Transport of Diverse Substrates into Malaria-infected Erythrocytes via a Pathway Showing Functional Characteristics of a Chloride Channel*  

(Received for publication, August 6, 1993)  

Kiaran Kirk‡, Heather A. Horner‡, Barry C. Elford‡, J. Clive Ellory‡, and Chris I. Newbold†  
From the ‡University Laboratory of Physiology, Oxford, OX1 3PT, United Kingdom and †Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United Kingdom  

Following infection by the malaria parasite, Plasmodium falciparum, human erythrocytes show increased permeability to a variety of low molecular weight solutes. In this study a number of anion transport blockers were identified as potent inhibitors of the transport of a wide range of solutes into human erythrocytes infected in vitro with P. falciparum. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), furosemide, and nitrate blocked the malaria-induced transport of monovalent cations, neutral amino acids, sugars, nucleosides, and monovalent anions. For all of the substrates tested the order of potency of these three inhibitors was the same (NPPB > furosemide > nitrate) and dose-response curves for the effect of these inhibitors on malaria-induced choline transport were similar to those for malaria-induced thymidine transport. The data suggest that much, if not all, of the high capacity (non-saturable) transport of low molecular weight solutes into P. falciparum-infected erythrocytes is via a single type of pathway. The broad specificity of the pathway, its non-saturability in the physiological concentration range, and its failure to distinguish between stereoisomers (L- and D-alanine) are consistent with its being a type of pore or channel. For those substrates for which quantitative influx measurements were made the malaria-induced (inhibitor-sensitive) transport was in the order: Cl⁻ > lactate > thymidine, adenosine > carnitine > choline > K⁺. The pathway is therefore anion-selective. The pharmacological and substrate-selectivity properties of the pathway show marked similarities to those of chloride channels in other cell types; this raises the possibility that the high capacity transport of small organic solutes may be an important and, as yet, largely unrecognized role for such channels in other tissues.

During the intra-erythrocytic development of the malaria parasite, Plasmodium falciparum, the red cell shows increased permeability to a wide range of structurally unrelated solutes via transport pathways that are functionally distinct from those known to operate in normal erythrocytes. Solutes which have been shown to enter infected cells via the new permeation pathways include sugars and sugar-alcohols (Ginsburg et al., 1985, 1986; Ginsburg, 1988), amino acids (Elford et al., 1985; Ginsburg et al., 1985), nucleosides (Gero et al., 1988, 1991; Gero, 1991; Gero and Upston, 1992), anions (Kutner et al., 1983; Ginsburg and Stein, 1987a; Cabantchik, 1990; Kanaani and Ginsburg, 1991; Elford and Finches, 1992; Poole and Halestrap, 1993), and cations (Bookchin et al. 1980; Elford et al., 1990a, 1990b; Kirk et al., 1991a, 1991b, 1992a). It has been suggested that the new pathways facilitate both the entry of metabolic and biosynthetic substrates into infected cells and the rapid exit of potentially harmful catabolites (Elford et al., 1985; Ginsburg et al., 1985; Ginsburg and Stein, 1987b; Sherman, 1988; Ginsburg, 1989; Cabantchik, 1990; Kanaani and Ginsburg, 1991; Poole and Halestrap, 1993). However, despite their possible physiological importance and chemotherapeutic potential (Cabantchik, 1989), neither the identity nor the biophysical nature of the parasite-induced pathways has been conclusively established.

In early studies of the enhanced permeability of human erythrocytes infected in vitro with P. falciparum it was suggested that the enhanced transport of monosaccharides and neutral amino acids might occur via simple positively charged pores which exclude cations (Kutner et al., 1983; Ginsburg et al., 1985). However, this model was rejected later in favor of one in which the transport of small organic solutes occurs via pathways formed from a mismatch between the lipids of the erythrocyte membrane and parasite-derived integral membrane proteins (Ginsburg and Stein, 1987b; Ginsburg, 1990). More recently it has been proposed that a combination of these mechanisms might operate, with hydrophilic substrates crossing the infected erythrocyte membrane via pores, and hydrophobic compounds permeating via protein-lipid interfaces (Cabantchik, 1990; Cabantchik et al., 1990). However, alternative explanations have also been postulated, ranging from perturbation of the arrangement of phospholipids in the host cell membrane (Taraschi et al., 1986; Joshi et al., 1987) to insertion (or activation) of heterogeneous, substrate-specific transporters (Elford et al., 1991; Poole and Halestrap, 1993).  

