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Parasite-induced permeation of nucleosides in *Plasmodium falciparum* malaria

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Abstract

A mechanism which mediates the transport of the nonphysiological nucleoside, L-adenosine, was demonstrated in *Plasmodium falciparum* infected erythrocytes and naturally released merozoites. L-Adenosine was not a substrate for influx in freed intraerythrocytic parasites or in normal human erythrocytes nor was L-adenosine transported in a variety of cell types including other parasitic protozoa such as *Crithidia luciliae, Trichomonas vaginalis, Giardia intestinalis*, or the mammalian cells, Buffalo Green Monkey and HeLa cells. L-Adenosine transport in *P. falciparum* infected cells was nonsaturable, with a rate of 0.13 ± 0.01 pmol/μl cell water per s per μM L-adenosine, yet the transport was inhibited by furosemide, phloridzin and piperine with IC_{50} values between 1–13 μM, distinguishing the transport pathway from simple diffusion. The channel-like permeation was selective as disaccharides were not permeable to parasitised cells. In addition, an unusual metabolic property of parasitic adenosine deaminase was found in that L-adenosine was metabolised to L-inosine by both *P. falciparum* infected erythrocytes and merozoites, an activity which was inhibited by 50 nM deoxycoformycin. No other cell type examined displayed this enzymic activity. The results further substantiate that nucleoside transport in *P. falciparum* infected cells was significantly altered compared to uninfected erythrocytes and that L-adenosine transport and metabolism was a biochemical property of Plasmodium infected cells and merozoites and not found in normal erythrocytes nor any of the other cell types investigated.

Keywords: Merozoite; L-Adenosine; Nucleoside transport; Parasitophorous duct; Metabolism; (*P. falciparum*)

1. Introduction

In mammalian cells, including erythrocytes, the transport of both physiological and cytotoxic nucleosides occurs via specific proteins in the cell membrane (reviews [1–3]). Nucleoside influx in erythrocytes is reversible, non-concentrative and potently inhibited by structural analogs of adenosine, such as 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine, NBMPR) and nitrobenzylthioguanosine, and by the vasodilators, dipyridamole and dilazep [1–3]. The mammalian transporter is specific for nucleosides and although it is tolerant of a variety of modifications to the purine ring, it is enantiomerically selective, in that the normal physiological structure of the ribose moiety (9-β-D-ribofuranosyl) is crucial for transport.

Recently we have determined that in order to obtain purine compounds from the extracellular media, the intraerythrocytic malaria parasite appears to induce specific nucleoside transport sites into the membrane of the infected host erythrocyte of both normal and drug resistant strains of malaria [4–6]. The new parasite-induced nucleoside transport sites are distinct from those of the normal host erythrocytic nucleoside transporter, by their insensitivity to NBMPR inhibition. The endogenous nucleoside transporter, which is potently inhibited by NBMPR, remains functional in infected cells and represents about 50% of the total nucleoside transport that occurs in the malarial infected cell [7].

In this paper we demonstrate that the parasite-induced nucleoside transport mechanism found in *P. falciparum* infected cells is a combination of nonsaturable and carrier-mediated transport. The nonsaturable component allows the entry into the cell of compounds which cannot enter via the endogenous host nucleoside transporter. We have examined herein the nonsaturable nature of nucleo-

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side transport in *P. falciparum* infected erythrocytes, free parasites and merozoites using the nonphysiological nucleoside isomer, L-adenosine (9-β-L-ribofuranosyl). This transport function was characteristically different to the normal erythrocytic nucleoside transporter and represents an unusual property of Plasmodium infection which may be targeted by new antimalarial strategies.

2. Materials and methods

2.1. Cell cultures

P. falciparum, FCQ-27 isolate, was maintained in culture using the standard techniques described by Trager and Jensen [8]. Cultures contained 2% hematocrit suspensions of parasitised human type O + erythrocytes in RPMI 1640 medium (ICN Biomedicals, CA USA), supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Sigma, MO, USA), pH 7.2, 25 mM $NaHCO_3$ and 10% (v/v) human serum and were maintained at 37° C in modular incubator chambers in a gas mixture of $CO_2/O_2/N_2$ (5:5:90%, v/v). Erythrocytes and human serum were provided by the New South Wales Red Cross Blood Transfusion Service from healthy volunteers. Infected erythrocytes in culture were synchronised using sterile D-sorbitol [9]. Mature trophozoite infected cells were concentrated using Percoll (Pharmacia, Sweden) gradients as described previously [5]. The resulting cells (> 80% parasitised with mature trophozoites) were washed three times to remove Percoll in phosphate-buffered saline containing glucose (PBSG) (1.8 mM KH_2PO_4, 5 mM K_2HPO_4, 0.9% NaCl (w/v), 5 mM D-glucose, pH 7.4) and resuspended to a concentration of $2 \cdot 10^8$ cells/ml in PBSG.

Highly purified naturally released merozoites (exoerythrocytic forms) were purified from schizont enriched synchronous parasite cultures by centrifugation of the culture media as described in [10]. Culture media were centrifuged at $440 \times g$ for 6 min to remove any infected or non infected erythrocytes. The resulting supernatant was subsequently centrifuged at $3000 \times g$ for 10 min to pellet the merozoites, washed twice in PBSG buffer and used immediately for experimentation. Freed intraerythrocytic parasites were isolated from mature trophozoites by 0.1% saponin lysis [11]. As described previously, parasites released by this method were consistently found to be enclosed within the PVM and were morphologically intact [11]. Giemsa-stained smears of merozoite preparations and free intraerythrocytic parasites indicated the complete absence of contaminating infected or uninfected erythrocytes.

