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Two functionally distinct organic osmolyte pathways in *Plasmodium gallinaceum*-infected chicken red blood cells

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Abstract

Red cells infected with the human malaria parasite *Plasmodium falciparum* have an increased permeability to a range of small, structurally unrelated solutes via a malaria-induced pathway. We report here a similar pathway present in parasitised red cells from chickens infected with the avian malaria parasite, *Plasmodium gallinaceum*. Parasitised cells showed a marked increase in the rate of influx of sorbitol (76-fold) and, to a lesser degree, taurine (3-fold) when compared with red cells from uninfected chickens. Pharmacological data suggest that both sorbitol and taurine are transported via a single malaria-induced pathway, which is sensitive to inhibition by 5-nitro-2-(3-phenylpropylamino)benzoic acid ($IC_{50} \sim 7$ μM). The malaria-induced pathway differed in its inhibition by a range of anion channel inhibitors when compared to the endogenous, volume-activated osmolyte pathway of chicken red cells. There were also differences in the selectivity of sorbitol and taurine by the two permeation routes. The data presented here are consistent with the presence of two distinct organic solute pathways in infected chicken red cells. The first is an endogenous volume-activated pathway, which is not activated by the parasite and the second is a malaria-induced pathway, similar to those that are induced by other types of malaria in other host species.

Keywords: Membrane permeability; Induced transport; Osmolyte pathway; Malaria

1. Introduction

As part of their life cycle, malarial parasites infect the red blood cells of their hosts. Once infected the host red cell membrane becomes increasingly permeable to a variety of small, structurally unrelated molecules. This is due to the induction of malaria-induced transport pathways [1–3].

In malaria-infected human red cells, the majority of these pathways, for a variety of substrates, have been shown to have identical characteristics and have been termed the malaria-induced new permeation pathways or NPP. They have identical pharmacological profiles for different solutes, are linear with substrate concentration up to millimolar levels, prefer anions, while still having a significant permeability to cations (the permeability of electroneutral solutes falling between that of anions and cations), prefer hydrophobic over hydrophilic solutes and are inhibited by a range of anion channel blockers [2,3].

The NPP share many similarities with volume-ac-

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tivated organic osmolyte pathways found in most eukaryotic cell types [4]. However, normal human red cells do not respond to cell swelling by activating organic osmolyte transport pathways, and so a direct comparison of the NPP and an endogenous volume-activated pathway has not been possible.

Chicken red cells were used during early studies of nutrient uptake and utilisation in malaria-infected red cells [5]. Other work reported that malaria-infected avian red cells have an increased uptake of glucose and amino acids [6,7], which suggests the presence of malaria-induced transport pathways. When coupled with data, which show chicken red cells also release taurine when subjected to a hypo-osmotic shock [8], these cells are obvious candidates to study both malaria-induced and volume-activated pathways in the same system.

In this work we have characterised the transport of sorbitol and taurine into uninfected and *P. gallinaceum*-infected chicken red cells. The aim of this study was to establish whether malaria-induced pathways are present in red cells from *P. gallinaceum*-infected chickens and, if so, to compare them with the endogenous volume-activated transport pathway of chicken red cells and the malaria-induced pathways of other types of malaria.

2. Materials and methods

2.1. Chemicals

[^{14}C]Sorbitol and [^{14}C]taurine were from Amersham Corp. Unlabelled sorbitol and taurine, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 1,9-dideoxyforskolin (1,9-ddfsk), 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), niflumate, and furosemide were obtained from Sigma. Tamoxifen was obtained from Calbiochem.

2.2. Parasite culture

Parasite culture was performed in accordance with national and local ethical guidelines. Chicks (4–6 days old) were injected intravenously with 0.2 ml of red cells infected with the malaria parasite *Plasmodium gallinaceum* at approximately 30% parasitaemia. At 5–10 days post-infection, when the parasitaemia was in the range 20–60%, the blood was collected via cardiac puncture into sodium heparin (20 units ml^{-1} of blood). For this procedure chicks were anaesthetised by intramuscular injection of between 0.1 and 0.3 ml (depending on age) of anaesthetic containing Rompan, Ketaset and phosphate buffered saline in a ratio of 1:2:3. Following the procedure the chicks were killed by cervical dislocation. Blood from age-matched uninfected chicks was obtained by the same method. Infected chicks yielded between 0.5 and 1.5 ml of red cells, whereas uninfected chicks typically yielded 1–3 ml of red cells. Where necessary, blood from different chicks was pooled to give the amount of cells required for each experiment. Red cells were then washed four times and resuspended in HEPES-buffered saline (HBS) containing 10 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 155 mM NaCl and 5 mM KCl (pH 7.4); 320 ± 5 mosmol (kg H_2O)$^{-1}$). The osmolality was measured using a freezing-point osmometer (Roebling, Germany). After the first wash the buffy coat was aspirated to remove peripheral blood leukocytes.

Cell counts were made using an improved Neubauer counting chamber. Parasitaemia was estimated from methanol-fixed Giemsa-stained smears.

