Enhanced choline and Rb⁺ transport in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*

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Human erythrocytes infected *in vitro* with the malaria parasite *Plasmodium falciparum* showed a markedly increased rate of choline influx compared with normal cells. Choline transport into uninfected cells (cultured in parallel with infected cells) obeyed Michaelis–Menten kinetics ($K_m \approx 11 \mu\text{M}$). In malaria-parasite-infected cells there was an additional choline-transport component which failed to saturate at extracellular concentrations of up to $500 \mu\text{M}$. This component was less sensitive than the endogenous transporter to inhibition by the *Cinchona* bark alkaloids quinine, quinidine, cinchonine and cinchonidine, but showed a much greater sensitivity than the native system to inhibition by piperine. The sensitivity of the induced choline transport to these reagents was similar to that of the malaria-induced (ouabain- and bumetanide-resistant) Rb⁺-transport pathway; however, the relative magnitudes of the piperine-sensitive choline and Rb⁺ fluxes in malaria-parasite-infected cells varied between cultures. This suggests either that the enhanced transport of the two cations was via functionally distinct (albeit pharmacologically similar) pathways, or that the transport was mediated by a pathway with variable substrate selectivity.

INTRODUCTION

Over the past 25 years it has been demonstrated that erythrocytes infected with the malaria parasite show enhanced membrane transport of a wide range of substrates, including anions, amino acids, sugars and nucleosides [see Ginsburg & Stein (1987) or Sherman (1988) for reviews]. However, there has been relatively little attention paid to the transport of cations across the membrane of the infected cell. In an early study, Dunn (1969) found a significant increase in the ouabain-insensitive 'leak' of ²²Na⁺ across the infected-cell membrane. Bookchin *et al.* (1980) found a similar increase in the ouabain-insensitive transport of ⁴²K⁺; however, it has been assumed generally that these increases reflect enhanced activity of endogenous transport systems (Ginsburg *et al.*, 1985; Ginsburg, 1988). In a recent study in which ⁸⁶Rb⁺ was used as a congener for K⁺, it was demonstrated that a large fraction of the enhanced ⁸⁶Rb⁺ influx into malaria-parasite-infected cells was via a pathway that remained operative in the presence of inhibitors of the pathways known to mediate K⁺ influx under physiological conditions (Kirk *et al.*, 1991). These results therefore suggested that the enhanced ⁸⁶Rb⁺ flux was mediated by a system that was absent (or at least inactive) in uninfected cells.

The present paper focuses on the enhanced transport of the organic cation choline across the membrane of the malaria-parasite-infected erythrocyte. The normal human erythrocyte possesses a (high-affinity, low-capacity) choline transporter which appears to follow Michaelis–Menten kinetics (Martin, 1977), though which does show some kinetic complexities (Krupka & Deves, 1980). After invasion of the erythrocyte by the malaria parasite, there is an increase in the rate of choline uptake (Elford *et al.*, 1990a,b). In the work reported here the kinetic and pharmacological characteristics of the induced transport are compared with those of the endogenous choline transporter as well as those of the malaria-induced Rb⁺-transport pathway.

(Aylesbury, Bucks., U.K.). Choline chloride (three-times-re-crystallized), cinchonine, cinchonidine, Hepes, ouabain, piperine, quinine and quinidine were from Sigma. Ionomycin was from Calbiochem. All buffer reagents were of analytical grade.

Bumetanide, ouabain, quinine and quinidine were added to cell suspensions as dilute solutions in saline. Cinchonine, cinchonidine and piperine were added as 13 mM solutions in dimethyl sulphoxide. Ionomycin was added as a 0.2 mM solution in dimethyl sulphoxide.