Crithidia luciliae was grown aerobically in RPMI 1640 medium containing 10% (v/v) foetal calf serum and 1% (w/v) L-glutamine at pH 7 in a 26° C incubator [12]. *Trichomonas vaginalis*, (WAA 38 strain) was cultured in modified TYM medium [13] without agar, but containing 14 mM D-glucose (replacing maltose) and 10% (v/v) heat-inactivated new born calf serum. *Giardia intestinalis* trophozoites (Portland 1 stock ATCC 30888) were grown under microaerophilic conditions at 37° C using TYI-S-33 medium with the modification that pooled human serum replaced foetal calf serum [14]. Parasitic protozoa at mid logarithmic phase were harvested and resuspended in PBSG. Buffalo Green Monkey (BGM) and HeLa 229 cells were cultured in DMEM (Cytosystems, NSW, Australia) containing 10% (v/v) foetal calf serum, 1% (w/v) L-glutamine, 30 mM D-glucose and 50 μg/ml gentamycin. Mammalian cells were harvested at exponential growth by trypsinisation. Cell numbers were assessed microscopically using a haemocytometer with Improved Neubauer ruling.

2.2. Nucleoside transport measurements

The radiolabelled substrates utilised in nucleoside transport assays; D-[2-^{3}H]adenosine (26 Ci/mmol), ^3H_2O (5 mCi/mmol), [U-^{14}C]sucrose (540 mCi/mmol) and inulin [^{14}C]carboxylic acid (9.4 mCi/mmol) were obtained from Amersham International, Buckinghamshire, UK, except for L-[8-^{3}H]adenosine (33 Ci/mmol) and unlabelled L-adenosine which were purchased from Moravek Biochemicals (CA, USA). Nucleoside transport over short time intervals at 22° C was measured by an established procedure as reported previously [15]. Transport assays were initiated when the cells ($2 \cdot 10^7$ in 100 μl) were added to Eppendorf centrifuge tubes containing 150 μl of an oil mixture with 100 μl of radiolabelled nucleoside permeant layered on top of the oil. The oil mix was adjusted for the different cell types used. For uninfected erythrocytes, *P. falciparum* infected cells and freed parasites, a mixture of silicon oils (Ajax Chemicals, Australia), designated 'Oil-A', was prepared by combination of three different oil grades as described in [15]. For *P. falciparum* merozoites a mixture of Oil-A and 200 (5cTs) silicon oils, 3:1 (v/v), was used. Transport assays for the parasitic protozoa *C. luciliae, T. vaginalis and G. intestinalis*, utilised a phthalate oil mix, prepared by combining di-n-butyl phthalate and di-iso-octyl phthalate (Ajax Chemicals, Australia), 8:2 (v/v). For the mammalian cells BGM and HeLa, a combination of 1:1 (v/v), di-n-butyl phthalate/di-iso-octyl phthalate was used. The density of all oil mixes was 1.03 g/ml, except for the oil mix of 1.02 g/ml used for merozoite transport.

Transport assays at 22° C, were terminated between 4 and 14 s by fast centrifugation ($16000 \times g$ for 15 s) which pelleted cells under the oil layer. Cell passage through the oil during centrifugation was calculated to take 3–4 s. Influx rates were determined from the initial linear portion of the transport data. 2'-Deoxycoformycin 50 nM was used in all transport experiments using D-adenosine to inhibit metabolism. Under the experimental conditions of the transport assay, using 1 μM L- or D-adenosine as the permeant, less than 5% of the nucleoside was metabolised within the cell, as determined by PEI chromatography

described below. Although adenosine transport in the presence of 2′-deoxycoformycin proceeded in the absence of metabolism, intracellular adenosine concentrations higher than the extracellular substrate concentration were detected in *P. falciparum* infected cells and may represent binding to cytosolic or one of the three membrane components in infected cells. This problem is often encountered in transport experiments [16,17]. Background radioactivity due to the extracellular volume of permeant present in the cell pellet was measured by control experiments containing [U-^{14}C]sucrose in the medium in place of the nucleoside permeant. Inulin [^{14}C]carboxylic acid was used as an extracellular volume marker for *C. luciliae*, *T. vaginalis*, and *G. intestinalis*. $^{3}H_2O$ was used to determine the total cell water space. The cell pellet was processed as described previously [15] and counted in a Packard Model 1900TR liquid scintillation spectrometer. To determine quenching of the radioactive samples, internal standards were introduced in each assay performed.

2.3. Transport inhibitor studies

All compounds (obtained from Sigma, USA), tested as potential inhibitors were dissolved in PBSG and preincubated with the cells for 15 min at 37° C. The cells were returned to room temperature before assaying for transport. Exceptions were piperine and carbonylcyanide *m*-chlorophenylhydrazone which were first dissolved in ethanol then diluted to the correct concentration in PBSG (< 0.5% (v/v) ethanol) before addition to the cells for preincubation. A saturated solution of nitrobenzylthioinosine (NBMPR) (Sigma, USA) in PBSG was obtained by stirring overnight at room temperature. The IC_{50} values for inhibition experiments were defined as the concentration of reagent required to inhibit transport by 50%.

2.4. Metabolism of L- and D-adenosine

Analysis of the metabolism of D- and L-adenosine was performed as previously described [5]. Whole cell preparations and lysates (freeze-thawed three times in PBSG) of *P. falciparum* infected cells, merozoites and normal erythrocytes were incubated at 37° C and the reactions initiated by adding cells or lysates to prewarmed radiolabelled substrate (final concentration, 50 μM). Lysates were not used experimentally for more than 1 h. Aliquots (200 μl) were removed immediately after mixing (zero time) and at appropriate intervals and the reactions terminated by addition of ice-cold 3 M perchloric acid (40 μl), followed by neutralisation with 4.5 M KOH in 1 M $KHCO_3$. Samples were analysed by ascending thin layer chromatography using Polyethyleneimine-impregnated (PEI) cellulose plates (Macherey Nagel, Germany). Briefly cell extracts (containing radiolabelled purine nucleosides, bases and nucleotides) (20 μl) were streaked onto the PEI plates and separated using butan-1-ol/acetic acid/water, 5:3:2 (v/v) as described in [18] with the following 'cold' and ^{14}C-labelled 'hot' markers (Amersham International, Buckinghamshire, UK) in separate lanes; D-adenosine, L-adenosine, inosine, hypoxanthine, IMP and ATP. Each lane containing the sample was sectioned into 0.5 cm squares and analysed by scintillation counting. Where ^{3}H-labelled substrates were used for PEI thin-layer chromatography the results were represented as direct counts (measured in cpm) occurring in the various bands of the plate due to the substantial quenching of the tritium label on PEI plates. Therefore, qualitative rather than quantitative results were derived from this data. Quenching of the signal was compensated in part by increasing the specific activity of the labelled substrate in the assay.