2.3. Iso-osmotic haemolysis measurements

The permeability of *P. gallinaceum*-infected chicken red cells to sorbitol was investigated using a semi-quantitative haemolysis method, as used previously [9].

An iso-osmotic solution of sorbitol was prepared by dissolving the compound to a concentration of approximately 320 mM in a solution containing 10 mM HEPES and 5 mM glucose (pH 7.4). The osmolality was then adjusted (by the addition of either sorbitol or the hypo-osmotic HEPES+glucose solution) to 320 ± 5 mosmol (kg H_2O)$^{-1}$.

Time course measurements commenced with the addition of a 0.2-ml aliquot of cell suspension to 3.8 ml of the iso-osmotic sorbitol solution (pre-warmed for at least 10 min) to give a cell concentration of approximately 0.5×10^8 cells ml^{-1}. All such experiments were carried out at 41°C. At predetermined intervals, 0.5-ml aliquots of the suspension were transferred to microcentrifuge tubes containing 0.5 ml of an ice-cold 'stopping solution' (400 mM

sucrose in H_2O). The tubes were centrifuged for 30 s ($10\,000\times g$) then 0.9 ml of the supernatant solution was transferred to another tube for the subsequent spectrophotometric (A_{540}) estimation of haemoglobin concentration.

Estimates of 100% haemolysis in each experiment were determined from three 0.1-ml samples, which were placed into 0.9 ml of 0.1% v/v Triton X-100 (to lyse the cells), and then centrifuged for 10 s ($10\,000\times g$), before spectrophotometric analysis.

2.4. Influx measurements

Estimates of the unidirectional influx rates for sorbitol and taurine were made from the uptake of [^{14}C]sorbitol and [^{14}C]taurine, respectively. All such measurements, unless stated otherwise, were carried out at 41°C. Influx commenced with the addition of cells suspended in HBS (pre-warmed for at least 10 min) to microcentifuge tubes containing radioisotope and unlabelled substrate (pre-warmed and at appropriate concentrations), giving a final activity of 0.3 μCi ml^{-1}, a final cell concentration of $1–4\times10^8$ cells ml^{-1}, and a final sample volume of 0.5–1.0 ml. At appropriate times aliquots (0.15 ml for time courses and 0.2 ml for fixed-time influxes) of the suspension were transferred to microcentrifuge tubes containing 1 ml of ice-cold HBS, which was layered over 0.3 ml of dibutylphthalate. The ice-cold solution was used to stop further influx and dilute the total radiolabel to reduce the extracellular radioactivity in the cell pellet. The tubes were then centrifuged ($10\,000\times g$, 5 s) to sediment the cells beneath the oil. The aqueous supernatant solution was removed by aspiration and the radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water. The dibutylphthalate was aspirated then the cell pellet was lysed with 0.1% v/v Triton X-100 (0.5 ml) and deproteinised by the addition of 5% w/v trichloroacetic acid (0.5 ml), followed by centrifugation ($10\,000\times g$, 10 min). Radioactivity was measured using a β-scintillation counter.

When required inhibitors were added to cell suspensions as stock solutions in dimethylsulphoxide at least 5 min before beginning an influx experiment. The final concentration of dimethylsulphoxide was always less than 0.5% and usually 0.1%.

For the study of the volume-activated pathway in chicken red cells, the pathway was activated by adding 0.4 ml H_2O to 0.6 ml of the cell suspension in HBS just prior to commencing each experiment. This reduced the osmolarity of the suspending solution from 320 to approximately 190 mosmol (kg H_2O)$^{-1}$. Preliminary experiments showed this pathway was activated within 1 min of H_2O addition (data not shown).

The radioactivity trapped in the extracellular space of the cell pellet was estimated in each experiment from the radioisotope present in pellets derived from samples taken within a few seconds (10–15 s) of combining the prewarmed cells with radiolabel. However, for sorbitol uptake experiments in infected cells, which occurred rapidly, separate samples were taken from experimental tubes that were first placed on ice for at least 10 min before radiolabel addition. The low temperature inhibited sorbitol transport and allowed the extracellular radioactivity to be measured.

Initial experiments involved uptake over a range of predetermined time intervals (see, e.g., Fig. 1). In later experiments sorbitol and taurine influx rates were estimated from the amount of radiolabel accumulated within fixed times that fell within the initial linear portion of the uptake time course. For sorbitol influx in uninfected chicken red cells in isotonic conditions, 15- or 60-min time points were used, and in hypotonic conditions, a 15-min time point was used. For sorbitol influx in infected red cells in isotonic conditions, a 1-min time point was used. For taurine influx in both uninfected and infected red cells in both isotonic and hypotonic conditions, a 15-min time point was used.

Unless specified otherwise the influx data for parasitised cells are corrected to 100% parasitaemia using the expression:

$$J_{\mathrm{pRBC}} = \frac{J_{\mathrm{measured}}\times 100\% - [(100\% - \%\mathrm{parasitaemia})\times J_{\mathrm{RBC}}]}{\%\mathrm{parasitaemia}}$$

where J_{pRBC} is the influx in parasitised red cells (i.e., cells infected at 100% parasitaemia), J_{measured} is the influx measured in a suspension infected at % parasitaemia (i.e., the percentage of red cells in the suspension that contain parasites), and J_{RBC} is the influx in the uninfected cells in the cell suspension, (esti-

mated from the influx measured in uninfected red cells from age-matched chickens).

