infection was not due to the constant removal and transformation of choline in PC by the parasite.

We thus forced the parasite to alter its PC biosynthesis *de novo* and looked for a possible consequent effect on choline entry. Inhibition of PC metabolism was carried out either by energy depletion (under glucose- or ATP-depletion conditions), or by re-directing PL metabolism in the presence of high ethanolamine concentration. The effect of high concentrations of chloroquine, a metabolic inhibitor, has also been investigated previously [34]. None of these conditions provoked any modifications in either the K_m or the V_{max} of choline transport in infected erythrocytes. Hence the increase in choline transport after infection is probably not related to its further metabolism.

Further experiments were developed to gain insight into the nature of the choline carrier (symmetric or asymmetric) and to determine whether re-orientation of the empty carrier is still the rate-limiting step of transport in erythrocytes, after infection. The two sets of experiments, using NEM or ethanolamine or imidazole, are mutually complementary and have made it possible to establish the essential features of the system.

In normal erythrocytes, NEM reacts with a thiol group of the inward-facing conformation of the carrier and leads to its inactivation [10,15,25]. We show here that NEM also inactivates the choline carrier of infected cells. Addition of an inhibitor outside the cell (DM) totally prevented this inactivation (Fig. 5b). This suggests that the carrier exists in two conformational states, as in normal erythrocytes, and that DM blocks the carrier in the outward-facing state, preventing the reaction of NEM with the inward-facing carrier. The fact that external choline accelerates carrier inactivation by NEM (Fig. 5a) reveals that accelerated exchange occurs [15] and that re-orientation of the free carrier remains the rate-limiting step of choline transport after infection. The addition of exogenous choline allows the carriers to accumulate in the inward-facing state, which is then quickly accessible to NEM.

The results of our experiments with ethanolamine or imidazole, which are capable of accelerating choline transport, also show that reorientation of the free carrier is a limiting step of choline transport. Indeed, in the presence of the freely diffusible ethanolamine, we observed an inhibition of choline entry at low choline concentrations, and an acceleration at high choline concentrations (see Fig. 6a). Present in both compartments (*cis* and *trans*), ethanolamine is able to interact with both the inward-facing and outward-facing carrier sites, and acts in the same way as 'competing substrate' [27]. In the *cis* compartment, both choline and ethanolamine compete for the carrier. In the *trans* compartment, where there is no choline, ethanolamine freely adds to the carrier and produces a *trans* acceleration (owing to the more rapid translocation of the complex than of the free carrier). For a given ethanolamine concentration, at choline concentrations lower than K_m , inhibition predominates. At increasing concentrations, choline competes more and more effectively with ethanolamine in the *cis* compartment, until the *cis*-inhibition balances the acceleration by ethanolamine in the

trans compartment (no-effect point). At saturating concentration, choline completely displaces ethanolamine from the carrier site and overcomes the competitive inhibition.

The point of no-effect is dependent on the ethanolamine concentration. At low concentrations, ethanolamine is both an inhibitor at the *cis* site and an activator at the *trans* site, but activation outweighs inhibition. At higher ethanolamine concentrations, activation is limited when the *trans* site is saturated, whereas the inhibitory effect of competing ethanolamine steadily increases. The transport rate reaches a peak, while the ethanolamine concentration rises and then falls steadily.

A similar asymmetric behaviour was found with imidazole, which is not structurally related to choline or ethanolamine. Imidazole also rapidly enters the normal erythrocytes by simple diffusion and can add to carrier sites on both sides of the membrane [27]. In *Plasmodium*-infected erythrocytes, imidazole is also able either to inhibit (*cis*-effect predominating) or to activate (*trans*-effect) choline transport, depending on the external choline concentration.

Thus, in infected erythrocytes, choline transport satisfies the criteria of an asymmetric carrier system, whose rate-limiting step involves re-orientation of the free carrier, as in normal cells. In this context, the increase in choline entry rate, as much as 10-fold, could result from either a modification in the carrier operation (consecutive to an activation of the limiting step in choline transport or to a secondary modification of the transport protein itself) or from an increased number of active carriers.

The increased rate of choline entry could be the consequence of an activation of the rate-limiting step. In the present study, we have shown that choline transport could be activated by various compounds (small choline analogues or choline itself). In infected erythrocytes, intracellular choline is almost absent from the parasite [8], and the transport rate increase cannot result from an augmentation in intracellular choline content after infection. Some other compounds released by the parasite and constantly exported outside the cells (such as parasite catabolites, including products from haemoglobin degradation [28]), which could be activators of the rate-limiting step, can probably also be ruled out. Indeed, assuming that these catabolites are capable of leaving as well as entering the cell, we have shown that pre-incubation of normal erythrocytes with supernatants from infected cells does not produce any significant modification in the $V_{\max}$. Furthermore, egress of waste products from the parasite highly increases with parasite maturation [28], but the $V_{\max}$ of choline transport appears to be similar from one parasite stage to another (results not shown).