2.5. In vitro incorporation of nucleosides

The incorporation of various radiolabelled nucleoside substrates into *P. falciparum* nucleic acid material under in vitro culture conditions was determined essentially as described by Desjardins et al. [19] for [G-^{3}H]hypoxanthine incorporation in a 96-well microtiter plate. Each substrate tested, ^{3}H-labelled L-adenosine, D-adenosine, uridine and guanosine, was added to parasitised cells at a concentration of 0.125 μM. Radiolabel present in parasite nucleic acid material was detected as described in [19].

2.6. Nucleoside transport into swollen erythrocytes

Normal erythrocytes were swollen to approximately the same volume size as *P. falciparum* infected cells (1.5-times) by placement in hypotonic buffer as described for fish erythrocytes [20]. Erythrocytes exposed to isotonic, Na^+-free buffer (150 mM KCl, 15 mM Hepes, 5 mM D-glucose, pH 7.4.) were washed four times (10 times volume) and incubated at room temperature for 1 h. Cells were then centrifuged (500 × *g* for 5 min) and 500 μl aliquots diluted to 2.5 ml in either isotonic or hypotonic buffer. Hypotonic buffer contained 70% isotonic and 30% 15 mM Hepes, 5 mM D-glucose, pH 7.4. The transport of nucleosides was examined in these cells and compared to normal cells suspended in isotonic solution and to *P. falciparum* infected cells. 2′-Deoxycoformycin (final concentration, 0.5 μM) was added to each assay to inhibit metabolism. NBMPR (1 μM) was also added to cells to block adenosine influx via the erythrocyte nucleoside transporter.

3. Results

3.1. Transport of L-adenosine in P. falciparum infected erythrocytes

The NBMPR-insensitive parasite-induced nucleoside transport system in the infected erythrocytes containing

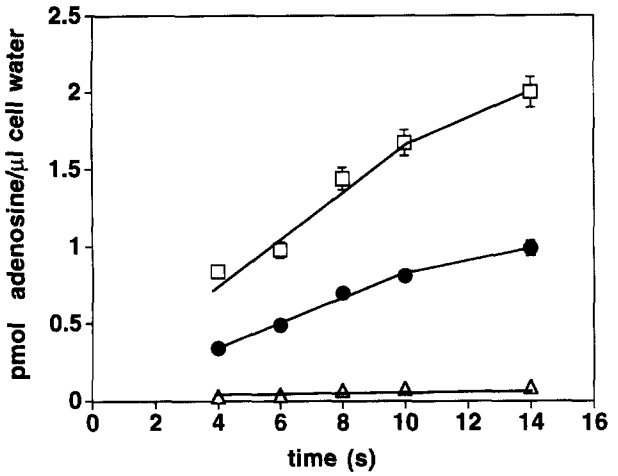

Fig. 1. Transport of L-adenosine in *P. falciparum* infected human erythrocytes. Transport of 1 µM L-[^{3}H]adenosine (●) measured over 14 s in *P. falciparum* trophozoite infected erythrocytes (85% parasitised) was compared to the transport of 1 µM L-[^{3}H]adenosine in normal human erythrocytes (△) and 1 µM D-[^{3}H]adenosine in the presence of 1 µM NBMPR in infected cells (□). Influx was measured by exposure of the cells to D- or L-[^{3}H]adenosine followed by centrifugation of the cells through an inert oil layer, which was calculated to take an additional 4 s. The results represent the means of triplicate experiments ± S.E.

85% trophozoites is represented in Fig. 1. The rate of parasite-induced transport of D-adenosine (1 µM) in the presence of NBMPR (1 µM) was 0.17 ± 0.01 pmol D-adenosine/µl cell water per s. The initial rate of transport of L-adenosine in the same preparations of trophozoite infected erythrocytes was 0.08 ± 0.006 L-adenosine pmol/µl cell water per s and represented approx. 50% of the total parasite-induced nucleoside transport. L-Adenosine was totally impermeable to uninfected human erythrocytes (Fig. 1).

Both 1 µM L-adenosine and D-adenosine were also transported into *P. falciparum* merozoites at similar rates of 0.07 ± 0.005 pmol L-adenosine/µl cell water per s and 0.06 ± 0.001 pmol D-adenosine/µl cell water per s, respectively (Fig. 2a). In merozoites neither L-adenosine or D-adenosine transport was inhibited by 1 µM NBMPR (data not shown). D-Adenosine was also transported into