To test whether uninfected red cells from infected chickens had similar transport properties to uninfected red cells from uninfected chickens, sorbitol transport was measured in uninfected and malaria-infected red cells subjected to a sorbitol lysis (see above), which ruptured the majority of infected cells but not uninfected cells (see Fig. 2B). In paired experiments, the influx of sorbitol, in the presence of a 10 mM external concentration, in uninfected and infected blood after this process was 0.10 ± 0.03 and 0.07 ± 0.01 mmol $(10^{12}$ cells h$)^{-1}$, respectively, in isotonic conditions (mean ± S.D.; $n = 3$). There was no statistical difference ($P = 0.30$; paired, two-tailed Student's *t*-test), which implies that their transport properties are essentially identical.

To rule out the possibility that white blood cells were the cause of the experimental data reported here, two further experiments were performed in blood before and after the removal of the buffy coat. In the first experiment, uninfected blood was used to investigate swelling-activated sorbitol transport. In paired experiments, the influx of sorbitol, in the presence of a 10 mM external concentration, in uninfected blood with and without its buffy coat was 0.38 ± 0.01 and 0.36 ± 0.02 mmol $(10^{12}$ cells h$)^{-1}$, respectively, after a hypotonic shock (mean ± S.D.; $n = 3$). There was no statistical difference ($P = 0.36$; paired, two-tailed Student's *t*-test), which implies that the increased presence of white blood cells has no effect on volume-activated transport measurements.

In the second experiment, infected blood was used in isotonic conditions. In paired experiments, the parasitaemia-corrected influx of sorbitol, in the presence of a 10 mM external concentration, in infected blood with and without its buffy coat was 6.7 ± 2.7 and 7.3 ± 1.8 mmol $(10^{12}$ cells h$)^{-1}$, respectively (mean ± S.D.; $n = 3$). There was no statistical difference ($P = 0.77$; paired, two-tailed Student's *t*-test), which implies that the increased presence of white blood cells has no effect on malaria-induced transport measurements. The data from these two experiments suggest that the observed increase of sorbitol transport in chicken blood after swelling or malaria infection cannot be caused by the presence of white blood cells.

3. Results

3.1. Sorbitol and taurine uptake in uninfected chicken red cells

Initially, the endogenous osmolyte pathways in uninfected chicken red cells were studied. Fig. 1 shows the uptake of sorbitol and taurine into uninfected chicken red cells in isotonic and hypotonic conditions. An unlabelled external concentration of 10

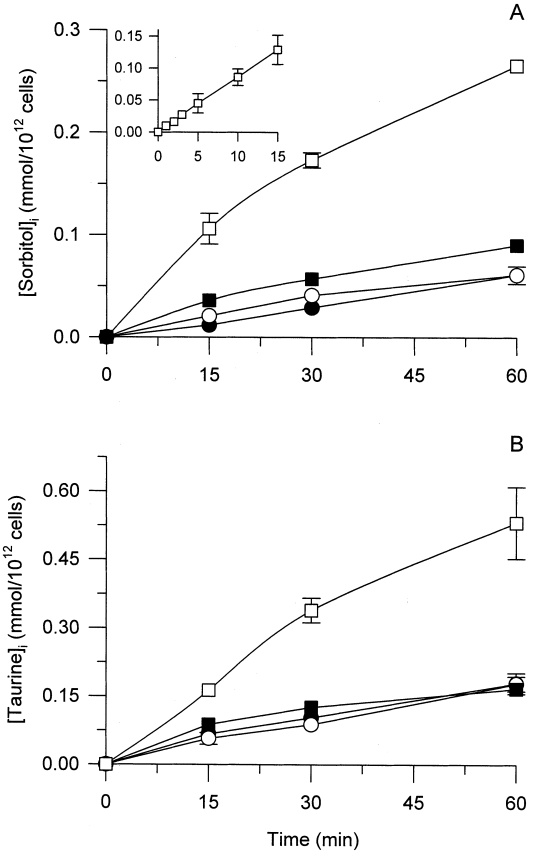

Fig. 1. Time courses for the uptake of sorbitol (A) and taurine (B) into uninfected chicken red cells in isotonic (circles) and hypotonic (squares) conditions measured in the absence (open symbols) and presence (closed symbols) of 0.1 mM NPPB. The extracellular sorbitol and taurine concentrations were 10 mM. Points represent means from three single experiments on red cells from a single chicken. Error bars indicate S.D. and, where not shown, fall within the symbols. Inset: uptake of sorbitol into uninfected chicken red cells in hypotonic conditions, where points represent means from single experiments on red cells from three different chickens.

mM was used for both sorbitol and taurine to saturate any carrier-type transporters. With the exception of sorbitol uptake in hypotonic conditions, time courses were linear for at least 30 min. For the former, uptake was shown to be linear for at least 15 min (Fig. 1A, inset).

