Transport of lactate and pyruvate in the intraerythrocytic malaria parasite, *Plasmodium falciparum*

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The mature, intraerythrocytic form of the human malaria parasite, *Plasmodium falciparum*, is reliant on glycolysis for its energetic requirements. It produces large quantities of lactic acid, which have to be removed from the parasite's cytosol to maintain the cell's integrity and metabolic viability. Here we show that the monocarboxylates lactate and pyruvate are both transported across the parasite's plasma membrane via a H⁺/monocarboxylate symport process that is saturable and inhibited by

the bioflavonoid phloretin. The results provide direct evidence for the presence at the parasite surface of a H⁺-coupled monocarboxylate transporter with features in common with members of the MCT (monocarboxylate transporter) family of higher eukaryotes.

Key words: glycolysis, osmoregulation, pH regulation.

INTRODUCTION

The intraerythrocytic form of the human malaria parasite, *Plasmodium falciparum*, lacks a functional citric acid cycle and is largely reliant on glycolysis to fulfil its very substantial energy requirements [1]. Human erythrocytes infected with mature (trophozoite-stage) parasites consume glucose up to two orders of magnitude faster than normal, uninfected erythrocytes [2,3], ultimately converting it to lactic acid [4,5]. Accumulation of lactic acid within the parasite cytosol would lead to a chronic decrease in intracellular pH (pH_i) and would threaten the osmotic stability of the cell. It would also interfere with the oxidation of glycolytically derived NADH to NAD⁺, which occurs through the conversion of pyruvate to lactate via lactate dehydrogenase [1]. For all of these reasons it is important that the parasite have an efficient means of clearing lactic acid from its cytosol.

In many cells, monocarboxylates such as lactate are transported across the plasma membrane via H⁺-linked monocarboxylate transporters (MCTs), which operate with a stoichiometry of 1:1 (i.e. 1 H⁺ per monocarboxylate anion; reviewed in [6]). A family of mammalian MCTs has been identified, as have related proteins from non-mammalian species. MCTs are susceptible to inhibition by a range of compounds, including the bioflavonoid phloretin, substituted aromatic monocarboxylates such as cinnamic acid derivatives, the thiol reagent *p*-chloromercuribenzenesulphonate (pCMBS) and anion transport inhibitors such as niflumic acid.

Two previous studies have investigated the transport of lactate across the plasma membrane of the intraerythrocytic form of *P. falciparum* parasites. Kanaani and Ginsburg [7], working with parasitized erythrocytes permeabilized with Sendai virus, presented evidence that the transport of L-lactate across the parasite's plasma membrane is linked to H⁺ flux and that it is rapid, insensitive to cinnamic acid derivatives or pCMBS and unaffected by the presence of the monocarboxylate pyruvate. However, the results do not permit any conclusions on whether the transport was carrier-mediated (as opposed to occurring via a process of simple diffusion of the protonated acid). In a subsequent study, evidence was obtained for the presence in the parasitized erythrocyte of a saturable and α-cyano-4-

hydroxycinnamate-sensitive H⁺-linked carrier, which was postulated to be located on the parasite's plasma membrane [8]. However, the experiments were performed exclusively on intact *P. falciparum*-infected erythrocytes, and it was not possible to distinguish unambiguously between transport across parasite and host cell membranes; neither was it possible to rule out a contribution of the endogenous erythrocyte MCT to the measured fluxes.

In this study we have used *P. falciparum* parasites 'isolated' from their host erythrocyte by a saponin permeabilization procedure to make direct measurements of the transport of L-lactate, D-lactate and pyruvate across the parasite's membrane. Our results confirm the earlier finding that the movement of these monocarboxylates is H⁺-linked [7,8] and demonstrate the presence at the parasite surface of a transporter that is both saturable and inhibited by phloretin.

EXPERIMENTAL

Materials

L-[¹⁴C]Lactic acid (specific radioactivity 154 mCi/mmol), [¹⁴C]sucrose (556 mCi/mmol), ³H₂O (18 mCi/mol) and ⁸⁶Rb⁺ (131 mCi/mmol) were from Amersham. 2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes, D-lactate from ICN, and *n*-butyrate from BDH. All other chemicals were from Sigma.

Parasite culture

P. falciparum parasites (FAF6, cloned from ITG2 [9]) were maintained *in vitro* as described previously [10], with modifications [11]. Parasite cultures were synchronized with sorbitol [12]. All experiments were performed on late-stage parasites (36–40 h after invasion) by using infected cells in which both the host erythrocyte membrane and the parasitophorous vacuole membrane (in which the intracellular parasite is enclosed [13]) were permeabilized with saponin (0.05 %, w/v) as described previously [14,15].