Parasite culture

Human erythrocytes (type O) infected with the ITO4 line of *Plasmodium falciparum* (Berendt *et al.*, 1989) were cultured as described previously (Elford *et al.*, 1985) in RPMI 1640 medium with 10% (v/v) human serum under decreased O₂ partial pressure (O₂/CO₂/N₂, 1:3:96). The culture medium was supplemented with D-glucose (10 mM), glutamine (2 mM) and gentamycin sulphate (25 mg·l⁻¹). Synchronous cultures containing 46–93% parasitized erythrocytes with 90–95% at the mature pigmented trophozoite stage (30–35 h post-invasion) were obtained by a combination of gelatin flotation (Pasvol *et al.*, 1978) and sorbitol lysis (Lambros & Vanderberg, 1979). Suspensions of uninfected erythrocytes from the same donor were incubated in parallel with the infected erythrocyte cultures under identical conditions. Cell concentrations were determined using a Coulter counter (Model Zm). Parasitaemia was estimated from methanol-fixed Giemsa-stained smears.

Flux measurements

Rates of choline and Rb⁺ entry into erythrocytes were estimated from the uptake of [¹⁴C]choline and ⁸⁶Rb⁺ respectively. ⁸⁶Rb⁺ in most cases served as a K⁺ congener and was added to cells suspended in solutions containing K⁺ (but no Rb⁺). In experiments in which the magnitudes of choline and Rb⁺ influx rates were compared directly, the suspension media contained Rb⁺ in place of K⁺ (Table 3 below).

For all experiments other than those giving rise to the inset to Fig. 2 (below), erythrocytes (both infected and uninfected) were washed four times in phosphate- and Hepes-buffered saline (which, unless stated otherwise, contained 134 mM-NaCl, 2.7 mM-KCl, 1.5 mM-KH₂PO₄, 8.1 mM-Na₂HPO₄ and 25 mM-Hepes,

EXPERIMENTAL

Materials

[methyl-¹⁴C]Choline chloride and ⁸⁶RbCl were from Amersham International. Bumetanide was a gift from Leo Laboratories

Fig. 1. Time courses for choline influx into infected (●) and uninfected (○) erythrocytes

The extracellular choline concentration was 50 μM . Both sets of data were averages of those from three separate experiments using cells from a single donor. The S.E.M. values fall within the symbols. The parasitaemia in the infected cell suspension was 72%. $[\text{Choline}]_i$ is the intracellular choline concentration.

pH 7.4) at 37 °C, then resuspended and combined with the appropriate reagent solutions in a 1.5 ml microcentrifuge tube to give $(1-4) \times 10^8$ erythrocytes $\cdot \text{ml}^{-1}$ for infected cells and $(2-10) \times 10^8$ erythrocytes $\cdot \text{ml}^{-1}$ for uninfected cells. The tubes were transferred to a 37 °C water bath at least 10 min before beginning the influx incubation to ensure temperature equilibration. Influx of radioisotope was commenced by the addition to the suspension of a solution of the isotope in saline (containing appropriate concentrations of unlabelled substrate) to give a final radioactivity of $\sim 2 \mu\text{Ci} \cdot \text{ml}^{-1}$. Influx was terminated by transferring aliquots (usually 145 μl) of the suspension to microcentrifuge tubes containing 800 μl of an ice-cold iso-osmotic Mops-buffered MgCl_2 solution (107 mM- MgCl_2 /10 mM-Mops, pH 7.4) layered over 250 μl of dibutyl phthalate. The tubes were centrifuged (10000 g , 30 s) to sediment the cells below the oil then the supernatant solution and dibutyl phthalate were aspirated. Traces of solution on the inner wall of the tube were removed with cotton dental rolls. The cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml), then deproteinized by the addition of 5% (w/v) trichloroacetic acid (0.5 ml) followed by centrifugation (10000 g , 10 min). The radioactivity in the supernatant solutions was measured by scintillation counting.

In most of the experiments reported here, initial influx rates were estimated from the radioactivity accumulated by the cells within a fixed incubation period. The amount of radioactivity trapped in the extracellular space of the cell pellet was estimated from a single (145 μl) sample taken from each flux suspension immediately after the addition of radioisotope, and processed as described above.