In this work we address the question of whether the malaria-induced, high capacity (non-saturable) transport of low molecular weight solutes into parasitized erythrocytes occurs via common routes or via heterogeneous, functionally distinct transport pathways. We have identified a number of potent inhibitors of the enhanced transport in malaria-infected erythrocytes (including the most effective inhibitor reported to date) and have shown that these compounds block the movement of a diverse range of solutes into infected cells. Our data are consistent with the hypothesis that much of the malaria-induced transport of cations, anions, amino acids, sugars, and nucleosides into parasitized cells occurs through a single type of pathway. This pathway behaves as an anion-selective chan-
Induced Transport in Malaria-infected Erythrocytes

Experimental Procedures

Materials—$^{86}$RbCl was from DuPont NEN. $[1^{4}C]$Choline chloride, $[1^{4}C]$Karnetine, $[1^{4}C]$Cadenosine, $[3^{5}H]$Thymidine, sodium $[1^{3}C]$Lactate, and Na$^{+}$Cl were from Amersham Corp. Ouabain, 4,4'-dioctyloxyanostilbene-2,2'-disulfonic acid (DIDS), and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) were from Sigma. Bumetanide was a gift from Leo Laboratories (Aylesbury, Buckinghamshire, UK). Nitrobenzylthioinosine (NBMPR) was a gift from Dr. J. W. Young (Department of Physiology, University of Alberta, Canada). 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPBA) was a gift from Prof. R. Greger (Physiologisches Institut der Albert-Ludwigs-Universität, Freiburg, Germany) and indacrinone (MK-196) was a gift from the late Dr. M. W. Wolowyk (Faculty of Pharmacy and Pharmaceutical Science, University of Alberta, Canada).

Cell Culture—Human erythrocytes (type O) infected with the D10 line of P. falciparum (Berendt et al., 1989) were cultured under conditions identical for at least 24 h prior to the experiment. Cell concentrations were determined using a Coulter counter (model ZF) or an improved Neubauer counting chamber. Parasitemia was estimated using methanol-fixed Giemsa-stained smears.

Radioisotope Influx Measurements—The unidirectional influx into infected and uninfected erythrocytes of the monovalent cations K$^+$ and choline, the zwitterion carnitine, the uncharged nucleosides adenosine and thymidine and the monovalent anions L-lactate and Cl$^-$ was estimated from the uptake of $^{86}$Rb$^+$, $[1^{4}C]$Choline, $[1^{4}C]$Karnetine, $[1^{4}C]$Cadenosine, $[3^{5}H]$Thymidine, sodium $[1^{3}C]$Lactate and Na$^{+}$Cl$^-$, respectively, into cells washed four times by centrifugation (5 min at 1000 x g) then resuspended in HEPES-buffered saline (125 mm NaCl, 5 mm KCl, 25 mm HEPES, 5 mm glucose; pH 7.4).

Influx time courses were obtained using one of two methods. In the first time course method cells in HEPES-buffered saline were combined with the appropriate reagents in a single microcentrifuge tube. The samples were pre-equilibrated to the required temperature then, at predetermined intervals, an aliquot of cell suspension (0.15 ml) was dispensed into each tube in turn. Immediately following the addition of the cells to the full tube in the series the samples were centrifuged (10,000 x g, 30 s) to sediment the cells below the oil and thereby terminate the flux. The time taken between starting the centrifuge and the termination of the flux was estimated to be 2 s (by extrapolation of data from short time course experiments).

In both methods, following sedimentation of the cells below the oil, the supernatant solution was aspirated, the radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water. The dibutyolphthalate was aspirated, then the cell pellet was lysed with 0.1% (w/v) Triton X-100 (0.5 ml) and deproteinized by the addition of 5% w/v trichloroacetic acid (0.05 ml), followed by centrifugation (10,000 x g, 10 min). Radioactivity was measured using a $\beta$-scintillation counter.

In some experiments initial unidirectional influx rates were estimated from the amount of radio label accumulated during a fixed incubation period that fell within the initial (approximately linear) portion of the influx time course. The incubation periods used were normally 3–5 min for choline influx at 37°C (see Fig. 1A), 10–30 min for choline influx at 22°C, 8–12 min for Rb$^+$ influx at 37°C (see Fig. 1B), and 4–5 s for thymidine influx at 22°C (see Fig. 6C). Fixed-period incubation experiments were carried out by using the rapidly transported substrate carried out by adding 0.15 ml of cell suspension to 0.15 ml of saline containing radiolabel, unlabeled substrate, and inhibitors as appropriate) layered over 0.2 ml of dibutyolphthalate in a microcentrifuge tube. 4–5 s later the tube was centrifuged (10,000 x g, 30 s) to sediment the cells below the oil and thereby terminate the flux. Fixed period choline and Rb$^+$ influx experiments commenced with the addition of radiolabel together with unlabeled substrate to a microcentrifuge tube containing cells together with the appropriate pharmacological reagents. The final sample volume was 0.5 ml. Influx was terminated at the appropriate time by transferring aliquots (0.11 ml) of the suspension to microcentrifuge tubes containing 0.8 ml of ice-cold stopping solution layered over 0.25 ml of dibutyolphthalate. The tubes were centrifuged immediately then processed for scintillation counting as outlined above.