Abbreviations used: BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; β_i, intracellular buffering capacity; MCT, monocarboxylate transporter; pCMBS, *p*-chloromercuribenzenesulphonate; pH_i, intracellular pH; ΔpH_i^{max}, maximum change in intracellular pH.

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Measurements of L-[¹⁴C]lactic acid influx and efflux

Measurement of the uptake of L-[¹⁴C]lactic acid into saponin-derived parasites was performed as described previously for other substrates [14]. All measurements were performed at 5 °C to slow the transport process sufficiently to allow its characterization.

In brief, saponin-derived parasites were suspended at $(0.5\text{--}1.9) \times 10^8$ cells/ml in a minimal saline solution (125 mM NaCl/5 mM KCl/20 mM glucose/25 mM Hepes/25 mM Mes/1 mM MgCl₂) at pH 7.1 or 5.5, then incubated with 0.2 μ Ci/ml L-[¹⁴C]lactic acid for a specified period. At the end of the incubation, an aliquot (0.2 ml) of the suspension was layered on 0.35 ml of dibutyl phthalate/dioctyl phthalate (5:4, v/v) in a 1.5 ml microcentrifuge tube and centrifuged (15800 g, 2 min) to separate the cells from the extracellular solution and thereby terminate the uptake of radiolabel.

In each experiment, the amount of radiolabel trapped in the extracellular space within the cell pellets was estimated by using the K⁺ congener ⁸⁶Rb⁺, which, in the presence of K⁺ channel blockers such as Ba²⁺ or Cs⁺, serves as an effective extracellular space marker (R. J. W. Allen, K. J. Saliba and K. Kirk, unpublished work). ⁸⁶Rb⁺ (typically 0.2 μ Ci/ μ l) was added to cells suspended (at the same concentration as that used in the L-[¹⁴C]lactic acid uptake experiments) in saline that contained either 5 mM BaCl₂ or 5 mM CsCl to inhibit ⁸⁶Rb⁺ uptake via K⁺ channels in the parasite's plasma membrane. Immediately after the addition of ⁸⁶Rb⁺, aliquots (0.2 ml) of the suspension were layered on 0.35 ml of the oil mix in a 1.5 ml microcentrifuge tube, then centrifuged to sediment the cells below the oil (as above).

In L-[¹⁴C]lactic acid efflux experiments, saponin-derived parasites were preloaded with L-[¹⁴C]lactic acid by suspension for approx. 5 min in saline at pH 5.5 and 5 °C, in the presence of the radiolabel (0.2 μ Ci/ml). The L-[¹⁴C]lactic-acid-loaded cells were then centrifuged (15800 g, 20 s) and resuspended in a solution at pH 7.1. At predetermined time intervals, aliquots of cells (0.4 ml) were transferred to 1.5 ml microcentrifuge tubes containing 0.35 ml of the oil mix, then centrifuged (15800 g, 2 min).

In both influx and efflux experiments the cell pellets beneath the oil layer were processed for scintillation counting as described previously [14].

In presenting the results of the L-[¹⁴C]lactic acid influx experiments, the amount of L-[¹⁴C]lactic acid taken up by the parasites is expressed in terms of the L-[¹⁴C]lactic acid 'distribution ratio', $[L\text{-}[^{14}\text{C}]\text{lactic acid}]_i/[L\text{-}[^{14}\text{C}]\text{lactic acid}]_o$ (i.e. the concentration of L-[¹⁴C]lactic acid inside the parasites relative to that in the extracellular solution). $[L\text{-}[^{14}\text{C}]\text{lactic acid}]_i$ was determined from the measured uptake of L-[¹⁴C]lactic acid and the intracellular water volume in the cell pellet, calculated from the parasite number and the water volume of a single saponin-derived parasite, estimated (using ³H₂O as described previously [14]) to be 20.5 ± 1.8 fl (mean $\pm$ S.E.M.; $n = 7$ experiments).

pH_i measurements with BCECF

The pH_i of the saponin-derived parasites was monitored at 5 °C with the intracellular fluorescent pH indicator BCECF, as described previously [15]. The parasites were suspended in a minimal saline solution containing 125 mM NaCl, 5 mM KCl, 20 mM glucose, 25 mM Hepes and 1 mM MgCl₂ at pH 7.1. In experiments monitoring the effect of the influx of monocarboxylates on pH_i, the monocarboxylates were added to the cell suspension as the sodium salt. Measurements were performed with a Perkin Elmer LS-50B spectrofluorimeter. With the use of a dual-excitation 'Fast Filter' accessory, the suspension of

BCECF-loaded parasites was excited successively at 440 nm and 495 nm and the fluorescence emission was measured at 520 nm.