Experiments investigating the concentration-dependence of choline influx into uninfected cells alone (the results of which are shown in the inset to Fig. 2 below) were carried out by using a multiple-wash technique (Fervenza *et al.*, 1991). Uninfected cells (preincubated for 48 h at 37 °C in RPMI 1640 with 10% human serum) were washed four times then resuspended in phosphate- and Hepes-buffered saline. Aliquots of the suspension (0.2 ml, in triplicate) were dispensed into microcentrifuge tubes then cooled to 4 °C and combined with ice-cold saline (0.2 ml) containing both unlabelled and ^{14}C -labelled choline at appropriate concentrations. The final cell concentration was $\sim 12 \times 10^8$ erythrocytes $\cdot \text{ml}^{-1}$. Choline influx was commenced by transferring the samples to a 37 °C water bath, where they remained for 5 min before being placed on ice for 2 min (Young & Ellory, 1982). The

cells were then washed four times in ice-cold Mops-buffered MgCl_2 solution, then processed for scintillation counting as described above.

Preliminary experiments were carried out in order to assess the extent to which ^{14}C choline was incorporated into the trichloroacetic acid precipitates under the conditions used in the flux experiments. Cell pellets (separated from the extracellular solution by centrifugation through dibutyl phthalate) were either resuspended in saline (1 ml) then transferred directly to scintillation fluid, or extracted with a mixture of water (50 μl), propan-2-ol (550 μl) and chloroform (350 μl) (Rose & Oklander, 1965). The ^{14}C choline concentrations in the whole-cell suspension and in the water/propan-2-ol/chloroform extract were within 3 and 5% (respectively) of that in the trichloroacetic acid-soluble supernatant solution for both malaria-parasite-infected and uninfected cells. Similarly, when trichloroacetic acid precipitates (from typical experiments with both infected and uninfected cells) were redissolved in formic acid (1 ml), then transferred to scintillation fluid, there was found to be no incorporation of the ^{14}C label. All of the radiolabel transported into the cells during the flux incubation period was therefore present in the trichloroacetic acid-soluble supernatant solutions.

RESULTS

Choline transport

Fig. 1 shows time courses for the uptake of choline (present in the extracellular solution at a concentration of 50 μM) into human erythrocytes infected with *Plasmodium falciparum* and into (co-cultured) uninfected erythrocytes. The infected cells showed a greatly enhanced rate of choline uptake. Uptake into infected erythrocytes appeared approximately linear with time for up to ~ 5 min; uptake into normal cells appeared linear for at least 10 min. In subsequent experiments initial choline influx rates were estimated from the amount of ^{14}C choline accumulated during a 5 min incubation period in the case of infected cells and a 10 min incubation period in the case of uninfected cells.

Fig. 2 shows the concentration-dependence of the initial rate of choline transport into infected and uninfected cells. In uninfected cells, the choline influx obeyed Michaelis-Menten kinetics, with a K_m of $11.4 \pm 1.1 \mu\text{M}$ ($\pm$ S.E.M., $n = 3$). In malaria-parasite-infected cells there was an additional choline-transport component which increased approximately linearly with increasing choline concentration up to 500 μM . The magnitude of this component was highly variable; in five similar experiments with cells from different synchronized cultures judged to be 25–35 h post-invasion (46–93% parasitaemia) the rate constant defining the linear component $\{k = (\text{induced flux})/[\text{choline}]_o\}$, where $[\text{choline}]_o$ is the extracellular choline concentration} varied from 0.017 to 0.151 μmol of choline $\cdot \text{h}^{-1} \cdot 10^{12}$ erythrocytes $\cdot \mu\text{M}^{-1}$.

Table 1 shows the effects of a range of pharmacological agents (known to have antimalarial activity) on the endogenous choline transporter of uninfected cells and on the malaria-induced pathway. The compounds showed marked differences in their effects on the two systems. The *Cinchona* bark alkaloids, quinine and quinidine and their stereoisomers, cinchonine and cinchonidine (100 μM), appeared to inhibit the endogenous transporter with greater efficacy than they did the malaria-induced transport component. In contrast, piperine (100 μM) caused a $78 \pm 3\%$ ($\pm$ S.E.M., $n = 5$) inhibition of the induced pathway, but reduced choline influx into uninfected cells by only $27 \pm 3\%$ ($n = 5$).