In all influx experiments the amount of radiolabel trapped in the extracellular space was determined using the cell pellets was centrifuged (10,000 x g, 30 s) to sediment the cells below the oil and thereby terminate the flux. Fixed period choline and Rb$^+$ influx experiments commenced with the addition of radiolabel together with unlabeled substrate to a microcentrifuge tube containing cells together with the appropriate pharmacological reagents. The final sample volume was 0.5 ml. Influx was terminated at the appropriate time by transferring aliquots (0.11 ml) of the suspension to microcentrifuge tubes containing 0.8 ml of ice-cold stopping solution layered over 0.25 ml of dibutyolphthalate. The tubes were centrifuged immediately then processed for scintillation counting as outlined above.

In all influx experiments the amount of radiolabel trapped in the extracellular space was determined using the cell pellets was centrifuged (10,000 x g, 30 s) to sediment the cells below the oil and thereby terminate the flux. Fixed period choline and Rb$^+$ influx experiments commenced with the addition of radiolabel together with unlabeled substrate to a microcentrifuge tube containing cells together with the appropriate pharmacological reagents. The final sample volume was 0.5 ml. Influx was terminated at the appropriate time by transferring aliquots (0.11 ml) of the suspension to microcentrifuge tubes containing 0.8 ml of ice-cold stopping solution layered over 0.25 ml of dibutyolphthalate. The tubes were centrifuged immediately then processed for scintillation counting as outlined above.
(10^{12} \text{ RBC-h}). In uninfected cells from the same donors the unidirectional choline influx measured under the same conditions ranged from 1.3 to 7.4 \mu\text{mol/(10}^{12} \text{ RBC-h)}, with a mean value of 3.8 \pm 0.4 \mu\text{mol/(10}^{12} \text{ RBC-h}).

A similar increase was seen in the basal permeability of the infected cell to \textsuperscript{86}Rb\textsuperscript{+} (which provides a measure of K\textsuperscript{+} permeability). Fig. 1B shows time courses for the influx of K\textsuperscript{+} into infected and uninfected cells pretreated with ouabain, bumetanide, and nitrendipine in order to inhibit the native erythrocyte Na\textsuperscript{+}/K\textsuperscript{+} pump, NaKCl\textsubscript{2} cotransporter, and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel, respectively (Ellory et al., 1992). In experiments with blood from 11 different donors, the unidirectional influx of K\textsuperscript{+} measured at 37°C in trophozoite-infected cell suspensions (70–95% parasitemia) in the presence of 100 \mu\text{mol/l ouabain, 100} \mu\text{mol/l bumetanide, and 10} \mu\text{mol/l nitrendipine} per ml to block the influx of \textsuperscript{37}Cl\textsuperscript{−} via the Na\textsuperscript{+}/K\textsuperscript{+} pump, NaKCl\textsubscript{2} cotransporter, and the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel, respectively.

Various membrane transport inhibitors were screened for their effect on the increased cation permeability of malarial-infected cells, and a number of potent inhibitors were identified. Somewhat surprisingly, these were all compounds familiar as anion transport inhibitors. The effect of selected anion transport inhibitors on the malaria-induced components of choline and K\textsuperscript{+} transport into infected erythrocytes is shown in Table I. DIDS (at a concentration far in excess of that usually necessary to block anion exchange via the band 3 protein; e.g. Schofield et al. (1992)) caused partial inhibition of malaria-induced choline and \textsuperscript{86}Rb\textsuperscript{+} influx. Bumetanide, MK-196, nitrendipine, furosemide, and NPPB were more effective inhibitors of the induced choline flux, and the latter four reagents also blocked most of the induced transport of \textsuperscript{86}Rb\textsuperscript{+} (Ellory et al., 1992). Dose-response curves for the effect of nitrendipine, furosemide, and NPPB on malaria-induced choline influx are shown in Fig. 2. The IC\textsubscript{50} values for the three reagents (i.e. the concentrations required to reduce the induced choline influx by 50%) were for nitrendipine, 20 \mu\text{mol/l; for furosemide, 5} \mu\text{mol/l; and for NPPB, 0.8} \mu\text{mol/l.}

\textbf{Malaria-induced Zwitterion Transport—}Having identified a number of potent inhibitors of parasite-induced cation transport we went on to test the effect of these reagents on the transport of many of the other classes of compounds to which infected cells show increased permeability. Screening was carried out using the semi-quantitative hemolysis method that has been used widely in previous studies of the permeability of infected erythrocytes (Ginsburg et al., 1985). Fig. 3A shows time courses for the hemolysis of malaria-infected erythrocytes suspended in an iso-osmotic solution of choline chloride. In the absence of inhibitors the infected cells lysed with a 1/50 value (i.e. the inverse of the half-time for hemolysis) of around 0.052 min\textsuperscript{−1}. Nitrendipine (100 \mu\text{mol/l}, furosemide (100 \mu\text{mol/l}), and NPPB (100 \mu\text{mol/l}) all protected against hemolysis, as might be expected if the hemolysis was due to influx of choline via the induced pathways characterized in Fig. 2.