In experiments monitoring the effect on pH_i of the efflux of lactate from the parasites, BCECF-loaded, saponin-derived parasites were suspended in a small volume (50 μ l) of 5 mM sodium lactate in a cuvette for approx. 15 min before the addition of 1.95 ml of saline [125 mM NaCl/5 mM KCl/20 mM glucose/25 mM Hepes/1 mM MgCl₂ (pH 7.1)]. This decreased the extracellular lactate concentration to approx. 0.125 mM, thereby creating an outwards lactate concentration gradient ($[L\text{-lactate}]_i/[L\text{-lactate}]_o \approx 40$).

In each experiment, BCECF fluorescence was calibrated with nigericin (0.7 μ M) added to cells suspended in solutions containing a high K⁺ concentration (130 mM KCl/20 mM glucose/25 mM Hepes/1 mM MgCl₂) and adjusted to pH values of 6.8, 7.1 and 7.8.

The buffering capacity of the parasites was determined (at 5 °C) by adding various concentrations (10–40 mM) of either weak base (NH₄Cl) or weak acid (propionic acid or butyric acid) to BCECF-loaded parasites. The buffering capacity of the parasite cytosol (β_i) was calculated from the difference between the observed change in pH_i and that predicted to occur in the absence of intracellular buffering, as described elsewhere [16,17]. β_i showed a non-linear dependence on pH_i, increasing exponentially as pH_i decreased (results not shown). Regression analysis of the data yielded a β_i of 32 mmol of H⁺/litre of cell water per pH unit at pH 7.21, the average baseline pH_i of the parasite at 5 °C (see the Results section). This value was used in the evaluation of H⁺ transport rates. In those experiments in which the baseline pH_i was affected by high concentrations of phloretin (200 μ M or more; see the Results section), the appropriate β_i was calculated and used accordingly.

RESULTS

H⁺/monocarboxylate symport

As shown in Figure 1(A), saponin-derived parasites suspended in a minimal saline solution of pH 7.1 accumulated L-[¹⁴C]lactic acid to a maximum concentration approximately double that in the extracellular medium. When the pH of the suspending medium was decreased to 5.5, the L-[¹⁴C]lactic acid distribution ratio increased to a maximum of approx. 17 (after approx. 5 min). These results show that an inwardly directed H⁺ gradient could induce lactate accumulation by the parasite, which is consistent with the hypothesis that lactate is transported together with H⁺ ions across the parasite's plasma membrane.

Figure 1(B) illustrates the effect of (unlabelled) L-lactate, as well as that of D-lactate and pyruvate, each at a concentration of 40 mM, on the pH_i of saponin-derived parasites loaded with BCECF and suspended at 5 °C in saline (pH 7.1). Under the conditions of the experiment, the resting pH_i of the parasites was in the range 7.03–7.36, with a value of 7.21 ± 0.02 (mean $\pm$ S.E.M.; $n = 22$). The addition of each of the three monocarboxylates to the suspending medium resulted in an immediate acidification of the parasite cytosol. The rates of acidification after the addition of 40 mM L-lactate and D-lactate were the same [122 ± 12 mmol H⁺/min per litre of cell water ($n = 7$) and 123 ± 14 mmol H⁺/min per litre of cell water ($n = 7$) respectively], and substantially higher than that after the addition of 40 mM pyruvate (27 ± 4 mmol H⁺/min per litre of cell water; $n = 5$). In each case the monocarboxylate-induced acidification was followed by a slow recovery of pH_i. The decrease in pH_i after the addition of each of the three monocarboxylates was consistent with the entry of these compounds into the parasite together with H⁺ ions.

Figure 1 H^+ /lactate symport into saponin-derived *P. falciparum* trophozoites

(A) Time courses for the uptake of L-[^{14}C]lactic acid by saponin-derived parasites in the presence and the absence of an inwardly directed [H^+] gradient. Parasites were suspended (at zero time) in a HEPES/MES-buffered solution, pH-adjusted to either 7.1 (○) or 5.5 (●) and containing L-[^{14}C]lactic acid (0.2 μ Ci/ml; 154 mCi/mmol). Results are from three separate experiments, each performed in duplicate, and are means $\pm$ S.E.M. Where not shown, error bars lie within the symbols. (B) Monocarboxylate-induced acidification of saponin-derived parasites loaded with the pH_i indicator BCECF. L-Lactate, D-lactate and pyruvate were each added at 40 mM. The traces are each from a single representative experiment.