Piperine is a lipophilic molecule, and a significant fraction of

Fig. 2. Concentration-dependence of choline influx into infected (●) and uninfected (○) erythrocytes

The main Figure shows results from a single experiment (in which the parasitaemia of the infected cell suspension was 93 %) which are representative of those obtained in five similar experiments with cells from different donors. The inset shows in more detail the concentration-dependence of choline influx into uninfected cells (incubated for 48 h in RPMI 1640 with 10 % human serum before carrying out the transport experiments). These data were averaged from separate experiments on cells from three different donors, and the curve was drawn using a simple Michaelis-Menten expression with $K_m = 11.4 \mu\text{M}$ and $V_{\text{max}} = 3.1 \mu\text{mol} \cdot \text{h}^{-1} \cdot 10^{12} \text{ erythrocytes}^{-1}$.

Table 1. Effect of quinine, quinidine, cinchonine, cinchonidine and piperine (100 μM) on choline influx via the endogenous transporter of uninfected cells and the induced pathway of malaria-parasite-infected cells

The extracellular choline concentration was 50 μM. Choline influx via the induced pathway was obtained by subtracting that measured in uninfected cells from that measured in parasitized cells for each of the drugs tested. Influx rates are expressed as a percentage of those measured in the absence of drug. Each result is averaged from *n* separate experiments, each of which was carried out on a different day using cells from a different donor.

Reagent	Choline influx (% of control)		<i>n</i>
	Endogenous pathway	Malaria-induced pathway	
Quinine	51 ± 7	70 ± 3	3
Quinidine	31 ± 9	66 ± 10	3
Cinchonine	69 ± 7	80 ± 9	3
Cinchonidine	45 ± 10	83 ± 11	3
Piperine	73 ± 3	22 ± 2	5

the drug added to the samples may have partitioned into the cell membranes. This being the case, it is possible that the marked difference between the effect of piperine on choline influx into infected and uninfected cells was due, at least in part, to the different cell concentrations used in the experiments with the two cell types. However, when the fractional inhibition by 100 μM-piperine of both the endogenous and induced pathways was plotted as a function of the cell concentrations used in five separate experiments [(1.2–3.2) × 10⁸ erythrocytes · ml⁻¹ in the case of infected cells and (2.2–6.8) × 10⁸ erythrocytes · ml⁻¹ in the case of uninfected cells] there was found to be little, if any, dependence of the degree of inhibition on the cell concentration for either cell type. This suggests that the apparent difference between the piperine-sensitivity of choline influx measured in

Table 2. Effect of quinine, quinidine, cinchonine, cinchonidine and piperine (100 μM) on Rb⁺ influx via the Gardos channel of uninfected cells and the induced (ouabain- and bumetanide-resistant) pathway of malaria-parasite-infected cells

All samples contained ouabain (100 μM) and bumetanide (100 μM). The Gardos channel was activated by the addition of ionomycin (1 μM) to uninfected cells suspended in Hepes-buffered saline containing 400 μM-Ca²⁺, 30 s before the addition of the ⁸⁶Rb⁺ solution. Initial Rb⁺ influx rates were estimated from the amount of ⁸⁶Rb⁺ accumulated during a 10 min incubation period in the case of infected cells and a 3–4 min incubation period in the case of uninfected cells to which ionomycin had been added (Kirk *et al.*, 1991). The magnitudes of the Ca²⁺-activated and parasite-induced fluxes were calculated by subtracting the residual ⁸⁶Rb⁺ influx measured in uninfected cells (in the absence of ionomycin) from that measured in uninfected cells pre-treated with ionomycin or in malaria-parasite-infected cells. Influx rates are expressed as a function of those measured in the absence of drug.