Sorbitol and taurine influxes in uninfected chicken red cells in isotonic conditions were measured to be 0.09 ± 0.03 and 0.17 ± 0.07 mmol $(10^{12}$ cells $h)^{-1}$, respectively (mean ± S.D.; $n = 4$) and in hypotonic conditions were measured to be 0.44 ± 0.09 and 0.70 ± 0.03 mmol $(10^{12}$ cells $h)^{-1}$, respectively (mean ± S.D.; $n = 4$). The data showed a 4–5-fold increase in the influx rates of both solutes in uninfected chicken red cells when the cells were placed in hypotonic conditions.

Further experiments with uninfected chicken red cells showed that sorbitol influx was linear with external concentration over the range of 0.01 to 100 mM in both isotonic and hypotonic conditions (data not shown).

3.2. *Effect of NPPB on sorbitol and taurine uptake in uninfected chicken red cells*

The observation of an increase in the uptake of sorbitol and taurine into chicken red cells in hypotonic conditions is similar to the situation in HeLa cells [10]. Hall and co-workers reported that the swelling-activated uptake of both solutes is inhibited by a range of anion transport blockers, including NPPB. We therefore tested the effect of this reagent on the transport of sorbitol and taurine into normal and hypotonically swollen chicken red cells (see Fig. 1).

In paired experiments 0.1 mM NPPB had no effect ($P > 0.38$; paired, two-tailed Student's t-test) on the transport of either solute in isotonic conditions. However, in hypotonic conditions 0.1 mM NPPB reduced the transport of each solute to levels only slightly higher than those measured under isotonic conditions.

The effect of NPPB on sorbitol and taurine transport in uninfected chicken red cells subjected to hypotonic shock was further studied by using a range of external concentrations from 0.1 to 333 μM. From this data it was possible to calculate IC_{50} values (the concentration of inhibitor at which the total influx of a solute is reduced by half) for the effect of NPPB on the transport of both solutes. However, before these were calculated it was first necessary to correct the data for the NPPB-insensitive routes for sorbitol and taurine, which were measured in isotonic conditions. This was performed by subtracting the influx results measured in isotonic conditions from the data produced in hypotonic conditions, in paired experiments (it was assumed that hypotonic shock had no effect on the NPPB-insensitive fluxes). The IC_{50} values for the effect of NPPB on sorbitol and taurine transport

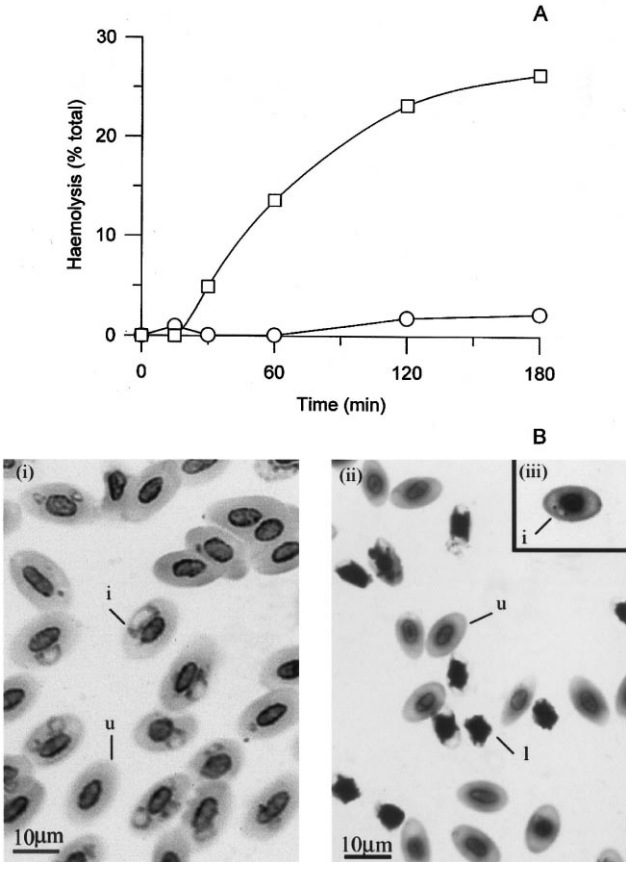

Fig. 2. Time courses for the haemolysis (A) of uninfected (○) and malaria-infected (□) chicken red cells suspended in an isotonic solution of sorbitol and the microscopic examination (B) of the infected red cells before (B(i)) and after (B(ii, iii)) the haemolysis experiment (u, uninfected; i, infected; l, lysed). The parasitaemia was 51% and the points are representative of those obtained in single experiments on red cells from three different chickens. Note that the percentage of haemolysis is not directly related to the percentage of cells lysed, as infected red cells contain less haemoglobin, and that infected red cells were difficult to find after sorbitol lysis.

were estimated to be 31 ± 6.6 and 32 ± 4.1 μM, respectively (mean ± S.D.; $n = 3$), and were found to be not statistically different ($P = 0.66$; paired, two-tailed Student's t-test).