Figure 2 Stoichiometry of H^+ /lactate influx in saponin-derived *P. falciparum* trophozoites

Comparison of $\Delta pH_i^{\max}$ induced by equimolar concentrations of L-lactate (○) and of butyrate (●). Results are from four separate experiments and are means $\pm$ S.E.M.

Stoichiometry of the transport process

The stoichiometry of lactate/ H^+ symport was investigated by comparing the maximum changes in pH_i ($\Delta pH_i^{\max}$) observed after the addition of increasing concentrations of L-lactate and the more lipophilic monocarboxylate butyrate to the extracellular medium, as has been described previously [18,19]. Butyrate crosses cell membranes predominantly via a process of simple diffusion of the undissociated (neutral) acid, doing so with an effective stoichiometry of 1 H^+ per butyrate molecule. As shown in Figure 2, there was no significant difference between the $\Delta pH_i^{\max}$ induced by equimolar concentrations of L-lactate and butyrate, which is consistent with the hypothesis that lactate and H^+ ions are transported across the parasite's plasma membrane with a stoichiometry of 1:1.

Kinetics of the transport process

Experiments similar to those shown in Figure 1(B) were performed with a range of different concentrations (1–40 mM) of L-

lactate, D-lactate and pyruvate. In each case the initial portions of the resulting pH_i traces were fitted to a first-order exponential equation and the initial slope (i.e. the initial rate of acidification of the cytosol) was calculated for each of the fitted curves [shown in Figure 3(A) for L-lactate]. This was multiplied by β_i to give an initial rate of H^+ influx.

For all three compounds, H^+ influx showed a non-linear dependence on the concentration of monocarboxylate (Figure 3B). In each case the results were fitted to a Michaelis–Menten equation, incorporating an additional linear component, the contribution of which was limited to a maximum of 20% of the influx measured at a monocarboxylate concentration of 40 mM. This corresponds to the approximate percentage of the total flux that was observed in parasites treated with 800 μ M phloretin (see below) and is therefore the maximum that can be attributed to a non-inhibitable transport process.

For L-lactate and D-lactate the estimated $V_{\max}$ values were similar: 94 ± 6 mmol H^+ /min per litre of cell water ($n = 7$) and 105 ± 8 mmol H^+ /min per litre of cell water ($n = 7$) respectively. However, the estimated K_m for L-lactate (3.8 ± 0.8 mM) was significantly lower than that for D-lactate (7.1 ± 0.9 mM; paired t test, $P = 0.013$), indicating a degree of stereoselectivity for the transport process. Pyruvate was transported at lower capacity and with lower affinity, with an estimated $V_{\max}$ of 34 ± 8 mmol H^+ /min per litre of cell water and an estimated K_m of 15.7 ± 3.3 mM ($n = 5$).

Inhibition of H^+ /monocarboxylate influx by phloretin

The bioflavonoid phloretin, an inhibitor of monocarboxylate transport in a wide range of cell types (see, for example, [19–21]), inhibited the influx of L-lactate and pyruvate into saponin-derived parasites.

Figure 4(A) illustrates the effect of 100 μ M phloretin on the influx of L-[^{14}C]lactic acid into saponin-derived parasites suspended in saline at pH 5.5. The data were fitted to a first-order exponential equation to permit the estimation of the initial rate of L-[^{14}C]lactic acid influx (i.e. the initial slopes of the influx time courses) in the presence and the absence of phloretin. At this concentration the bioflavonoid decreased the initial influx rate by $46 \pm 17\%$ (paired t test, $P = 0.046$; $n = 4$).

Figure 4(B) shows full dose–response curves for the inhibition by phloretin of the L-lactate-induced and pyruvate-induced influx

Figure 3 Kinetics of H^+ /lactate symport in saponin-derived *P. falciparum* trophozoites