Reagent	Rb ⁺ influx (% of control)		<i>n</i>
	Gardos channel	Malaria-induced pathway	
Quinine	1 ± 1	75 ± 1	3
Quinidine	10 ± 5	73 ± 2	3
Cinchonine	80 ± 12	84 ± 2	3
Cinchonidine	73 ± 3	77 ± 6	3
Piperine	12 ± 4	34 ± 1	3

infected and uninfected cells is unrelated to differences in cell concentrations for the two cell types, but reflects a real difference in the sensitivity of the endogenous and induced transport pathways to the inhibitor.

Rb⁺ transport

Table 2 shows the effects of piperine and the four *Cinchona* alkaloids on the influx of ⁸⁶Rb⁺ through the Ca²⁺-activated K⁺ channel (the Gardos channel) of uninfected cells and via the induced (ouabain- and bumetanide-resistant) pathway of infected cells. Quinine and quinidine were effective inhibitors of the Gardos channel, but were relatively ineffective at inhibiting the malaria-induced Rb⁺ pathway. Cinchonine and cinchonidine inhibited both pathways only slightly. As has been reported previously (Kirk *et al.*, 1991), piperine was an effective inhibitor of both systems, though in these experiments it showed a somewhat greater efficacy against the Gardos channel.

Comparison of malaria-induced choline and Rb⁺ transport

It is clear from a comparison of the data of Tables 1 and 2 that the inhibitor-sensitivities of the malaria-induced choline- and Rb⁺-transport pathways are very similar. In particular, the enhanced fluxes of the two cations were effectively inhibited by piperine, and this raises the question as to whether the piperine-sensitive fluxes in the infected cells might be attributed to a single transport pathway. In this case there might be expected to be a strict correlation between the magnitudes of the induced choline and Rb⁺ fluxes in cells from different cultures. Table 3 compares the magnitude of the piperine-sensitive choline and Rb⁺ fluxes in five separate experiments using cells from different donors under conditions in which the piperine-sensitive fluxes were attributable almost exclusively to the malaria-induced pathway(s). In these experiments, infected cells were suspended in a K⁺-free solution, and RbCl or choline chloride were added at a concentration of 1 mM, so that the ‘driving force’ for the influx of the two ions was

Table 3. Comparison of the relative magnitudes of the malaria-induced choline and Rb⁺ fluxes in cells from five different donors

Malaria-infected cells were washed and suspended in a K⁺-free solution (140 mM-NaCl/1.5 mM-NaH₂PO₄/8.1 mM-Na₂HPO₄/25 mM-Hepes/10 mM-glucose, pH 7.4) at 37 °C, and RbCl or choline chloride were added (with trace amounts of either ⁸⁶Rb⁺ or [¹⁴C]choline) at a concentration of 1 mM. Ouabain (100 µM) was added to all samples. The piperine-sensitive flux was calculated in each case from the difference between the influx measured in the presence or absence of piperine (100 µM). Under these conditions the endogenous choline transporter was fully saturated (see Fig. 2) and, in all cases, it contributed less than 3.6% of the total measured choline uptake. The Rb⁺ uptake in the uninfected cells was as much as 30% of that measured in the infected cells under these conditions, but was entirely insensitive to piperine. [The flux measured in uninfected cells in the presence of 100 µM-piperine was 103 ± 2% (*n* = 4) of that measured in its absence.] The piperine-sensitive choline and Rb⁺ fluxes measured in malaria-infected cells may therefore both be attributed almost exclusively to the induced transport pathways.

Donor	Parasitaemia (%)	Piperine-sensitive influx (µmol · h ⁻¹ · 10 ¹² erythrocytes ⁻¹)		Choline influx Rb ⁺ influx
		Choline	Rb ⁺	
A	78	110	58	1.90
B	46	53	50	1.06
C	77	13.3	79	0.17
D	72	103	48	2.16
E	86	117	48	2.44

equivalent. The ratio of the induced choline flux to the induced Rb⁺ flux varied widely between experiments, from 0.17 to 2.44.