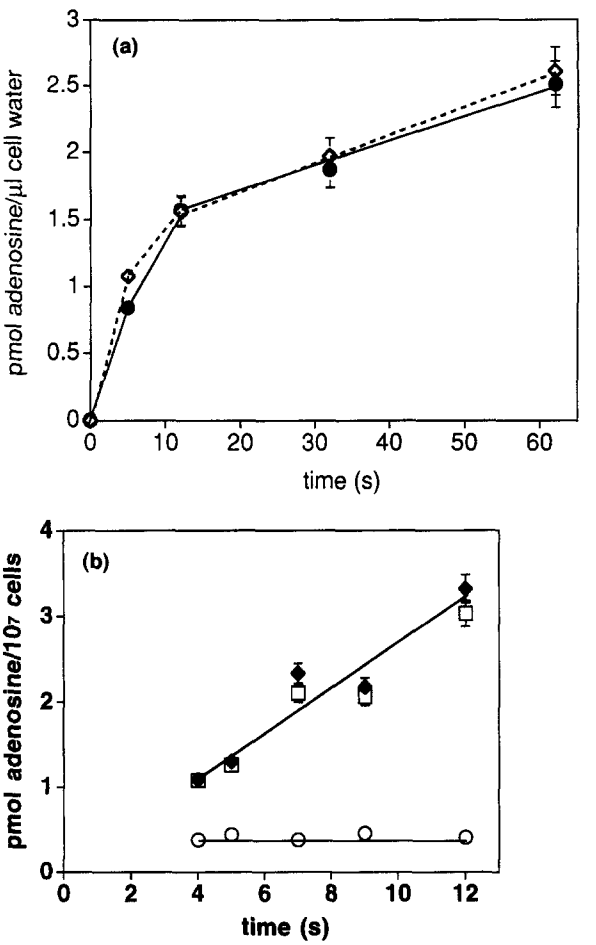

Fig. 2. Influx of D-and L-[^{3}H]adenosine in *P. falciparum* merozoites and free parasites. (a) Transport of 1 µM L-[^{3}H]adenosine (●) and 1 µM D-[^{3}H]adenosine (◇) was determined in merozoites over 60 s. (b) Transport of 1 µM L-[^{3}H]adenosine (○),1 µM D-[^{3}H]adenosine (◆) and 1 µM D-[^{3}H]adenosine in the presence of 10 µM NBMPR (□) was also determined in saponin freed *P. falciparum* trophozoites over 12 s. Assays were carried out as described in Fig. 1. The data are representative of at least three experimental results ± S.E.

saponin-freed *P. falciparum* parasites at a rate of 0.21 ± 0.05 pmol D-adenosine/10^7 cells (Fig. 2b). NBMPR (1 or 10 µM) had no significant effect on the transport of

Table 1
Comparison of D- and L-adenosine influx in different cell types

Cell type	Initial rate of transport (pmol/µl cell water per s) · 10^2	
	D-adenosine	L-adenosine
P. falciparum + 1 µM NBMPR	17 ± 0.9	8.0 ± 0.6
P. falciparum merozoite	6 ± 0.3	7.0 ± 0.1
Human erythrocyte	20 ± 1	0.6 ± 0.03
Human erythrocyte in hypotonic buffer	19 ± 2	0.5 ± 0.05
C. luciliae	69 ± 3	0.3 ± 0.02
G. intestinalis	16 ± 0.8	< 0.1
T. vaginalis	11 ± 0.6	< 0.1
Buffalo African Green Monkey	4 ± 0.2	0.2 ± 0.01
HeLa 229	3 ± 0.2	< 0.1

Influx rates of L- and D-[^{3}H]adenosine (1 µM) in different cell types was determined by calculation of initial transport over 3–5 s. Various parasitic protozoa, such as *C. luciliae*, *T. vaginalis* and *G. intestinalis* and mammalian cells, Buffalo Green Monkey and HeLa cells were examined for transport of both isomers and compared to *P. falciparum*. The values represent the mean of at least three identical experiments ± S.E.

D-adenosine into free parasites (Fig. 2b). However, influx of 1 μM L-adenosine into saponin-freed *P. falciparum* trophozoites did not occur (Fig. 2b) even up to incubation times of 20 min (data not shown). Non permeation of sucrose further determined that the membranes of the saponin-freed *P. falciparum* parasites remained intact and that nonspecific leaks were not formed by the isolation procedure.

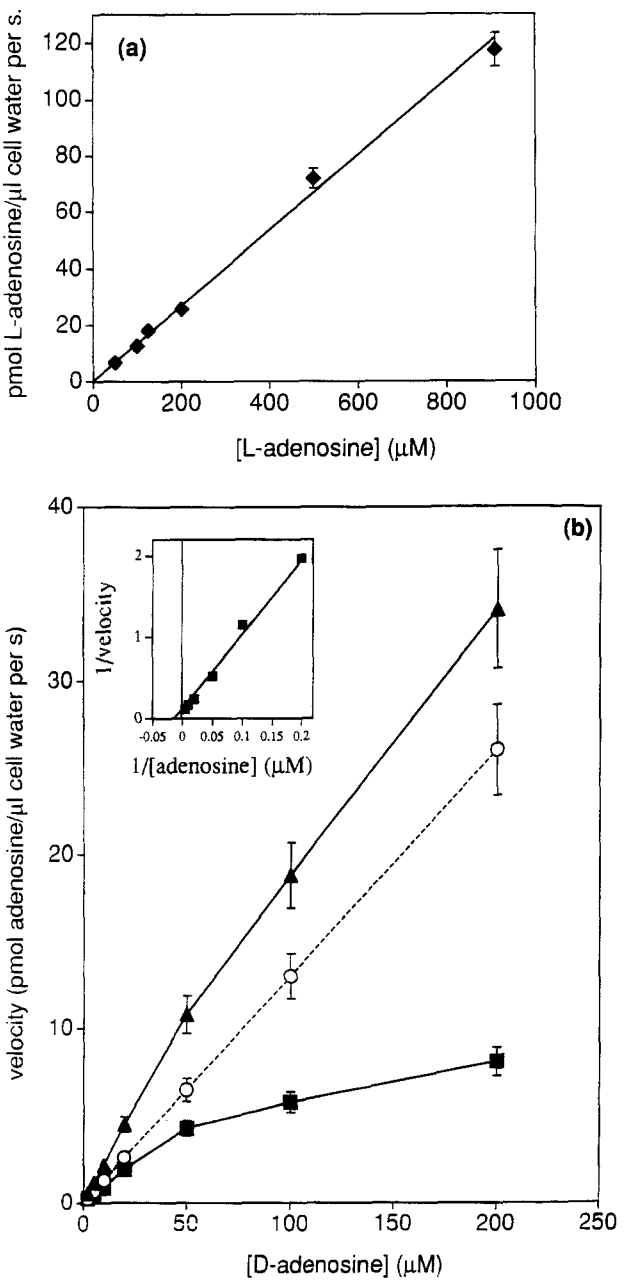

Fig. 3. The effect of substrate concentration on the rates of adenosine transport in *P. falciparum* infected cells. (a) The velocity of L-[^{3}H]adenosine transport (◆) in infected cells (85–90% parasitised) was measured over 7 s time periods in the presence of varying concentrations of unlabelled substrate (10–910 μM). (b) The velocity of D-[^{3}H]adenosine transport (2–200 μM) was measured in the same cells as in (a) in the presence of 10 μM NBMPR (▲). The nonsaturable adenosine rate (represented by L-adenosine, (○)) was subtracted from the observed rate of parasite D-adenosine transport to yield a corrected D-adenosine rate (■). The inset represents the Lineweaver-Burk plot of the corrected values. Values shown are the average of three replicate experiments ± S.E.