Assuming, as the data suggests, that both sorbitol and taurine are using the same volume-activated pathway, the permeability ratio (calculated by dividing the averaged volume-activated, NPPB-sensitive sorbitol influx by the averaged volume-activated, NPPB-sensitive taurine influx) for sorbitol relative to taurine was measured to be 0.6.

3.3. Sorbitol and taurine uptake in infected chicken red cells

To begin with, a semi-quantitative haemolysis assay [11] was used to study the permeability of malaria-infected chicken red cells. Fig. 2A shows a representative haemolysis time course for malaria-infected (51% parasitaemia) and uninfected chicken red cells suspended in an isotonic solution of sorbitol. The data show that uninfected chicken red cells did not undergo any significant haemolysis over the 3-h time course. However, malaria-infected chicken red cells underwent a partial haemolysis, which in this case levelled off after approximately 25%.

The data suggested that a sub-population of the infected blood had an increased permeability to sorbitol and so a microscopic examination of Giemsa stained cells at the end of the time course was performed (Fig. 2B). This revealed that uninfected and very early developmental-stage infected red cells remained intact. However, there was a total lack of intact mature malaria-infected red cells.

The $1/t_{50}$ value (the inverse of the time taken for the measured haemoglobin to reach half its maximum value) for sorbitol transport into malaria-infected chicken red cells was 0.030 ± 0.009 min^{-1} (mean ± S.D.; $n = 3$).

Fig. 3A shows the marked increase in the uptake of sorbitol in malaria-infected chicken red cells compared to uninfected chicken red cells in isotonic conditions, using a radiolabelled sorbitol tracer assay. The external concentration of sorbitol was 10 mM and the parasitaemia in this particular experiment was 50%. The time course for sorbitol uptake into malaria-infected chicken red cells was linear for at least 1 min (see Fig. 3A, inset).

Parasitaemia-corrected results (see Section 2) showed that the rate of sorbitol influx in infected chicken red cells was 6.6 ± 1.5 mmol $(10^{12}$ cells h$)^{-1}$ (mean ± S.D.; $n = 6$), which is 76 times higher than the influx of sorbitol in uninfected chicken red cells in isotonic conditions. It should be noted that the cultures were not synchronous and that the haemolysis data suggested that the induced transport of sorbitol did not occur until partway through the cellular phase. The results were corrected for total parasitaemia and so probably underestimate the true influx values. Fig. 3B shows that sorbitol influx in infected chicken red cells was linear with external concentration over the range of 0.01 to 100 mM in isotonic conditions.

There was also a slight increase in the uptake of taurine into infected chicken red cells in isotonic conditions. In the presence of 10 mM external taurine, the uptake was linear with time for at least 15 min (data not shown) and was measured to be 0.59 ± 0.11 mmol $(10^{12}$ cells h$)^{-1}$ (parasitaemia-corrected; mean ± S.D.; $n = 7$), which is approximately three times higher than the influx of taurine in uninfected chicken red cells in isotonic conditions.

In four paired experiments, using infected blood with parasitaemias of 49%, 50%, 54% and 60%, the uptake of taurine into infected chicken red cells (0.40 ± 0.06 mmol $(10^{12}$ cells h$)^{-1}$ (mean ± S.D.; $n = 4$)) was significantly higher than into uninfected chicken red cells (0.17 ± 0.07 mmol $(10^{12}$ cells h$)^{-1}$ (mean ± S.D.; $n = 4$)) in isotonic conditions ($P = 0.03$; paired, two-tailed Student's t-test). The taurine influx in infected chicken red cells was also further increased when the cells were subjected to hypotonic shock. The taurine influx remained significantly higher in infected chicken red cells (0.83 ± 0.08 mmol $(10^{12}$ cells h$)^{-1}$ (mean ± S.D.; $n = 4$)) compared with uninfected chicken red cells (0.67 ± 0.05 mmol $(10^{12}$ cells h$)^{-1}$ (mean ± S.D.; $n = 4$)) in hypotonic conditions ($P = 0.04$; paired, two-tailed Student's t-test; $n = 4$). Furthermore, the difference between the influx measured in infected cells in hypotonic and in isotonic conditions (i.e., the swelling-induced component of taurine influx) was found not to be

statistically different ($P = 0.34$; paired, two-tailed Student's t-test) from the value calculated for the swelling-induced component of taurine influx in uninfected chicken red cells.