The remaining panels of Fig. 3 show time courses for the hemolysis of malaria-infected erythrocytes suspended in iso-

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Induced choline influx</th>
<th>Induced K\textsuperscript{+}Rb\textsuperscript{+} influx</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diiodosuccinimide (DIDS)</td>
<td>54 ± 3\textsuperscript{**} (n=3)</td>
<td>57 ± 4 (n=3)</td>
<td>100</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>45 ± 2\textsuperscript{**} (n=6)</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>MK-196</td>
<td>35 ± 4\textsuperscript{**} (n=3)</td>
<td>49 ± 10\textsuperscript{*} (n=3)</td>
<td>100</td>
</tr>
<tr>
<td>Niflumate</td>
<td>16 ± 2\textsuperscript{**} (n=4)</td>
<td>31 ± 9\textsuperscript{*} (n=6)</td>
<td>100</td>
</tr>
<tr>
<td>Furosemide</td>
<td>7 ± 1\textsuperscript{**} (n=7)</td>
<td>22 ± 4\textsuperscript{**} (n=9)</td>
<td>100</td>
</tr>
<tr>
<td>NPPB</td>
<td>7 ± 2\textsuperscript{*} (n=4)</td>
<td>17 ± 4\textsuperscript{*} (n=4)</td>
<td>100</td>
</tr>
</tbody>
</table>

*In all cases the induced flux measured in the presence of each of the transport inhibitors was significantly less than that measured in their absence (paired t test; \textsuperscript{*}p < 0.05; \textsuperscript{**}p < 0.01; \textsuperscript{***}p < 0.001).

The induced flux was calculated by subtracting the influx rate measured in uninfected erythrocytes (cultured in parallel with infected cells) from that in infected cells from the same donor. Induced influx rates (mean values from n experiments, each on cells from different donors, mean ± S.E.) are expressed as a percentage of those measured in the absence of inhibitor. Cholinefluxes were measured at an extracellular choline concentration of 1 \mu\text{mol/l, under which conditions the influx of choline into infected cells was generally 20-fold higher than that into uninfected cells. K\textsuperscript{+}Rb\textsuperscript{+} influxes were measured in the presence of ouabain (100 \mu\text{mol/l}) and bumetanide (100 \mu\text{mol/l}) to inhibit the endogenous Na\textsuperscript{+}/K\textsuperscript{+} pump and the NaKCl\textsubscript{2} cotransporter, respectively; under these conditions the influx of \textsuperscript{86}Rb\textsuperscript{+} into infected cells was typically 10-fold higher than that into uninfected cells.**
tested niflumate, furosemide, and NPPB caused a reduction in the rate of hemolysis, consistent with their having reduced the osmotic solutions of a variety of amino acids. The l/50 values in and all choline chloride, (B) L-glutamine, uptake of substrate through the induced transport route. In all the absence of inhibitors were 0.056 min⁻¹ for L-glutamine, all 37 °C. For each substrate the data are averaged from between 9 and 6 different experiments on cells from different donors (mean ± S.E.). Symbols: O, control; A, NPPB; ▲, furosemide; ◦, niflumate.

osmotic solutions of a variety of amino acids. The l/50 values in the absence of inhibitors were 0.056 min⁻¹ for L-glutamine, 0.080 min⁻¹ for L-serine, 0.11 min⁻¹ for L-threonine, 0.30 min⁻¹ for L-alanine, 0.31 min⁻¹ for D-alanine, 0.55 min⁻¹ for L-valine, and 1.15 min⁻¹ for L-methionine. The time course for hemolysis of cells in iso-osmotic L-alanine was similar to that in iso-osmotic D-alanine which implies that the induced transport mechanism was not stereoselective. For all of the amino acids tested niflumate, furosemide, and NPPB caused a reduction in the rate of hemolysis, consistent with their having reduced the uptake of substrate through the induced transport route. In all cases 100 μM NPPB provided almost full protection against hemolysis throughout the duration of the experiment. Furosemide at 100 μM was somewhat less potent and niflumate at 100 μM slightly less effective. The order of efficacy of the three inhibitors was therefore the same as that seen for their inhibition of malaria-induced choline influx (Fig. 2).

Fig. 4A shows time courses for the influx of L-carnitine, another electroneutral zwitterion, into normal and malaria-infected erythrocytes (measured from l-¹⁴C)carnitine uptake). There was no significant influx of L-carnitine into uninfected cells over a 4-min period (when presented at an extracellular concentration of 1 μM). By contrast, L-carnitine was transported into malaria-infected erythrocytes via a pathway that was blocked by furosemide (100 μM). Fig. 4B shows the corresponding hemolysis experiment. Infected cells suspended in an iso-osmotic carnitine solution lysed with a l/50 value of 0.030 min⁻¹. Niflumate (100 μM), furosemide (100 μM), and NPPB (100 μM) each provided full protection against hemolysis for up to 60 min.