In contrast to *P. falciparum* infected cells and merozoites (Figs. 1 and 2a), other parasitic protozoa, such as *C. luciliae, T. vaginalis* and *G. intestinalis*, and the mammalian cells, BGM and HeLa, failed to demonstrate any transport 1 μM L-adenosine (Table 1). In all instances the transport rate of L-adenosine was negligible with a rate less than the 0.006 pmol L-adenosine/μl cell water per s. However all the above cells transported D-adenosine (Table 1).

3.2. Kinetics of the parasite-induced transporter

L-Adenosine transport showed nonsaturability in *P. falciparum* trophozoite infected cells up to concentrations of approx. 1 mM with a rate of 0.14 ± 0.01 pmol/μl cell water per s per μM L-adenosine (Fig. 3a). In comparison, Fig. 3b shows the concentration dependence of the parasite-induced D-adenosine transport in *P. falciparum* infected cells which is NBMPR-insensitive. The data are consistent with a two component system comprising a combination of carrier-mediated transport and a nonsaturable component which appeared to be the component responsible for L-adenosine transport.

A proportion of NBMPR-insensitive D-adenosine transport in infected cells inhibited by phloridzin and furosemide (see below) was found to be equal to that transport represented by L-adenosine (data not shown). Hence nonsaturable D- and L-adenosine transport appeared to proceed via the same permeation mechanism. Thus, with the assumption that D-adenosine was therefore transported through the L-adenosine site, subtraction of the nonsaturable L-adenosine rate from the total D-adenosine transport and analysis of the resulting data by nonlinear regression resulted in a K_m for D-adenosine of 103 ± 12.7 μM and a V_{max} = 12.2 ± 0.7 pmol/μl cell water per s per μM D-adenosine (Fig. 3b, inset).

In addition to mathematically subtracting the L-adenosine transport rate to derive kinetic parameters for the parasite-induced saturable component, phloridzin and furosemide (0.1–1 mM) were used to inhibit nonsaturable transport at high D-adenosine concentrations. However, these reagents at the above concentrations also interacted with the saturable component and hence kinetic data for the parasite saturable transport system in isolation could not be obtained with these inhibitors.

3.3. Substrate specificity of the parasite-induced D-adenosine transporter

Competition studies using physiological and nonphysiological purine nucleosides were used to compare the normal erythrocyte nucleoside transporter and the saturable parasite-induced D-adenosine transporter (Table 2). In un-

infected erythrocytes, D-adenosine transport was inhibited by the physiological purine nucleosides, guanosine and inosine and also by nonphysiological nucleosides with a variety of modifications to the purine ring structure such as tubercidin, N^6-methyladenosine, sangivamycin (7-deaza-7-amidoadenosine) and 8-azidoadenosine (Table 2). A variety of compounds with alterations in the ribose moiety, 2′-deoxyadenosine, 5′-deoxyadenosine, erythroadenosine, 8-bromo-3-β-D-furanosyladenine and 5′-deoxy-5′-*S*-isobutylthioadenosine (SIBA), also inhibited normal host nucleoside transport of D-adenosine. L-adenosine, acycloguanosine (acyclovir), hypoxanthine 9-β-D-arabinofuranoside, adenine 9-β-D-arabinofuranoside (ara-A) and sinefungin and the purine bases, adenine, hypoxanthine, guanine and xanthine, demonstrated no competition with normal erythrocyte nucleoside transport of D-adenosine.

In contrast, the inhibition profile of the saturable NBMPR-insensitive parasite-induced D-adenosine transporter was considerably altered. Of the purine nucleosides tested only 2′-deoxyadenosine inhibited (> 50%) the parasite-induced D-adenosine transport. Guanosine, at the lower concentration of 250 μM competed for transport moderately well, however inosine was not inhibitory. Blockage of D-adenosine transport in parasitised cells by tubercidin, N^6-methyladenosine, sangivamycin and 8-azidoadenosine was significantly decreased compared to normal cells. L-Adenosine and all other nucleosides with alterations to the ribose moiety had no effect on D-adenosine transport. However, a significant deviation from the properties of the normal host nucleoside transporter, was the observation that the purine bases, hypoxanthine and adenine, were also inhibitors (50–60%) indicating that the saturable D-adenosine parasite transport system, which was NBMPR insensitive, had a different substrate specificity to the endogenous erythrocyte nucleoside transporter.

3.4. Characterisation of L-adenosine permeation

As significant transport of L-adenosine was found in *P. falciparum* infected cells and merozoites (Figs. 1 and 2a) whilst uninfected erythrocytes and other cell types remained impermeable to 1 μM L-adenosine (Table 1), the potential for the development of cytotoxic agents which would selectively enter only malaria infected cells led us

Table 2
Effect of purine compounds on D-adenosine transport in *P. falciparum* infected and normal erythrocytes