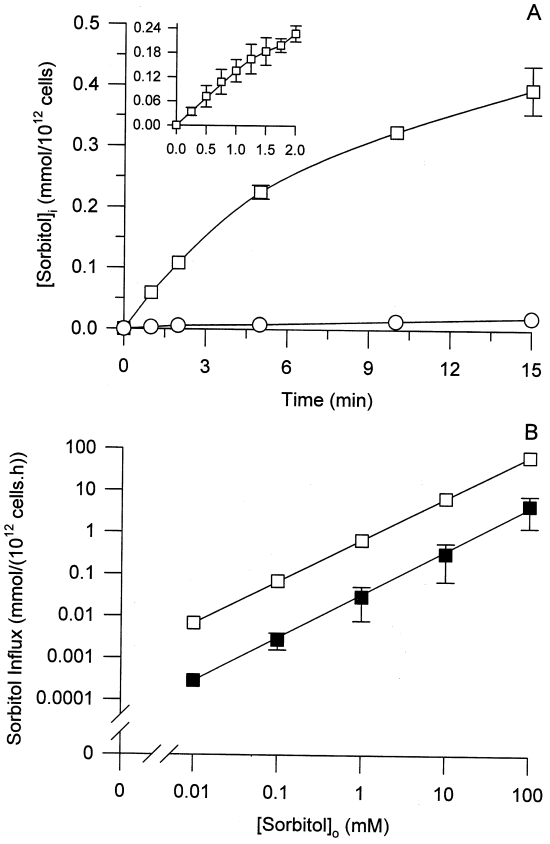

Fig. 3. Time courses (A) for the uptake of sorbitol into uninfected (○) and malaria-infected (□) chicken red cells in isotonic conditions and the concentration dependence (B) of the influx of sorbitol in malaria-infected chicken red cells measured in the absence (□) and presence (■) of 0.1 mM NPPB. For the main time course for infected red cells the parasitaemia was 50%, the extracellular sorbitol concentration was 10 mM, and the points represent means from three single experiments on red cells from a single chicken. Inset: uptake of sorbitol into infected chicken red cells in isotonic conditions. For the concentration dependence and the time-course inset, points represent means from single experiments on red cells from three different chickens and were recalculated equivalent to 100% parasitaemia. Error bars indicate S.D. and, where not shown, fall within the symbols.

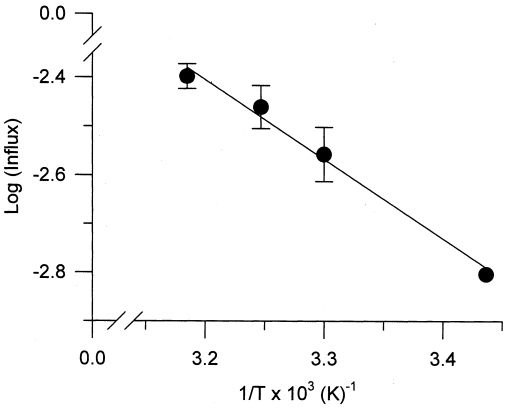

Fig. 4. Arrhenius plot constructed from the temperature dependence of the influx of sorbitol in malaria-infected chicken red cells. The extracellular sorbitol concentration was 10 mM. Values were recalculated equivalent to 100% parasitaemia. Points represent means from single experiments on red cells from three different chickens. Error bars indicate S.D. and, where not shown, fall within the symbols.

3.4. *Effect of NPPB on sorbitol and taurine uptake in infected chicken red cells*

NPPB (0.1 mM) was also found to inhibit the influx of sorbitol (see Fig. 3B) and taurine (data not shown) in infected chicken red cells in isotonic conditions. The IC_{50} values for the effect of NPPB on sorbitol and taurine transport in infected chicken red cells were estimated to be 6.2 ± 2.8 and 7.3 ± 0.6 μM, respectively (mean ± S.D.; $n = 3$), and were not found to be statistically different ($P = 0.67$; paired, two-tailed Student's t-test). However, a comparison of the IC_{50} values for sorbitol and taurine transport in infected chicken red cells in isotonic conditions, with those for sorbitol and taurine transport in uninfected chicken red cells in hypotonic conditions showed a significant difference between them ($P < 0.02$; paired, two-tailed Student's t-test).

Assuming, as the data suggest, that both sorbitol and taurine are using the same malaria-induced pathway, the permeability ratio for sorbitol relative to taurine was measured to be 17.

3.5. *Temperature dependence of sorbitol transport in infected chicken red cells*

Sorbitol influx in malaria-infected red cells de-

Table 1
A comparison of malaria-induced and swelling-activated pathways

	Malaria-induced pathway		Swelling-activated pathway	
	Chicken (*P. gallinaceum*)	Human (*P. falciparum*)[a]	Chicken red cell	HeLa cell[b]
Selectivity (sorbitol/taurine)	17	0.2[c]	0.6	0.5
Sensitivity (IC_{50} (μM))	NPPB (7), niflumate > furosemide ≫ tamoxifen, 1,9-ddfsk, DIDS	NPPB (0.8) > furosemide (5) > niflumate (20) > DIDS (100)	Tamoxifen (10) > NPPB (32), niflumate, 1,9-ddfsk, DIDS ≫ furosemide	Tamoxifen, DIDS (4[d]), NPPB, niflumate, 1,9-ddfsk ≫ MK-196, verapamil
Kinetics	Non-saturable 0–100 mM sorbitol	Non-saturable 0–0.5 mM choline, 0–10 mM adenosine	Non-saturable 0–100 mM sorbitol	Non-saturable 0–50 mM sorbitol, taurine, thymidine, choline, K^+

[a]Ref. [12], unless stated otherwise.
[b]Ref. [10], unless stated otherwise.
[c]Calculated using data from [13,14].
[d]Ref. [15].

creased with decreasing temperature. An Arrhenius plot (Fig. 4) revealed the energy of activation (E_a) for the process to be 32 ± 2 kJ mol^{-1} or 8 ± 1 kcal mol^{-1} (mean ± S.D.; $n = 3$).