Malaria-induced Sugar Transport—As with the induced transport of amino acids, the malaria-induced transport of a variety of sugars was inhibited by niflumate, furosemide, and NPPB. Fig. 5 shows time courses for the hemolysis of malaria-infected erythrocytes suspended in iso-osmotic solutions of the 5-carbon sugar D-ribose, the 6-carbon sugars D-galactose and D-glucose, the 6-carbon sugar-alcohol D-sorbitol, and the disaccharide sucrose.

In the ribose solution the infected cells lysed with a l/50 value of 0.78 min⁻¹; the rate of hemolysis was reduced by niflumate, more so by furosemide, and more so by NPPB. Hemolysis in the 6-carbon sugar solutions was slower than in the ribose solution; the l/50 values were 0.26 min⁻¹ in the D-sorbitol solution, 0.10 min⁻¹ in the D-glucose solution, and 0.088 min⁻¹ in the D-galactose solution. In each of these solutions hemolysis was prevented almost completely during the time scale of the experiments by the three inhibitors, although infected cells in iso-osmotic sorbitol solution containing 100 μM niflumate showed some hemolysis after 60 min. As has been shown previously (Ginsburg et al., 1983), malaria-infected cells did not lyse in an iso-osmotic sucrose solution.

Malaria-induced Nucleoside Transport—A number of recent studies have focused on the enhanced permeability of malaria-infected erythrocytes to nucleosides (reviewed by Gero and Upston (1992)). We therefore tested the effects of the three inhibitors identified in this study on the transport into infected cells of the purine nucleoside adenosine and the pyrimidine nucleosides thymidine and uridine. Fig. 6 shows time courses for the uptake of (A) adenosine and (B) thymidine (each presented at an extracellular concentration of 1 μM) by normal and malaria-infected erythrocytes. The cells were pretreated with 20 μM NBMPR to inhibit the endogenous erythrocyte nucleoside transporter. For both adenosine and thymidine, influx into infected cells was markedly enhanced and was inhibited by niflumate, more so by furosemide and more so by NPPB.

Also shown in Fig. 6 are time-courses for hemolysis of infected cells suspended in iso-osmotic solutions of (C) thymidine
and (D) uridine. Qualitatively, the pattern of inhibition is the same as that seen in the previous hemolysis experiments, the three inhibitors provided protection against hemolysis in the order niflumate < furosemide < NPPB. Quantitatively, it is clear from Fig. 6, C and D, that hemolysis of infected cells in iso-osmotic thymidine and uridine solutions was less sensitive to inhibition than was hemolysis of infected cells in iso-osmotic solutions of the other classes of substrate tested (Figs. 3–5). For parasitized cells suspended in an iso-osmotic thymidine solution, niflumate and furosemide caused only a moderate reduction in the rate of hemolysis, and even in the presence of NPPB there was significant hemolysis after 12 min. (Fig. 6C). One possible explanation for the variation in inhibitor potency in the different iso-osmotic solutions is that different classes of substrate use distinct transport pathways, all sensitive to inhibition by niflumate < furosemide < NPPB, but with different IC\textsubscript{50} values in each case. An alternative possibility is that the uptake is mediated by a single pathway whose inhibitor sensitivity varies between the different iso-osmotic solutions, perhaps as a result of the more hydrophobic substrates (such as thymidine and uridine) competing with the inhibitors for occupancy of the transport pathway when presented at the very high concentrations used in the hemolysis experiments (i.e. 300 mm).

To distinguish between these possibilities we compared dose-response curves for the effect of the three inhibitors on the influx of \textsuperscript{3}H\textSubscript{2}thymidine with those for their effect on the influx of \textsuperscript{14}C\textSubscript{2}choline into malaria-infected cells under identical extracellular conditions. For these (paired) experiments, cells were suspended in physiological saline containing both 1 mm thymidine and 1 mm choline (i.e. more than two orders of magnitude lower than the concentrations used in the hemolysis experiments). As is shown in Fig. 7, for each inhibitor, the dose-response curves for the two different substrates were very similar, and there was no significant difference (p > 0.15, paired t test) between the relative induced fluxes of the two substrates at any of the inhibitor concentrations tested. Thus, although the inhibitors were much less effective at protecting parasitized cells against hemolysis in an iso-osmotic thymidine solution than in an iso-osmotic choline solution, a quantitative comparison of the effect of the inhibitors on the flux of thymidine and choline into malaria-infected erythrocytes under the same extracellular conditions reveals no difference in the pharmacological sensitivity of the induced transport of these two substrates. This is consistent with the view that a single pathway mediates the malaria-induced transport of choline and thymidine and that the differences between the inhibitor sensitivities in the thymidine and choline hemolysis experiments are due to the different effects of very high concentrations of these two solutes on the pathway.

The kinetics of thymidine transport into malaria-infected erythrocytes was investigated, and the results are shown in Fig. 8. Furosemide-sensitive influx of thymidine into infected erythrocytes increased linearly with concentration up to 10 mm, confirming the non-saturability of the furosemide-sensitive pathway over a concentration range far in excess of normal physiological values.