DISCUSSION

Measurements of choline influx into human erythrocytes infected with *Plasmodium falciparum* suggest that there is present, in the host-cell membrane, an additional transport pathway that is both kinetically and pharmacologically distinct from the choline transporter of normal erythrocytes. The pathway did not saturate at choline concentrations of up to 500 µM and showed a much greater sensitivity than the native system to inhibition by piperine. The pharmacological characteristics of the induced choline-transport pathway were very similar to those of the malaria-induced Rb⁺-transport pathway that has been described previously (Kirk *et al.*, 1991). However, the relative magnitudes of the malaria-induced (piperine-sensitive) choline and Rb⁺ fluxes varied widely between cell cultures. The simplest explanation for this variation is that the piperine-sensitive Rb⁺ and choline fluxes were mediated by separate (albeit pharmacologically similar) pathways. However, it is possible that the enhanced fluxes were via a single pathway which had a substrate selectivity that varied between different experiments as a consequence of variations in particular (and unknown) properties of the cells from different donors.

Normal erythrocytes have little capacity for phospholipid biosynthesis and have very low activities of enzymes associated with choline metabolism. By contrast, malaria-parasite-infected cells have a very active *de novo* Kennedy pathway by which choline is metabolized to phosphatidylcholine (Ancelin & Vial, 1986, 1989). Ancelin & Vial (1989) have demonstrated that, in monkey erythrocytes infected with *Plasmodium knowlesi*, the transport of choline into the host cell is a rate-limiting step in the

biosynthesis of phosphatidylcholine by the parasite. The malaria-induced pathway described here might therefore play an essential role in ensuring an adequate supply of choline to the intracellular parasite.

The physiological role of the induced K⁺ pathway may relate to its ability to mediate net K⁺ efflux and thereby counter the swelling effect imposed by such factors as the presence of the growing parasite within the cell, the alteration of the cell membrane permeability and the generation of osmotically active molecules in the cytosol by the digestion of haemoglobin (Ginsburg, 1988). It has been suggested recently that the Gardos channel may be activated in infected cells and that it may play a primary role in volume regulation of the host cell (Ginsburg, 1990). Furthermore, it was proposed that the known sensitivity of the channel to inhibition by quinine may, in part, underlie the drug's antimalarial activity (Ginsburg, 1990). It is clear from Table 2 that the enhanced (ouabain- and bumetanide-resistant) Rb⁺ 'leak' in infected cells was much less sensitive than the Ca²⁺-activated Gardos channel to inhibition by either quinine or quinidine. Thus, if the enhanced Rb⁺ leak is attributable to the Gardos channel, the properties of this pathway in the infected cell must be somewhat different from those of the channel in the uninfected cell.

The increased permeability of erythrocytes to anionic and electroneutral substrates on infection of the cell by the malaria parasite is well documented and has been attributed to transport pathways that are distinct from the endogenous transport systems (Ginsburg & Stein, 1987; Ginsburg, 1988). It has generally been considered that the malaria-induced pathways are inaccessible to cations and that any increase in cation permeability is attributable to increased activity of the native transporters (Ginsburg, 1988). In the present study we have shown that the characteristics of the induced transport of the cations choline and Rb⁺ are different from those of the endogenous transport systems. It therefore seems likely that, contrary to the accepted view, the enhanced transport of cations across the infected cell membrane is not attributable to increased capacity of native transport systems but represents transport through pathways that are absent from the normal erythrocyte.

The biophysical nature of the malaria-induced transport pathways remains to be elucidated. The observation that the pathway is prone to inhibition argues against the enhanced fluxes being attributable to general membrane damage or disruption. The failure of the induced choline transport to saturate at concentrations far in excess of the normal physiological plasma concentration (~13 µM; Bligh, 1952) is suggestive of a pathway having the properties of a pore or channel rather than those of a carrier (such as the endogenous choline transporter). Similar characteristics have been demonstrated for the transport of many other solutes across the membrane of malaria-infected cells (e.g. Ellory *et al.*, 1988; Gati *et al.*, 1990) though, in all cases, the identities of the induced pathways are yet to be established.

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