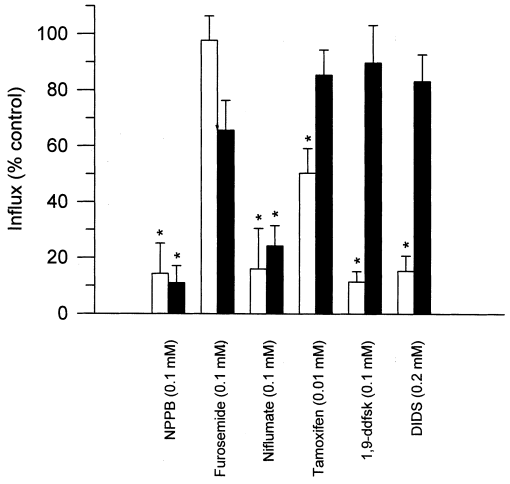

Fig. 5. Effect of anion channel inhibitors on the hypotonically activated influx of sorbitol in uninfected chicken red cells (open bars) and on the malaria-induced influx of sorbitol in infected chicken red cells (closed bars). Bars represent means from single experiments on red cells from at least three different chickens. Significant inhibition: $*P < 0.05$; paired, two-tailed Student's *t*-test. Error bars indicate S.D.

3.6. *Effect of anion channel inhibitors on sorbitol transport in uninfected and malaria-infected chicken red cells*

As a final test to distinguish between the endogenous volume-activated sorbitol transport and the malaria-induced sorbitol transport pathways, a range of anion channel inhibitors, which have been shown to inhibit volume-activated osmolyte transport in HeLa cells [10] and malaria-induced pathways in infected human red cells [12], were tested for their effect on the two pathways.

Fig. 5 shows their effect at single inhibitory concentrations. It can clearly be seen that there are several differences. In particular, DIDS and 1,9-ddfsk were very good inhibitors of volume-activated sorbitol influx but weak inhibitors of malaria-induced sorbitol influx.

4. Discussion

4.1. *Volume-activated osmolyte transport in chicken red cells*

Normal (uninfected) chicken red cells in hypotonic conditions showed an increased permeability to both

sorbitol and taurine compared to red cells in isotonic conditions. The data for enhanced taurine transport (measured as unidirectional influx) support that of a previous study, which shows a similar increase in the efflux of taurine from chicken red cells in hypotonic conditions [8]. However, the previous investigation fell short of characterising the hypotonically induced transport pathway.

The pharmacological data presented here show that taurine and sorbitol are likely to use the same swelling-activated pathway in chicken red cells as they both have an identical sensitivity to NPPB ($IC_{50} \sim 31$ μM). This pathway has also been shown to have a linear concentration dependence up to millimolar levels of external sorbitol, to have a preference for taurine over sorbitol (a permeability ratio ($P_{sorbitol/taurine}$) of 0.6), and to be inhibited by a range of anion channel blockers (see Fig. 5). The data are almost identical to those reported by Hall and coworkers [10] for swelling-activated transport in the human cancer cell line, HeLa cells (see Table 1). Such transport pathways (swelling-activated organic osmolyte channels) have been characterised in a range of eukaryotic cell types, from protozoa to mammalian [4].

4.2. *Malaria-induced osmolyte transport in chicken red cells*

Malaria-infected chicken red cells were also shown, using haemolysis and radiolabel tracer experiments, to have enhanced sorbitol and, to a lesser degree, taurine transport. However, for several reasons, the data are consistent with the parasite inducing a new transport pathway, such as those reported in human and murine infected red cells [12,16] and not activating the endogenous volume-activated osmolyte pathway.

Firstly, the observed increases in the transport of sorbitol and taurine into malaria-infected chicken red cells occurred in isotonic conditions (i.e., a hypotonic shock was not necessary to activate the flux). Secondly, the malaria-induced pathway has a marked preference for sorbitol over taurine, whereas the volume-activated pathway has a slight preference for taurine over sorbitol. Thirdly, the two pathways have different pharmacological profiles when measured using a range of different anion channel inhibitors (see Table 1).

It could be argued that the increased taurine transport measured in malaria-infected red cells (but not the increased sorbitol transport) was occurring via the endogenous volume-activated pathway, as the induced taurine influx was small and, in fact, slightly lower than that measured in hypotonically swollen uninfected red cells. However, the high NPPB-sensitivity of the taurine transport route suggests a different pathway from that activated in hypotonically swollen chicken red cells. Furthermore, taurine influx was further stimulated in infected cells when subjected to a hypotonic shock and this additional increase of taurine uptake was almost identical in magnitude to the increase measured in uninfected cells subjected to a hypotonic shock.