(A) Time-dependent change in pH_i of saponin-derived parasites in the initial period after the addition of increasing concentrations of L-lactate. ΔpH_i is the difference between the resting pH_i before the addition of L-lactate (an average of the pH_i values obtained over the 10 s immediately before lactate addition) and the pH_i at a given time t after L-lactate addition. The data obtained at each L-lactate concentration were fitted to the first-order equation $\Delta pH_i = \Delta pH_i^{\max}(1 - e^{-kt})$, where $\Delta pH_i^{\max}$ is the maximum decrease in pH after the addition of lactate and k is the first-order rate constant for the acidification. The success of the curve-fitting depended on the time of addition of lactate being known with a high level of precision; this was determined by extrapolation of the first-order equation fitted to the original data. The results shown are from a single representative experiment. (B) Concentration dependence of the initial rate of H^+ influx induced by L-lactate ($\circ$), by D-lactate ($\bullet$) and by pyruvate ($\blacksquare$). The initial rate of monocarboxylate-induced H^+ influx at each monocarboxylate concentration was estimated by multiplying the initial rate of acidification [determined from the initial slope of the fitted curves shown in (A); i.e. $\Delta pH_i^{\max}k$] by β_i (i.e. H^+ influx = $\beta_i \Delta pH_i^{\max}k$). The results for each monocarboxylate were fitted to a Michaelis–Menten equation incorporating a linear component (k_d), which was restricted to contribute a maximum of 20% of the influx measured at a monocarboxylate concentration of 40 mM (see the text) [H^+ influx = $V_{\max}[\text{monocarboxylate}]/(K_m + [\text{monocarboxylate}]) + k_d[\text{monocarboxylate}]$]. For L-lactate, $K_m = 3.8 \pm 0.8$ mM, $V_{\max} = 94 \pm 6$ mmol H^+ /min per litre of cell water and $k_d = 0.8 \pm 0.3$ min $^{-1}$; for D-lactate, $K_m = 7.1 \pm 0.9$ mM, $V_{\max} = 105 \pm 8$ mmol H^+ /min per litre of cell water and $k_d = 0.8 \pm 0.3$ min $^{-1}$; for pyruvate, $K_m = 15.7 \pm 3.3$ mM, $V_{\max} = 34 \pm 8$ mmol H^+ /min per litre of cell water and $k_d = 0.11 \pm 0.01$ min $^{-1}$. The results are from seven (L-lactate and D-lactate) and five (pyruvate) separate experiments, and are means $\pm$ S.E.M.

Figure 4 Effect of phloretin on H^+ /lactate influx in saponin-derived *P. falciparum* trophozoites

(A) Uptake of L-[^{14}C]lactic acid into saponin-derived parasites suspended in medium of pH 5.5 in the presence ($\bullet$) and the absence ($\circ$) of 100 μ M phloretin. Results are from three separate experiments, each performed in duplicate, and are means $\pm$ S.E.M. The data are fitted to the equation $L-[^{14}C]\text{lactic acid distribution ratio} = a(1 - e^{-kt})$, where a is the maximum L-[^{14}C]lactic acid distribution ratio, k is the rate constant for the process and t is the time after the addition of L-[^{14}C]lactic acid. The initial influx rate (given by ak) measured in the presence of phloretin (i.e. 12.0 ± 2.9 min $^{-1}$) was significantly lower than that measured in its absence (i.e. 22.2 ± 3.6 min $^{-1}$; $P = 0.046$, paired t test). (B) Dose–response curves for the effect of phloretin on the initial rate of H^+ influx induced by 40 mM L-lactate ($\circ$) and by 40 mM pyruvate ($\bullet$) in BCECF-loaded parasites. The results are from three separate experiments (means $\pm$ S.E.M.) and were fitted by non-linear least-squares regression to the equation H^+ influx (% of control) = $b + c/[1 + ([\text{phloretin}]/IC_{50})^d]$, where ' H^+ influx' denotes the initial rate of monocarboxylate-induced H^+ influx, expressed as a percentage of that measured in the absence of inhibitor, b , c and d are fitted parameters ($b + c$ is the maximum H^+ influx and d is the minimum H^+ influx) and IC_{50} is the concentration of phloretin at which inhibition of H^+ influx is half-maximal (i.e. at which H^+ influx = $b + 0.5c$). The estimated IC_{50} values for the inhibition by phloretin of the L-lactate-induced and pyruvate-induced H^+ influx were 74 ± 8 and 66 ± 19 μ M respectively.

of H^+ ions into BCECF-loaded parasites. Each monocarboxylate was added at 40 mM and the extracellular pH was 7.1. H^+ influx rates were calculated as described for Figure 3. In all experiments the phloretin was added 2–10 min before the monocarboxylate. High concentrations of phloretin (200 μ M or more) were found to cause a time-dependent decrease (0.05–0.25 pH units) in the pH_i of the saponin-derived parasites. The pH_i was allowed to

stabilize before the addition of either L-lactate or pyruvate; in each case the β_i used in the calculation of the initial H^+ influx rate was that appropriate for the measured starting pH_i .