Malaria-induced Anion Transport—Recently, two groups have reported a marked increase in the permeability of the malaria-infected erythrocyte to lactate (Kanaani and Ginsburg...
Induced Transport in Malaria-infected Erythrocytes

Fig. 7. Dose-response curves for the effects of NPPB (A), furosemide (B), and niflumate (C) on the malaria-induced influx of thymidine (solid lines) at 22 °C. Cells were suspended in a standard HEPES-buffered saline containing 1 mM thymidine and 20 μM NBMPR, as well as 1 mM choline. The induced flux was calculated by subtracting that measured in uninfected erythrocytes (cultured in parallel with infected cells) from that measured in infected cells from the same donor. The data comprising each curve were averaged from five different experiments on cells from different donors (mean ± S.E.). The data shown are from a single experiment, representative of that obtained in several similar experiments on cells from different donors.

Fig. 8. Concentration-dependence of thymidine influx into uninfected erythrocytes (•) and malaria-infected erythrocytes (○) in the presence of niflumate (•) and absence (open symbols) of furosemide (○). The data shown are from a single experiment, representative of that obtained in two similar experiments on cells from different donors.

Fig. 9. A, time courses for the influx of lactate into uninfected erythrocytes (○) and malaria-infected erythrocytes (△) in the absence of induced transport inhibitors (○) and in the presence of NPPB (△), furosemide (■), or NBMPR (▲), each at a concentration of 100 μM. The extracellular lactate concentration was 1 mM, and the temperature was approximately 22 °C. All samples contained 10 μM DIDS and 100 μM p-chloromercuribenzene sulfonic acid. B, time courses for the influx of Cl⁻ into uninfected erythrocytes (○) and malaria-infected erythrocytes (△) and in the presence of niflumate (■), furosemide (■), or NPPB (▲), each at a concentration of 100 μM. The extracellular Cl⁻ concentration was 150 mM, and the temperature was approximately 22 °C. All samples contained 10 μM DIDS. For both substrates the data shown are from a single experiment, representative of that obtained in several similar experiments on cells from different donors.

DISCUSSION

A wide variety of low molecular weight solutes has been shown previously to permeate malaria-infected erythrocytes via high capacity, non-saturable pathways that are functionally different from the transporters of normal cells (reviewed by Cabantchik (1990), Ginsburg (1990), and Gero and Upston (1992)). In the present study we have carried out experiments to address the question of whether the phenomenon is mediated by a single type of pathway or whether there are heterogeneous, substrate-specific transport pathways involved. In our initial experiments a number of anion transport blockers were identified as potent inhibitors of the enhanced cation permeability of malaria-infected cells. Three of these, NPPB, furosemide, and niflumate, were subsequently tested for their effect on the malaria-induced transport of neutral amino acids, sugars, nucleosides, and anions, using a combination of isosmotic hemolysis and radioisotope flux techniques. For each class of substrate tested the induced permeability was inhibited by the three inhibitors in order of potency: NPPB > furosemide > niflumate. The relative efficacy of the inhibitors at blocking hemolysis varied somewhat for the different isosmotic solutions tested (Figs. 3-6). However, a quantitative comparison of the effect of the inhibitors on the induced influx of thymidine and choline, substrates showing quite different
transport rates and inhibitor sensitivities in iso-osmotic hemolysis experiments, revealed no difference in the pharmacological sensitivity of the pathways by which these two disparate solutes entered infected cells in physiological media (Fig. 7). The explanation consistent with the pharmacological data obtained in this study is that most of the induced transport of cations, anions, amino acids, monosaccharides, and nucleosides into malaria-infected erythrocytes occurs via a single type of pathway. The functional characteristics of the pathway, its broad specificity, its non-saturability, and its failure to distinguish between stereoisomers (L- and D-alanine), are quite unlike those of a conventional transporter but are those expected of a pore or channel (Ellory et al., 1988; Stein, 1990). For those substrates for which quantitative influx measurements were made the magnitude of the malaria-induced (inhibitor-sensitive) transport was in the order: \( \text{Cl}^- > \text{lactate} > \text{thymidine} > \text{adenosine} > \text{carnitine} > \text{choline} > K^+ \) (Fig. 10). The pathway is therefore anion-selective.