The haemolysis data presented here suggest the increased permeation was only evident in mature parasitised red cells, as uninfected and younger parasitised red cells were still seen as intact whole cells after 3 h of incubation in isotonic sorbitol solution. This phenomenon is also true of parasitised human red cells and enabled Lambros and Vanderberg [17] to synchronise human parasite cultures by lysing mature forms (although they did not realise the mechanism of action at the time). The data are also consistent with the stage-dependent activation of induced pathways, as reported for K^+ [18] and L-glutamine [19] in malaria-infected human red cells.

Like the *P. falciparum*-induced pathway in human red cells [12,18], uptake of solutes via the *P. gallinaceum*-induced pathway characterised here was linear with substrate concentration up to millimolar levels, allowed the transport of structurally unrelated solutes, possessed a low E_a, which is indicative of channel/pore like pathways [20], and was inhibited by both NPPB and niflumate. However, there were two significant differences between the malaria-induced pathways found in infected chicken and human red cells. Firstly, the *P. falciparum*-induced pathway is more sensitive (at least 10-fold) to furosemide and NPPB compared with the *P. gallinaceum*-induced pathway. Secondly, the *P. falciparum*-induced pathway has a preference for taurine over sorbitol, whereas the *P. gallinaceum*-induced pathway prefers sorbitol over taurine (see Table 1).

4.3. Roles for the malaria-induced osmolyte transport in chicken red cells

The usual rationale offered for malaria-induced transport pathways is that they enable the internal parasite to obtain nutrients from the blood plasma. In support of this, Saliba and co-workers [21] have recently reported that a *P. falciparum*-induced pathway in infected human red cells is the only possible route for the parasite to obtain pantothenate, a precursor of co-enzyme A. However, the study presented here concentrated on the transport of two organic osmolytes, which are regarded as important solutes in the process of volume regulation.

The host cell has to contend with the volume increase caused by the growing parasite and the production of large quantities of amino acids from the degradation of haemoglobin by the parasite [22]. If these processes are not regulated the host cell would swell and lyse. The fact that this does not happen and yet the endogenous volume-activated pathway is not activated in infected chicken red cells suggests that the parasite does control the host cell volume to stop swelling via a separate transport process. Although some cell types such as those found in the renal medulla can accumulate sorbitol as an osmolyte [23], it is unlikely to be present at a significant level in chicken red cells. However, taurine has been reported to be present at a concentration of 1–3 mM in chicken red cells [24]. Although low, if this pool is increased by the presence of other permeable amino acids, derived from parasite metabolism, it would produce an enhanced level of osmolytes that could use the induced pathway for volume regulation.

4.4. Nature of the malaria-induced osmolyte transport in chicken red cells

It has yet to be shown whether malaria-induced pathways of this type are parasite-derived proteins, which are exported to the host cell membrane, or parasite-activated/modified, endogenous proteins (e.g., the anion exchanger band 3 for which there is indirect evidence in infected human red cells [25,26]). Recently, in his extensive review, Kirk suggested that the malaria-induced pathway characterised in infected human red cells could be an endogenous swelling-activated osmolyte pathway [3]. However, the data presented here rule out the simple activation of the classical endogenous volume-activated pathway in chicken red cells by the parasite but do not rule out the activation of a parasite-modified form of a host pathway.

Interestingly, Kirk and co-workers [12], using radiolabelled tracer experiments, reported that the transport of a wide range of solutes in *P. falciparum*-infected human red cells was via a single novel pathway with the characteristics of a chloride channel. More recently, Desai and co-workers [27], using the patch-clamp method, have found a novel voltage-dependent anion channel in *P. falciparum*-infected human red cells. These two pathways have identical properties, which provides strong evidence that they are the same transport protein. However, in parallel to the present flux study, in which we have presented evidence for a new malaria-induced solute pathway, we have used the patch-clamp method on uninfected and malaria-infected chicken red cells. We found no evidence for a novel anion-conductive pathway in infected chicken red cells [28]. Although there was enhanced conductance of anions, as well as cations, measured in infected cells, this correlates with upregulation of endogenous ion channels.

Three endogenous ion channels were characterised during this work [28]. Of these, only one, a large conductance anion channel, has a similar pharmacological profile to the malaria-induced pathway characterised here. However, this anion channel is only active in 10% of the infected cells patched under physiological conditions and is inactive at membrane potentials between +40 and −40 mV. This raises the possibility that the malaria-induced pathway in chicken red cells characterised here is for electroneutral transport of solutes only.

The principal advantage of the present use of avian red cells is to be able to compare directly the native volume-activated and parasite-derived transport pathways. The differences are significant enough to rule out activation of the volume-activated osmolyte pathway as the source of the malaria-induced permeability. Beyond this it is not possible to establish whether the malaria-induced pathway is a host or parasite-derived protein, a subject presently unresolved and the source of considerable debate [2,3]. It is likely that we must await resolution of the molec-

ular identities of these pathways before this question finally can be resolved.

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