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Biochimica et Biophysica Acta 1463 (2000) 88–98

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Increased permeability of the malaria-infected erythrocyte to organic cations

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Received 7 June 1999; received in revised form 21 September 1999; accepted 21 September 1999

Abstract

The human malaria parasite, *Plasmodium falciparum*, induces in the plasma membrane of its host red blood cell new permeation pathways (NPP) that allow the influx of a variety of low molecular weight solutes. In this study we have demonstrated that the NPP confer upon the parasitised erythrocyte a substantial permeability to a range of monovalent organic (quaternary ammonium) cations, the largest having an estimated minimum cross-sectional diameter of 11–12 Å. The rate of permeation of these cations showed a marked dependence on the nature of the anion present, increasing with the lyotropicity of the anion. There was no clear relationship between the permeation rate and either the size or the hydrophobicity of these solutes. However, the data were consistent with the rate of permeation being influenced by a combination of these two factors, with the pathways showing a marked preference for the relatively small and hydrophobic phenyltrimethylammonium ion over larger or less hydrophobic solutes. Large quaternary ammonium cations inhibited flux via the NPP, as did long-chain *n*-alkanols. For both classes of compound the inhibitory potency increased with the size and hydrophobicity of the solute. This study extends the range of solutes known to permeate the NPP of malaria-infected erythrocytes as well as providing some insight into the factors governing the rate of permeation. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Membrane permeability; Induced transport; Channel; Selectivity; *Plasmodium falciparum*

1. Introduction

Approximately halfway through the intraerythrocytic phase of the life cycle of the malaria parasite, *Plasmodium falciparum*, the infected red cell undergoes a marked increase in its permeability to a diverse range of low molecular weight solutes (reviewed in [1–3]). The available data are consistent with the view that much of the increased traffic of small solutes is via new (parasite-induced) permeation pathways (NPP) of a single type [4]. In early studies of these pathways it was proposed that the NPP are permeable to anions and neutral compounds but exclude cations [5,6]. However, they have subsequently been shown to have a significant permeability to the inorganic cations Rb^+ and K^+ [4,7] as well as to at least one organic cation, the phospholipid headgroup precursor, choline [4,8].

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PII: S0005-2736(99)00187-X

Several previous studies have considered the relationship between the physical properties of solutes and their rate of permeation via the NPP. In early studies of the enhanced permeability of parasitised erythrocytes it was postulated (on the basis of the apparent impermeability of the pathways to sucrose) that the NPP take the form of size-selective pores with a diameter of approximately 7 Å [5,9]. In a subsequent analysis of the rate of permeation of a wide range of solutes it was suggested that the NPP show little if any ability to discriminate between solutes of different sizes and it was postulated that the NPP are formed at the protein-lipid interface of proteins inserted by the parasite in the host cell membrane [6]. However, it has also been argued that for a series of electroneutral polyols the rate of permeation does decrease with solute size, in a manner that is consistent with the presence in the parasitised cell membrane of pores having a diameter of around 7.3 Å [1].

The induction of NPP in malaria-infected erythrocytes presents a number of chemotherapeutic opportunities. In particular, the NPP offer a route by which cytotoxic compounds might be targeted into parasitised cells, while remaining excluded from normal cells [10,11]. The inherent permeability of normal biological membranes to cations is substantially lower than that to similarly sized uncharged or negatively charged solutes [12,13]. In seeking to identify cytotoxic agents that do not permeate the plasma membrane of normal cells, cationic compounds therefore offer significant advantages over their neutral or anionic counterparts.

In this study we have investigated the permeability of *P. falciparum*-infected human erythrocytes to a range of structurally related organic (quaternary ammonium) cations, with the aim of extending our knowledge of the types of solutes to which the NPP are permeable, as well as gaining some insight into the factors that govern the rate of transport of cations via the NPP.

2. Materials and methods

2.1. Chemicals

The quaternary ammonium compounds used in this study were: tetramethylammonium (TMA); tetraethylammonium (TEA); methyltriethylammonium (MTEA); phenyltrimethylammonium (PhTMA); tetrapropylammonium (TPA); tetrabutylammonium (TBA); tetrapentylammonium (TPeA); and choline. All were obtained as chloride, bromide and (in the case of choline) iodide salts from Aldrich.

2.2. Calculation of cylindrical diameters, van der Waals volumes and octanol/water partition coefficients

The cylindrical diameters of the solutes of interest were calculated using Nemesis modeling software (Oxford Molecular) and are given in Table 1. To obtain these dimensions molecular structures were constructed on the screen, arranged manually into as long and narrow a conformation as possible (i.e. that conformation presenting the minimal longitudinal cross-sectional area), and then subjected to an

Table 1
Physical properties of the quaternary ammonium compounds used in this study

Cation	M_r	Mean cylindrical diameter (Å)	van der Waals volume (Å^3)	Equivalent spherical diameter (Å)	Log ($PC_{octanol/water}$)
TMA	74	6.7	96	5.7	−3.06
TEA	161	7.9	162	6.8	−2.82
MTEA	130	7.3	145	6.5	−2.88
PhTMA	136	6.7	149	6.6	−2.05
Choline	104	6.7	121	6.1	−3.71
TPA	247	10.7	229	7.6	−1.98

M_r is the relative molecular weight. The mean cylindrical diameter is the diameter of the narrowest cylinder able to enclose the longest, thinnest, stable configuration of each molecule. The equivalent spherical diameter is the diameter of a sphere having a volume equivalent to the van der Waals volume of each molecule. $PC_{octanol/water}$ is the octanol/water partition coefficient of each compound.