Anion-selective channels are widespread in the membranes of eukaryotic cells. Their physiological functions are generally assumed to relate to their permeability to the inorganic Cl\(^-\) ion, and they are commonly referred to as "chloride channels." The pharmacology of the induced pathway of malaria-infected erythrocytes is similar to that of chloride channels in other cell types. NPPB was identified originally as an inhibitor of Cl\(^-\) conductance in rabbit kidney (Wangemann et al., 1986), and it is, for many Cl\(^-\) channels, the most potent inhibitor yet identified. The IC\(_{50}\) of around 0.8 \(\mu\)M obtained here for the inhibition by NPPB of malaria-induced choline transport suggests that it is the most potent inhibitor of malaria-induced transport reported to date and falls within the range found previously for Cl\(^-\) channels in other cells (Wangemann et al., 1986; Paulmichl et al., 1992; Cabantchik and Greger, 1992). Furosemide, which has been shown previously to block the transport of adenosine into mouse erythrocytes infected with *Plasmodium yoelii* (Gati et al., 1990), has been suggested to inhibit Ca\(^{2+}\)-activated Cl\(^-\) channels in rat lacrimal glands (Evans et al., 1986), and it has been reported that both furosemide and bumetanide block Cl\(^-\) channels in bovine kidney (Pope et al., 1991). Niflumate blocks Ca\(^{2+}\)-activated Cl\(^-\) channels in *Xenopus oocytes* (White and Aylwin, 1990), and MK-196, which blocked malaria-induced cation transport (Table 1), inhibits Cl\(^-\) conductance in frog skin (Nagel et al., 1985) as well as volume-activated Cl\(^-\) channels in Ehrlich ascites tumor cells (Aabin and Hoffmann, 1986). DIDS which, at a concentration of 100 \(\mu\)M, caused partial (40–50\%) inhibition of the malaria-induced choline and \(^{86}\)Rb\(^+\) influx (Table 1) has a similar effect (i.e., partial blockage at 100 \(\mu\)M) on an anion-selective channel in human platelets (Manning and Williams, 1989), and it also inhibits Cl\(^-\) channels in a variety of epithelial cells (summarized by Gregor (1990) and by Frizzell and Cliff (1992)) and in lymphocytes (Sarkadi et al., 1985).

The simplicity consistent with the pharmacology, the substrate selectivity of the malaria-induced pathway bears a striking resemblance to that of chloride channels in other tissues. Although anion-selective, the induced pathway has a significant permeability to both inorganic and organic cations. This is a characteristic of many types of chloride channel (Francioli and Petris, 1990): for example, the "background chloride channel" of hippocampal neurons is permeable to monovalent inorganic cations (Francioli and Nonner, 1987) and has an even greater permeability to the organic cation, Tris (Francioli and Petris, 1992); the anion-selective channel from human platelets is permeable to \( K^+ \) and to the much larger tetraethylammonium ion (Manning and Williams, 1989); similarly, the "Ca\(^{2+}\)-activated Cl\(^-\) channel" of *Xenopus oocytes* is permeable not only to monovalent inorganic cations, but also to the divalent cation, Ca\(^{2+}\) (Young et al., 1984).

The similarities between the substrate selectivity of the malaria-induced pathway and chloride channels elsewhere also extend to the permeability pattern seen for organic solutes. A comparison of the rates of hemolysis of infected cells (Figs. 3–6) in iso-osmotic organic solute solutions suggests that the malaria-induced pathway shows a preference for more hydrophobic compounds. For the amino acids (as has been noted previously by Ginsburg et al. (1985) and by Ginsburg and Stein (1987b)), the presence of a (hydrophilic) hydroxyl group decreased the apparent rate of transport via the malaria-induced pathways (cf. valine *versus* threonine, alanine *versus* serine; Fig. 3) whereas the presence of (hydrophobic) methyl groups enhanced uptake (cf. valine *versus* alanine, threonine *versus* serine; Fig. 3). Of all the amino acids tested, methionine, the least water-soluble, showed the highest rate of induced transport (Fig. 3H). A similar trend is seen for the pyrimidine nucleosides; thymidine, with one less hydroxyl group and one more methyl group than uridine, permeated the infected cell much more rapidly (cf. Fig. 6, C and D). The observation that adenosine permeated the pathway (Fig. 6A) while sucrose, a similarly sized but much more hydrophilic molecule, did not (Fig. 6A) is again consistent with the view that hydrophobicity is an important determinant of permeation rate.

A number of chloride channels have been shown to accommodate organic ions, including anionic amino acids, polyols such as gluconate, and monocarboxylate acids such as lactate (Pollard et al., 1991; Valverde et al., 1992; Halm and Frizzell, 1992; Roy and Malo, 1992; Banderali and Roy, 1992; Rasola et al., 1992). There has been relatively little attention paid to the factors governing organic substrate selectivity; however, there is evidence that at least one such channel, the background Cl\(^-\) channel of hippocampal neurons, shares with the malaria-in-
duced transport pathway a preference for more hydrophobic solutes. The permeability of the background Cl⁻ channel to benzoate is nearly three times that to Cl⁻ itself, suggesting that the transport of organic substrates via this channel involves hydrophobic interactions between the solutes and components of the channel (Franciolini and Nonner, 1987). The results obtained in the present study, and previously (Ginsburg et al., 1985) are consistent with the same being true of the malaria-induced pathway. Such interactions may also be important in the mechanism by which the inhibitors identified in this study (all of which contain hydrophobic groups) block the pathway.