At the highest concentrations tested, phloretin inhibited the L-lactate-induced and pyruvate-induced H^+ influx rate by approx. 80%. The dose–response curves for the two monocarboxylates were very similar, which is consistent with the sharing of a

Figure 5 Efflux of H^+ /lactate from saponin-derived *P. falciparum* trophozoites

(A) Time courses for the efflux of L-[^{14}C]lactic acid from saponin-derived parasites in the presence (●) and the absence (○) of 100 μM phloretin. Parasites were preloaded with L-[^{14}C]lactic acid by suspension for 5 min in saline solution of pH 5.5 containing the radiolabel (0.2 μCi/ml), then sedimented by centrifugation and resuspended (at zero time) in medium at a pH of 7.1 (with or without phloretin). The results are from three separate experiments, each performed in duplicate, and are means $\pm$ S.E.M. The data were fitted to the equation $[L-^{14}C]lactic\ acid]_i = [L-^{14}C]lactic\ acid]_{i,t=\infty} + f e^{-kt}$, where $[L-^{14}C]lactic\ acid]_{i,t=\infty}$ is the amount of L-[^{14}C]lactic acid remaining in the cells once efflux is complete, f is the fraction of L-[^{14}C]lactic acid lost from the cells as a result of the efflux, k is the rate constant for the process and t is the time from the commencement of efflux. The initial efflux rate (given by f/k) measured in the presence of phloretin (i.e. $113 \pm 8\ min^{-1}$) was significantly lower than that measured in its absence (i.e. $201 \pm 13\ min^{-1}$; $P = 0.012$, paired t test). (B) Effect of lactate efflux on pH_i of saponin-derived parasites loaded with BCECF. Cells were preloaded with lactate by suspension for 15 min in medium containing 5 mM lactate at 5 °C. The suspension was then diluted (at the points marked by the arrows) by the addition of medium containing either 0 or 5 mM lactate, yielding a final extracellular lactate concentration of either approx. 0.125 mM (in which case $[L-lactate]_i/[L-lactate]_o \approx 40$) or 5 mM (in which case $[L-lactate]_i/[L-lactate]_o \approx 1$) respectively. The traces are from a single experiment, representative of those obtained in traces from four separate experiments on different cell preparations.

common transporter by the two compounds. Results from each experiment were fitted to a sigmoidal curve, permitting the estimation of IC_{50} values for phloretin; for the L-lactate-induced and pyruvate-induced H^+ influx these were 74 ± 8 and $66 \pm 19\ \mu M$ (means $\pm$ S.E.M.; $n = 3$) respectively.

Attempts to test a number of other MCT inhibitors were unsuccessful. The coloured cinnamic acid derivative α -cyano-4-hydroxycinnamate interfered with the BCECF fluorescence signal when added to the cuvette at 5 mM, whereas niflumic acid seemed to act as a protonophore, decreasing the baseline pH_i of BCECF-loaded parasites to levels similar to the extracellular pH when added at concentrations below that required to cause significant inhibition of lactate-induced H^+ influx.

Efflux of lactic acid from saponin-derived parasites

Although measuring the influx of lactate (and the concomitant influx of H^+ ions) permits the characterization of the transport process, it is important to recognize that *in vivo* there is a net efflux of lactate from the *P. falciparum* trophozoite [5].

Figure 5(A) shows time courses for the efflux of L-[^{14}C]lactic acid from saponin-derived parasites suspended in the presence and the absence of 100 μM phloretin. The results were fitted to a first-order exponential equation to permit the estimation of the initial rate of L-[^{14}C]lactic acid efflux. The initial rate of efflux in the presence of 100 μM phloretin was decreased by $44 \pm 5\%$ relative to the control (paired t test, $P = 0.012$; $n = 3$).

Figure 5(B) shows the effect on pH_i of a net efflux of L-lactate from saponin-derived parasites. Parasites were preloaded with L-lactate by incubating them for approx. 15 min in saline containing 5 mM L-lactate. At zero time the cells were diluted by the addition of a 40-fold excess of either lactate-free saline (left-hand trace) or saline containing 5 mM lactate (right-hand trace). In the former case the extracellular lactate concentration was decreased to approx. 0.125 mM, thus creating an initial outward lactate concentration gradient ($[L-lactate]_i/[L-lactate]_o$) of approx.

40 (see the Experimental section). The parasites responded to the dilution by undergoing a rapid and pronounced alkalinization, followed by a partial recovery of pH_i . In contrast, for parasites preloaded with 5 mM L-lactate and then exposed to saline having the same lactate concentration (under which conditions $[L-lactate]_i/[L-lactate]_o \approx 1$), there was little effect on pH_i .

The alkalinization observed in the presence (but not in the absence) of an outwardly directed lactate concentration gradient (Figure 5B) is consistent with a net loss of H^+ ions from the cell caused by the efflux of lactate (down its concentration gradient). Together, the results in Figure 5 indicate that the efflux of L-lactate from the *P. falciparum* trophozoite is, like the influx of monocarboxylates, via a H^+ -coupled process, inhibited by phloretin.