Compound	% Inhibition of transport	
	human cells	*P. falciparum* infected cells + NBMPR [a]
(i) Physiological purine nucleosides		
D-Adenosine [b]	93 ± 2	52 ± 7
Inosine	51 ± 2	16 ± 2
Guanosine (250 μM)	52 ± 7	39 ± 4
(ii) Nonphysiological purine nucleosides with changes in the purine ring structure		
Tubercidin	68 ± 2	11 ± 7
N^6-Methyladenosine	73 ± 7	35 ± 1
Sangivamycin	65 ± 2	0
8-Azidoadenosine	62 ± 6	22 ± 1
(iii) Nonphysiological purine nucleosides with changes in the sugar moiety		
L-Adenosine	21 ± 2	10 ± 2
2′-Deoxyadenosine	69 ± 3	52 ± 9
5′-Deoxy-5′-*S*-isobutylthioadenosine (SIBA)	78 ± 5	23 ± 2
8-Bromo-3-β-D-furanosyladenine	69 ± 4	11 ± 1
Erythroadenosine	55 ± 3	21 ± 2
5′-Deoxyadenosine	52 ± 3	27 ± 3
2′,3′-Dideoxyadenosine	39 ± 2	28 ± 3
Acycloguanosine (Acyclovir)	11 ± 1	27 ± 5
Hypoxanthine 9-β-D-arabinofuranoside	4 ± 0.1	12 ± 1
Adenine 9-β-D-arabinofuranoside (Ara-a)	1 ± 0.1	19 ± 1
Sinefungin	0	9 ± 1
(iv) Physiological purine bases		
Adenine	20 ± 5	62 ± 5
Hypoxanthine	26 ± 1	52 ± 2
Guanine (200 μM)	8 ± 0.2	32 ± 0.3
Xanthine (200 μM)	0	14 ± 1

1 μM D-[^{3}H]adenosine transport over 4 s was determined in both normal and *P. falciparum* infected cells (70% parasitised) in the presence of various purine and pyrimidine compounds added simultaneously to cells with the permeant solution. In these experiments cells were also preincubated with 0.5 μM 2′-deoxycoformycin. Inhibition studies in infected cells were additionally assayed in the presence of 1 μM NBMPR. The results represent the average of at least four determinations ± S.E.

[a] In *P. falciparum* infected cells, D-adenosine transport plus competitors was determined in the presence of 1 μM NBMPR to inhibit the endogenous nucleoside transporter.

[b] All compounds used at 500 μM except noted otherwise.

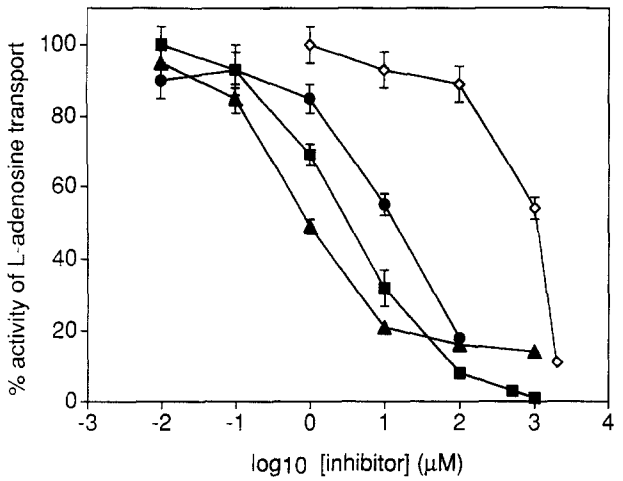

Fig. 4. Inhibition of L-adenosine transport. Furosemide (▲), phloridzin (■), piperine (●) and quinine (◇) were tested for inhibitory action against 1 μM L-[^{3}H]adenosine influx (5 s) in *P. falciparum* (80% parasitised) over a wide concentration range (10 nM–1 mM). The IC_{50} concentrations, representing that concentration causing 50% inhibition of L-[^{3}H]adenosine influx were found to be; furosemide, 1 μM, phloridzin, 3 μM, piperine, 13 μM and quinine, 1000 μM. The experimental values represent the average of three determinations ± S.E.

to a further characterisation of the L-adenosine transport system. In addition, compounds which block this pathway in *P. falciparum* infected cells may deprive the parasite of essential nutrients needed for replication.

The effects of various reagents on the rate of L-adenosine transport in *P. falciparum* infected erythrocytes was examined to discriminate between a transport mediated process, which would exhibit inhibition, or via a simple diffusion mechanism. As shown in Fig. 4, furosemide, phloridzin and piperine were excellent inhibitors of L-adenosine transport in *P. falciparum* infected cells with IC_{50} values of 1, 3 and 13 μM, respectively. These reagents were required in at least 10–1000-fold greater concentrations to block the transport of D-adenosine in normal erythrocytes, which had IC_{50} values of 1 mM, 500 μM and 100 μM, respectively (data not shown). By contrast, quinine appeared to inhibit L-adenosine transport in *P. falciparum* infected cells (IC_{50} = 1 mM, Fig. 4) and D-adenosine transport in normal human erythrocytes equally (IC_{50} = 1 mM).

Other compounds tested, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (500 μM), bumetanide (1 mM), verapamil (1 mM) and carbonylcyanide *m*-chlorophenylhydrazone (50 μM), blocked L-adenosine transport by 30–43% in *P. falciparum* infected cells but also blocked D-adenosine transport in uninfected erythrocytes to the same extent. The transport of L-adenosine into *P. falciparum* infected cells was not inhibited by the sulfydryl reagents, *p*-chloromercuriphenylsulfonic acid, (2 mM) iodoacetamide (2 mM) or *N*-ethylmaleimide (2 mM), suggesting the absence of thiol group/s involvement in L-adenosine influx. Incubation of infected cells with KCN (2 mM) to deplete energy reserves also had no effect on L-adenosine transport.

3.5. Transport into swollen uninfected erythrocytes

Recent publications have suggested that changes in the integrity of the erythrocyte, such as cell volume alterations, may be responsible in part for the increased permeability of *P. falciparum* infected cells [7,20,21]. Malaria infected erythrocytes have increased cellular volumes of approx. 1.5-times (61.4 ± 3 fl) compared to uninfected erythrocytes (42 ± 2 fl) [7]. Hence the effect of hypotonic swelling of normal human erythrocytes was examined to determine whether increased cell volume was responsible for the nonsaturable L-adenosine influx in *P. falciparum* infected cells. Normal human erythrocytes, hypotonically swollen by reducing the osmolarity to approx. 70% of the physiological value (and calculated to be approx. 1.5-times the volume of cells contained in isotonic medium) were assayed for L-adenosine transport as described above. However, L-adenosine was not transported into swollen normal human erythrocytes to any significant extent with a rate ≤ 0.006 pmol/μl cell water per s. In addition, no changes in D-adenosine influx were found in hypotonically swollen cells (rate = 0.19 ± 0.02 pmol/μl cell water per s) compared to normal unswollen erythrocytes (Table 1).