Ionic modifications take place in the infected-cell cytosol; notably there is a marked cation imbalance, i.e. a loss of K^+ and gain of Na^+ [35], as well as acidification of the intracellular pH [36]. However, these modifications should have an inhibitory effect on choline transport, rather than the observed increase. Indeed, in normal erythrocytes, choline entry declines with K^+ [27,37] and with the intracellular pH [38], but is rather independent of the Na^+ gradient [37]. All of these cation modifications are accentuated with parasite maturation, and some of them, i.e. K^+ [35], also take place in uninfected erythrocytes, whereas we found no modifications in the kinetic characteristics of choline entry, either during parasite maturation or between uninfected and normal erythrocytes.

Furthermore, all of the ionic modifications occurring after infection are accompanied by a substantial change in the electrical potential across the parasite membrane, which may account for the choline accumulation and the observed transport-rate increase. But the fact that, in normal erythrocytes, an increase of up to 31.1 mV in membrane potential (i.e. more than 13-fold)

had no effect on the carrier partition and flux ratio $[(\bar{v}^T/\bar{v})_{si} \rightarrow o]$ [38] probably negates this possibility.

Modifications (i.e. phosphorylation or glycosylation) of the carrier protein could also lead to functional changes after infection. A great variety of glycoproteins and phosphoproteins are synthesized during the parasitic cycle [39–41]. Phorbol-ester-stimulated phosphorylation of the glucose transporter of normal erythrocytes is linked to a stimulation of glucose uptake [42]. Since phorbol esters are known to stimulate choline incorporation into PC in various cell systems [29], we treated infected erythrocytes with PMA, but no effect on choline entry was observed. Nevertheless, it cannot be excluded that in infected cells the choline carrier has already reached a degree of phosphorylation that could not be further enhanced, or that eventual phosphorylation could involve protein kinases other than protein kinase C.

The general disturbance of the membrane properties, related to the infection by *Plasmodium*, could also contribute to form an appropriate environment allowing faster choline transport. The parasite provokes major modifications in the structure and composition of the membrane protein and lipid constituents of the erythrocyte, with notable alterations in PL molecular species [43]. Altogether, these drastic changes are associated with alterations in biochemical and physical membrane properties [44]. They certainly contribute to the appearance of new permeation pathways [32], but also to the augmentation of membrane fluidity [45], which is capable of inducing alterations in transport activity [46].

The other alternative to be considered is an augmentation of carrier number after infection. The fact that the rate constant of inactivation by NEM (k_o) was not modified after infection, whereas a large increase in $V_{\max}$ occurs, lends support to this hypothesis. This possibility also includes an unmasking of pre-existing carrier sites, which are hidden in normal erythrocytes, but unmasked after *Plasmodium* penetration into erythrocytes.

It has been shown that, as the blast cell matures into an erythrocyte, the number of transport systems for certain amino acids [47] or nucleosides [48] decreases. It is possible to delay or even inhibit the loss of nucleoside transporters during reticulocyte maturation, by ATP depletion or using metabolic inhibitors [48]. These data further suggest that there could be an intracellular pool of latent transporters in reticulocytes, which would be exposed under particular conditions. Immature erythrocytes are able to synthesize PC from choline [49] and may possess a larger number of active choline carriers than do erythrocytes. In this context, malarial invasion of erythrocytes could provoke a demasking of remnant sites that would have been masked during the maturation of the normal erythrocyte, whose biosynthetic needs are very limited or even non-existent [49].

A last possibility could be the synthesis of new carrier molecules. In the infected erythrocyte, insertion of new proteins (synthesized by the parasite itself) in the erythrocyte plasma membrane is now well established [50]. Thus the parasite could synthesize and export new choline carrier molecules toward the host membrane, resulting in increased choline permeability. In this case, it is particularly notable that the main characteristics of these newly synthesized carriers (in terms of affinity, effectors, asymmetry, rate-limiting step) would be very close to that of the normal cell.

In conclusion, we show that, after infection, choline entry into the erythrocytes remains strictly controlled. This occurs via a facilitated-diffusion mechanism which displays temperature-dependence, saturability with choline and specificity, and is inhibited by NEM, suggesting the presence of a thiol group involved in choline carrier activity. Inhibition by impermeant alkyltrimethylammoniums also suggests the existence of a

hydrophobic region adjacent to the active outward-facing site of the choline carrier. Results showing the dual effect of ethanolamine or imidazole on choline transport, depending on the choline concentration, and the possibility of preventing NEM inactivation by DM, fill the requirements of a cyclic model of an asymmetric carrier. The possibilities of producing a *trans*-acceleration with ethanolamine or imidazole, and of accelerating carrier inactivation by NEM with external choline, further suggest that re-orientation of the empty carrier is the rate-limiting step of choline transport. The increase, as much as 10-fold, in choline carrier activity after infection is not linked to the high rate of choline transformation by the parasite. The mechanisms for the large increase in choline carrier activity after infection by *Plasmodium* remain to be clarified (activation owing to the general disturbance of membrane properties and/or modifications in the nature or number of carriers?), but is probably related to the high amount of PC needed by the parasite for its membrane biogenesis. This demonstrates the acute adaptiveness of the parasite, which is able to modify the properties of its host cell to achieve its intracellular survival.

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