Whether the functional similarities between the transport pathway characterized here and chloride channels elsewhere are based on a genuine structural resemblance remains to be established. So too does the location and the physiological function(s) of the induced pathway. In the conventional view of the malaria-infected red cell the intracellular parasite is enclosed within both the parasitophorous vacuole and erythrocyte membrane, with compounds entering and leaving the parasite via the erythrocyte cytoplasm. In this model the induced channel is presumed to be situated at the red cell surface where it may facilitate the rapid entry of nutrients and the rapid exit of metabolic wastes from the infected cell cytoplasm. An alternative view, for which evidence is accumulating, is that the intracellular parasite may actually have direct access to the extracellular solution (Bodhammer and Bahr, 1973; Elford et al., 1985; Pouvelle et al., 1991; Loyerovsky et al., 1993), either via a "metabolic window" (Bodhammer and Bahr, 1973) or a "parasitophorous duct" (Pouvelle et al., 1991). This remains controversial (e.g. Sherman and Zidovetzki, 1992; Haldar and Uyetake, 1992; Fujioka & Aikawa, 1993); however, it does raise the possibility that solutes might enter the parasite directly, without passing through the erythrocyte cytoplasm. The solute pathway characterized here may therefore be either in the parasite membrane (with access being provided via a duct) or located at a "window" formed at a point of contact between the parasite, parasitophorous vacuole, and erythrocyte membranes. In either case it might be involved in the trafficking of metabolites and catabolites directly between the parasite cytoplasm and the extracellular medium, although the question then arises of how the parasite maintains its ionic and biochemical composition in the presence of this high capacity "leak" pathway.

Another alternative is that the non-saturable, high capacity pathway characterized here is in the erythrocyte membrane but is not primarily involved in the supply of nutrients to the parasite. Instead, it may play a "housekeeping" role in the host erythrocyte, perhaps being involved in cell volume control. In this context it is perhaps significant to note that the pathway shows marked functional similarities to chloride channels in other cell types and is therefore unlikely to be a simple defect in the lipid bilayer or a consequence of general membrane damage. This is relevant not only to our understanding of mechanisms of solute trafficking in malaria-infected cells but to the possible physiological roles of chloride channels elsewhere. Although, among the solutes tested here, the malaria-induced pathway showed the highest permeability to Cl⁻, it had a significant permeability to a wide variety of both charged and uncharged organic compounds, and it is these, rather than Cl⁻, that are likely to be the physiologically relevant substrates. This raises the question of whether the same might be true of chloride channels in other tissues. As has been discussed, a number of chloride channels have been shown to accommodate organic ions (and indeed, in at least one case, to show a pronounced preference for large hydrophobic substrates). However, there is little information available regarding the permeability of such channels to uncharged solutes (which are not amenable to study using electrophysiological techniques), and there is little recognition of a physiological role for the permeability of such channels to anything other than Cl⁻. The data obtained in the present study focus attention on the possibility that anion-selective channels might provide effective routes for the high capacity transport of low molecular weight organic substrates and, furthermore, that this might be an important aspect of their physiological function.

Acknowledgment—We thank Steven Cullford for skilled technical assistance in a number of the early experiments in this study.

REFERENCES


Induced Transport in Malaria-infected Erythrocytes

hydrophobic solutes; and it is inhibited by a range of chloride channel blockers, including furosemide, niflumate, MK-196 and, most effectively, NPPB (Kirk et al., 1992c). The functional characteristics of the pathway in fish erythrocytes are those of a volume-activated chloride channel although, as with the malaria-induced pathway, the molecular identity of this system remains to be established.

In summary, the pharmacological data obtained in this study are consistent with the hypothesis that much of the induced, non-saturable transport of low molecular weight solutes into malaria-infected erythrocytes occurs via a single type of pathway. This pathway shows marked functional similarities to chloride channels in other cell types and is therefore unlikely to be a simple defect in the lipid bilayer or a consequence of general membrane damage. This is relevant not only to our understanding of mechanisms of solute trafficking in malaria-infected cells but to the possible physiological roles of chloride channels elsewhere. Although, among the solutes tested here, the malaria-induced pathway showed the highest permeability to Cl⁻, it had a significant permeability to a wide variety of both charged and uncharged organic compounds, and it is these, rather than Cl⁻, that are likely to be the physiologically relevant substrates. This raises the question of whether the same might be true of chloride channels in other tissues. As has been discussed, a number of chloride channels have been shown to accommodate organic ions (and indeed, in at least one case, to show a pronounced preference for large hydrophobic substrates). However, there is little information available regarding the permeability of such channels to uncharged solutes (which are not amenable to study using electrophysiological techniques), and there is little recognition of a physiological role for the permeability of such channels to anything other than Cl⁻. The data obtained in the present study focus attention on the possibility that anion-selective channels might provide effective routes for the high capacity transport of low molecular weight organic substrates and, furthermore, that this might be an important aspect of their physiological function.