3.6. Metabolism of L-adenosine in P. falciparum infected erythrocytes

The ability of *P. falciparum* infected erythrocytes to metabolise L-adenosine was also investigated. D-Adenosine metabolism was examined in lysates of both normal human and *P. falciparum* infected cells as positive controls. Human erythrocyte and infected cell lysates incubated with 50 μM D-[^{3}H]adenosine at 37° C demonstrated that after 10 and 20 min that there was substantial metabolism of adenosine to hypoxanthine and purine nucleotides, with no label remaining as either unreacted substrate or as inosine.

Cell lysates and intact cells exposed to 50 μM L-[^{3}H]adenosine, rather than D-adenosine, demonstrated significantly different metabolic profiles. In uninfected human erythrocytes L-adenosine was not metabolised, with all radiolabel remaining as unreacted substrate even after 20 min at 37° C (Fig. 5a). However, both lysed and whole *P. falciparum* trophozoites deaminated L-adenosine to form inosine continually for 20 min after which equal proportions of the label could be detected as L-adenosine and L-inosine (Fig. 5b). Complete inhibition of 50 μM L-adenosine metabolism in lysates of *P. falciparum* infected cells at 37° C was demonstrated using 50 nM 2′-deoxycoformycin (Fig. 5c). Under the same conditions merozoites, both lysed and intact, were also found to metabolise 50 μM L-adenosine to L-inosine and additionally this metabolism was inhibited by 50 nM 2′-deoxycoformycin (data not shown).

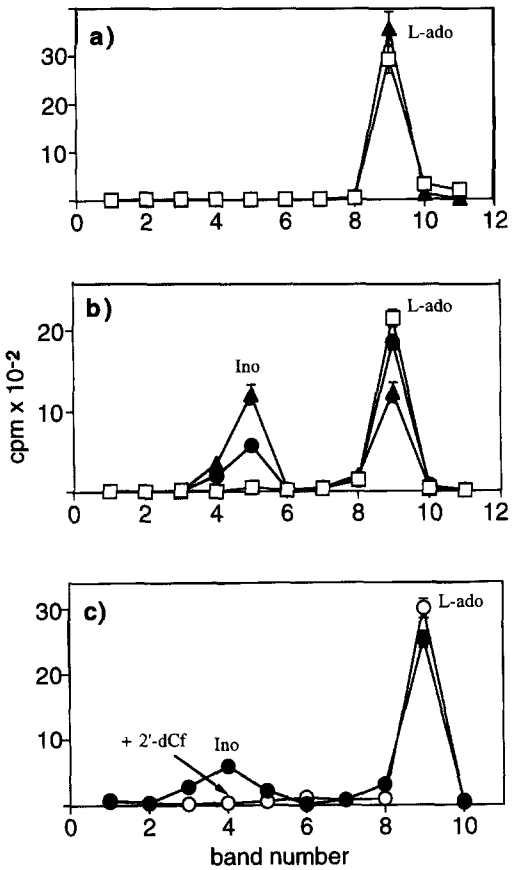

Fig. 5. The metabolism of L-[^{3}H]adenosine compared in normal human (a) and *P. falciparum* infected cells (b). L-[^{3}H]Adenosine (50 μM) was added to the cell lysates and reactions terminated after 0 (□), 10 (●), and 20 min. (△). The amount of radiolabel incorporated into products was analysed by thin-layer chromatography on PEI cellulose plates. L-Adenosine (50 μM) metabolism over 10 min in the presence of 0.5 μM 2'-deoxycoformycin was also examined in *P. falciparum* infected (c). The results are representative of three identical assays ± S.E. Unlabelled and ^{14}C-labelled markers corresponded to the following band numbers; nucleotides = 1 and 2, L-inosine = 5 (a, b) and band 4 in (c), hypoxanthine = 7, and D- and L-adenosine = 9.

Metabolism of L-adenosine in *P. falciparum* infected cells in culture was additionally examined over a longer time range (40 h) and under in vitro culture conditions conducive to parasite intraerythrocytic development and replication. However, whereas D-adenosine and guanosine were readily incorporated by the Plasmodium parasite into nucleic acid material (0.45 ± 0.1 and 0.35 ± 0.05 pmol substrate incorporated per 10^7 cells, respectively), less than 0.05 pmol L-adenosine was incorporated per 10^7 cells. A comparison of L-adenosine metabolism in other cell types demonstrated that similarly to normal erythrocytes, no metabolism of L-adenosine occurred in the lysates of *C. luciliae*, BGM, HeLa or *E. coli* although D-adenosine was metabolised to nucleotides by these cell lines (data not shown). These observations further indicate the unusual ability of *P. falciparum* infected cells and merozoites to metabolise a nucleoside containing the 9-β-L-ribofuranosyl group.

4. Discussion

In normal human erythrocytes the dependence of rates of D-adenosine transport on D-adenosine concentration shows a simple saturable transport system with a K_m of 25 μM [1]. This transport is potently inhibited by NBMPR. However, similar nucleoside transport experiments with *P. falciparum* infected erythrocytes, in the presence of NBMPR (used to totally block endogenous host nucleoside transport) revealed kinetics which could be resolved into two components of permeation. The first component represented a saturable, mediated system with a lowered affinity for D-adenosine (K_m = 103 μM) and an altered substrate specificity compared to the endogenous nucleoside transporter. Whilst the endogenous host nucleoside transporter was inhibited by all nucleosides, the saturable parasite transporter was only inhibited by 2'-deoxyadenosine, adenine and hypoxanthine. The NBMPR-insensitivity and the altered substrate specificity, in which a base competed with D-adenosine transport, suggested that the parasite-induced saturable transporter was distinct from the endogenous host nucleoside transporter.