DISCUSSION

A H^+ -coupled MCT in the parasite's plasma membrane

The intraerythrocytic malaria parasite has a vigorous anaerobic metabolism. It generates high levels of lactic acid that must be removed from the cell to avoid its build-up to cytotoxic levels. In a previous study of the transport of radiolabelled lactate in *P. falciparum*-infected human erythrocytes permeabilized with Sendai virus, Kanaani and Ginsburg [7] obtained evidence that lactate crosses the parasite's plasma membrane in conjunction with H^+ ions. In this early study it was found that neither cinnamic acid derivatives, pCMBS nor pyruvate (the latter at an unspecified concentration) inhibited lactate influx; it was concluded that lactic acid was transported across the parasite's plasma membrane either via a process of free diffusion of the undissociated acid or via a novel H^+ /lactate symport mechanism. In a subsequent study in which the influx of both L-lactate and D-lactate was measured in intact *P. falciparum*-infected human erythrocytes, Cranmer et al. [8] presented further evidence for the involvement of a H^+ gradient in the transport of lactate across

the parasite's plasma membrane, as well as for the process not being highly stereoselective (differing in this respect from the transport of lactate across the membrane of normal, uninfected human erythrocytes [22,23]). It was postulated that the transport of lactate across the parasite's plasma membrane might be via a H^+ -linked transporter; however, there was no direct evidence for this.

The present study confirms the involvement of H^+ ions in the transport of lactate across the parasite membrane (demonstrating both H^+ -induced lactate accumulation and lactate-induced H^+ flux) and provides evidence that the transport occurs predominantly via a H^+ /monocarboxylate symporter. The findings that lactate and pyruvate transport are both saturable (albeit with low affinity; Figure 3) and inhibitable (by the bioflavonoid phloretin; Figures 4 and 5A) are consistent with a transporter-mediated process, as is the finding that there is a significant (approx. 2-fold) difference in the affinity of the process for L-lactate and D-lactate (Figure 3B).

The similarity of the dose-response curves for the inhibition of the transport of L-lactate and pyruvate by phloretin (Figure 4B) is consistent with the two monocarboxylates sharing a common transporter across the parasite membrane, although the involvement of more than one transporter cannot be ruled out. In any case, the process operates with a stoichiometry of 1 H^+ per monocarboxylate (L-lactate) anion. It carries monocarboxylates both into and out of the intracellular parasite, and is likely to serve as the major route for the efflux of metabolically derived lactic acid from the parasite.

Comparison of the parasite transporter with mammalian MCTs

The functional characteristics of the transport process described here show both similarities to and differences from those of the mammalian MCTs. The finding that the estimated V_{max} for the transport of L-lactate (94 mmol H^+ /min per litre of cell water) across the parasite's plasma membrane is higher than that for pyruvate (34 mmol H^+ /min per litre of cell water) is consistent with what has been found for other cell types [19,20,24,25]. The finding that pyruvate is transported into the parasite with a higher K_m (15.7 mM) than that for L-lactate (3.8 mM) contrasts with the situation in a range of mammalian cell types [19,20,24–26] and for the cloned transporters MCT1 and MCT2 [21,27–29], which transport pyruvate with higher affinity (i.e. a lower K_m) than L-lactate. However, giant sarcolemmal vesicles from skeletal muscle fibres, containing predominantly MCT4 (with some MCT1), have been shown to transport pyruvate with a higher K_m than that for lactate [30], and the same is true of MCT4 expressed in *Xenopus* oocytes [31,32].

The degree of stereoselectivity among the mammalian MCTs is somewhat variable. MCT1 is stereospecific for L-lactate (with the K_m for L-lactate being an order of magnitude lower than that for D-lactate), although not for several other monocarboxylates [6]. A recent study characterizing MCT4 expressed in *Xenopus* oocytes (using BCECF to monitor H^+ -linked monocarboxylate influx) reported a strong stereoselectivity for L-lactate over D-lactate [32]. However, another report characterizing the same transporter in the same expression system (but measuring the influx of L-[^{14}C]lactic acid), showed a much lower degree of stereoselectivity for lactate, with the K_m for L-lactate being just 2–3-fold lower than the K_i for the effect of D-lactate on L-lactate influx [31]. This is similar to the situation found here in the malaria parasite. In this context it is relevant to note that an estimated 6–7% of the lactate produced by the parasite is in the form of D-lactate [5]; the ability of the parasite to transport D-lactate is therefore of physiological significance.