In this study we have concentrated on the second nonsaturable component of NBMPR-insensitive nucleoside permeation in *P. falciparum* infected erythrocytes and merozoites, represented by L-adenosine transport. This component of transport was nonsaturable to concentrations above normal physiological conditions, was not stereospecific in that it accepted the β-L-enantiomer but was discriminatory in that *P. falciparum* infected cells remained impermeable to sucrose. Inhibition studies suggested that this transport occurred via a pathway distinct from simple diffusion as inhibitors of various mammalian transport processes were effective in blocking the influx of L-adenosine in infected cells. Furosemide, an anion transport inhibitor [22], and phloridzin, a known inhibitor of renal Na^+ coupled glucose transport and ion exchange in erythrocytes [22], substantially inhibited L-adenosine transport in *P. falciparum* infected cells with similar potency (IC_{50} = 1 μM and 3 μM respectively). In addition, two inhibitors of cation transport, piperine in *P. falciparum* infected erythrocytes [23] and quinine in mammalian cells [24], both blocked L-adenosine influx in *P. falciparum* infected cells, although quinine (IC_{50} = 1 mM) was required in much higher concentrations than piperine (IC_{50} = 13 μM). However, other reagents which interact with ion channels had little effect on L-adenosine transport in *P. falciparum* infected cells; compounds such as bumetanide, also a loop diuretic, and DIDS, an inhibitor of ion influx in mammalian cells [25]. In addition, the *P. falciparum* L-adenosine transport mechanism did not appear to involve reactive sulfydryl groups.

In normal erythrocytes (Fig. 1) and in a variety of cell lines, both mammalian and parasitic (Table 1), the influx of L-adenosine was negligible indicating that the nucleoside transporter of these cells would not accept the β-L-en-

antiomer. It appeared that L-adenosine influx was a characteristic unique to the malaria infected cell and merozoites, although the transport of both stereoisomers of alanine have also been described in *P. falciparum* infected cells [26]. A similar L-adenosine transport mechanism has also been shown in mouse erythrocytes infected with *P. yoelii* which was also inhibited by furosemide but not by phloridzin [21].

It has been well documented that the intraerythrocytic malarial parasite has the ability to significantly alter the permeability properties of the host cell membrane [28]. What is still not clear is whether alterations of the host cell transport systems or whether parasite-specific transport mechanisms are inserted into the host membrane to account for the altered transport of various nutrients. The inhibitors of nonsaturable L-adenosine transport in *P. falciparum* infected cells, namely furosemide, phloridzin, piperine and quinine, have all been found to block *P. falciparum* infected cell induced transport of other nutrients (reviewed in [23,27–29]), in agreement with the proposal that many nutrients pass through the infected host cell membrane via a single type of parasite-induced transport system [30]. However, it should be noted that not all nutrients are similarly altered in their transport across Plasmodium infected cells, and thus the parasite transporting system must have some degree of selectivity.

For nutrients and other compounds several routes of entry into the intraerythrocytic parasite are possible. They may enter into the host cytoplasm via the host erythrocyte membrane, cross the parasitophorous vacuole membrane (PVM), which surrounds the intraerythrocytic parasite, followed by transport across the parasite plasma membrane (PPM) (conventional model). Alternatively they may also have direct access to the internal parasite PPM via 'metabolic windows' [31] or via the recently proposed 'duct' [32] or by some other means. Recent data from Loyevsky et al. [33] has suggested that certain compounds can enter the parasite without any access via the host erythrocyte cytoplasm.

Our finding that L-adenosine could not permeate the freed intraerythrocytic parasite which is encompassed by the PVM, but that influx into the parasite was demonstrated in both the infected erythrocyte and merozoites suggested that L-adenosine may have direct access into the parasite. The merozoite was used with the assumption that its membrane represented the PPM of the intraerythrocytic parasite (at least in the immature stage) and hence L-adenosine appeared permeable to the PPM. We used artificially released trophozoites from infected erythrocytes to determine the permeability of L-adenosine through the PVM. These released trophozoites were sucrose impermeable but transported D-adenosine at normal rates. As L-adenosine could not permeate the freed intraerythrocytic parasite and with the assumption that the PVM is intact in 'freed' parasites, L-adenosine may enter the parasite of the infected cell via some sort of direct access.

In addition to the unusual transport properties of L-adenosine, both *P. falciparum* infected cells and merozoites were able to metabolise this compound. The significant deamination of L-adenosine which occurred in *P. falciparum* infected cells and merozoites was inhibited by 2′-deoxycoformycin, a specific inhibitor of *P. falciparum* adenosine deaminase [34]. In normal erythrocyte lysates L-adenosine was not metabolised indicating that it was not accepted as a substrate for human erythrocytic enzymes, nor was it metabolised in lysates of other cell types. Although Plasmodium purine nucleoside phosphorylase has a significantly different substrate specificity profile compared to the erythrocytic enzyme [35,36] L-adenosine was not metabolised further than L-inosine. Additionally, L-adenosine did not appear to be acted upon by either the human or parasitic kinases (Table 2).

The significance of the selective metabolism of L-adenosine in infected erythrocytes and merozoites and the Plasmodium specific transport of L-adenosine indicate the chemotherapeutic potential of specifically designed purine analogues based on these unique characteristics. Purine permeation is essential for the survival of the intraerythrocytic parasite, as *P. falciparum* and other malarias are incapable of de novo purine synthesis and hence are dependent on transport of exogenous purines as part of the purine salvage process. A number of nucleoside analogs with altered ribose components, some of which may enter the infected cell via the L-adenosine permeation site, have been shown to produce significant cytotoxicity against malaria including 3′-deoxyadenosine (cordycepin) [37,38], adenine arabinoside (ara-A) [39,40], 5′-deoxy-5′-*S*-isobutyladenosine (SIBA), [41] and Sinefungin [42]. The existence in Plasmodium infected erythrocytes of a parasite-induced permeation site which accepts nucleosides with a significant ribose moiety alteration, provides the opportunity for an antimalarial strategy by which cytotoxic compounds could be specifically directed into infected cells while remaining impermeable to normal mammalian cells.

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