The IC_{50} for inhibition by phloretin of the transport of L-lactate across the parasite's plasma membrane ($74 \pm 8 \mu M$) was somewhat higher than that reported for most other systems. MCT1 and MCT2 expressed in *Xenopus* oocytes have IC_{50} values for phloretin inhibition of L-lactate transport of 28 μM [29] and 14 μM [21] respectively. Mouse Ehrlich–Lettré tumour cells (containing only MCT1), have a $K_{0.5}$ of 4.4 μM for the inhibition of L-lactate transport by phloretin. The kidney cell lines COS (containing both MCT1 and MCT4) and NBL1 (containing only MCT4) have $K_{0.5}$ values of 8.4 and 3.2 μM respectively for phloretin [20], whereas MCT4, when expressed in *Xenopus* oocytes, has an IC_{50} of approx. 40 μM for the inhibition of lactate transport by phloretin [31,32].

It is both interesting and significant to note that the mammalian MCT to which the transporter described here shows the closest functional resemblance (with regard to its kinetic characteristics, stereoselectivity and phloretin sensitivity) is MCT4 [31,32]. MCT4 is found in cells such as white muscle and white blood cells [20], which, like the *P. falciparum* trophozoite, rely on high rates of glycolysis to meet their energy requirements and therefore need an effective mechanism for the export of lactic acid from the cell cytosol [6].

Physiological roles of the H^+ /lactate transporter

The efflux of lactic acid from the intracellular parasite is important for a number of reasons. The generation of lactic acid within the parasite cytosol represents a substantial acid load. The primary H^+ extrusion mechanism in the parasite is a V-type H^+ -ATPase located on the parasite's plasma membrane [15,33]. However, the H^+ /lactate transport pathway described here serves as a second pathway by which glycolytically derived H^+ ions are exported from the parasite, driven by the efflux of lactate down its concentration gradient.

Another likely role for the pathway is in osmoregulation. The generation of two lactate molecules from each glucose molecule consumed has significant osmotic implications for the parasite. The osmoregulatory role of monocarboxylate transport was highlighted in a study on another protozoan, *Trypanosoma brucei* [34]. These parasites, like *Plasmodium*, rely on glycolysis to fulfil their energetic requirements. Unlike *Plasmodium*, however, they lack lactate dehydrogenase and they therefore produce pyruvate as the major end product of metabolism. In that study [34] it was shown that inhibition of pyruvate efflux by a cinnamic acid derivative resulted in the retention and accumulation of pyruvic acid within the parasites, leading to osmotic swelling and, ultimately, to cell lysis.

A third role for the lactate transporter of the parasite might be in maintaining the redox status of the parasite. The *P. falciparum* trophozoite contains only a single mitochondrion [35] and is reliant on the reduction of pyruvate to lactate and the concomitant oxidation of NADH, to enable the continued flux of glucose through glycolysis. The much higher capacity of the plasma membrane transporter for lactate over pyruvate (Figure 3B) might therefore be significant in ensuring that the concentration of pyruvate in the cell remains in excess of that of lactate. MCT4, which seems to be adapted for highly glycolytic cells (such as white skeletal muscle [20]), has been postulated to have a similar role [31,32].

Summary of lactate transport in the *P. falciparum*-infected erythrocyte

The intracellular malaria parasite generates large quantities of the glycolytic end product lactic acid, which leaves the parasite,

across the plasma membrane, via a H^+ /lactate symporter. *In vivo*, lactate effluxing from the parasite enters the so-called parasitophorous vacuole in which the intracellular parasite is enclosed. The parasitophorous vacuole membrane is thought to be freely permeable to small molecules such as lactate owing to the presence in this membrane of high-capacity non-selective pores [36]. Lactate diffuses through these pores into the erythrocyte cytosol, from which it leaves via a combination of the transporters that operate in the normal erythrocyte and new anion-selective channels induced by the parasite in the infected erythrocyte membrane [8,37,38].

The efflux of lactic acid, both from the intracellular parasite and from its host erythrocyte, is important for the biochemical and physiological integrity of the parasite. The pathways involved therefore hold considerable attraction as potential antimalarial drug targets. Lactate transport is a fundamental 'housekeeping' process in most, if not all, human cells; it remains to be established whether the processes involved in the export of lactic acid from the malaria parasite are sufficiently different from those operating in the host for them to be viable drug targets. The present study has shown there to be both similarities and differences between the functional characteristics of the transporter(s) in the parasite's plasma membrane and the mammalian MCTs. The molecular identity of the parasite MCT remains to be established.

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