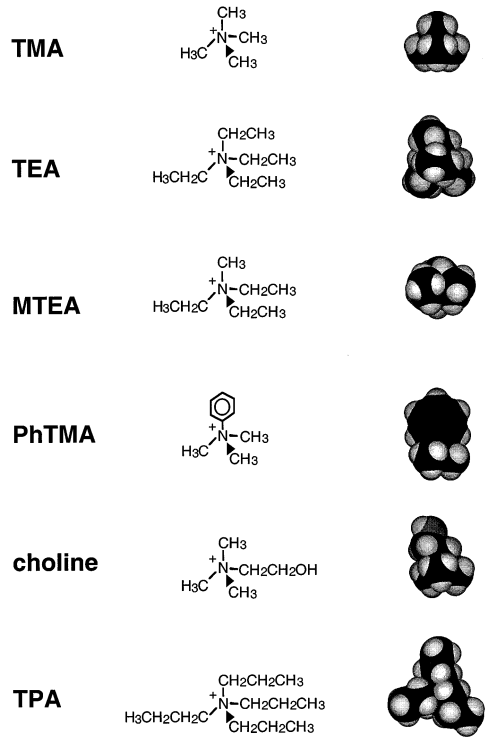

Fig. 1. Chemical structures and space-filling model representations of the thinnest, stable configurations of the quaternary ammonium cations used in this study.

energy minimisation procedure to obtain the longest, thinnest, stable configuration of each molecule. For each molecule the procedure was repeated a number of times, from different initial starting configurations. The dimensions cited in Table 1 are the length and diameter of the narrowest cylinder able to enclose the narrowest energy-minimised structure. Chemical structures of the different test solutes, together with space-filling model representations of the energy-minimised structures are shown in Fig. 1.

The van der Waals volumes of the various solutes were calculated as outlined by Bondi [14] and are listed in Table 1. For the purpose of this calculation van der Waals radii were taken from Bondi [14] and bond lengths from the CRC Handbook of Chemistry and Physics. The 'equivalent spherical diameter' of each solute was calculated as the diameter of a sphere having a volume equivalent to the van der Waals volume.

Octanol/water partition coefficients, used here as a measure of solute hydrophobicity and listed in Table 1, were taken, where possible, from experimentally determined values [15]. For those compounds for which experimental data were not available the partition coefficients were calculated from the measured values of structurally related compounds using the FRAGMENT method [15].

2.3. Parasite culture

Human erythrocytes (type O) infected with the ITO4 line of *P. falciparum* [16] were cultured under 1% O_2, 3% CO_2, 96% N_2 in RPMI 1640 culture medium (Gibco), supplemented with D-glucose (10 mM), glutamine (2 mM), HEPES (40 mM), gentamicin sulphate (25 mg/l), and human serum (8.5% v/v, pooled from different blood donors, National Blood Services, South West, Bristol, UK). All experiments were carried out using trophozoite-infected cells (36–40 h post-invasion), synchronised by a combination of sorbitol haemolysis [17] and gelatin flotation [18]. Parasitised cells were harvested from culture immediately prior to experimentation, either by gelatin flotation or by centrifugation on Percoll as described elsewhere [19]. The former method yielded suspensions of 40–70% parasitaemia and the latter method suspensions of 80–96% parasitaemia.

In experiments comparing infected with uninfected cell suspensions, uninfected erythrocytes from the same donor were incubated in parallel with *P. falciparum*-infected erythrocyte cultures under identical conditions for at least 24 h prior to the experiment. In order to ensure that the infected and uninfected cell suspensions were exposed to comparable conditions the uninfected cells were either subjected to gelatin flotation or centrifuged on a Percoll layer (as appropriate) prior to experimentation.

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

2.4. Influx measurements

The permeability of trophozoite-infected erythrocytes to compounds of interest was measured using three different techniques, with all experiments carried out at 37°C.

2.4.1. Iso-osmotic haemolysis measurements

Initial measurements of the rate of permeation of a

number of the quaternary ammonium compounds were made using a semi-quantitative haemolysis method that has been used previously to study the enhanced permeability of malaria-infected erythrocytes to low molecular weight solutes [4,5]. Iso-osmotic solutions of halide salts of the different cations of interest were prepared by dissolving them to a concentration of approximately 160 mM in a solution containing 10 mM HEPES (pH 7.4) + 5 mM glucose, then adjusting the osmolality (by the addition of either the salt or the hypo-osmotic HEPES + glucose solution) to 300 ± 3 mOsm (kg H_2O)$^{-1}$.

Time courses commenced with the addition of a 0.2 ml aliquot of cell suspension to 3.3 ml of the iso-osmotic solutions of the different test solutes to give a cell concentration of approximately 0.5×10^8 cells/ml. 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 then 0.9 ml of the supernatant solution was transferred to another tube for the subsequent spectrophotometric (A_{540}) estimation of haemoglobin concentration.

In all such experiments the A_{540} value corresponding to full haemolysis of trophozoite-infected erythrocytes was estimated from the final A_{540} value achieved in the supernatant solution from infected cells suspended in an iso-osmotic sorbitol and/or $CsNO_3$ solution for 180 min.

2.4.2. ^1H NMR influx measurements

Quantitative estimates of the rates of influx of a number of (non-labelled) quaternary ammonium compounds into parasitised cells were made using ^1H NMR spectroscopy to estimate the concentration of these compounds in extracts prepared from cells preincubated with the compounds of interest.

For initial time course experiments with a number of the solutes, cells were suspended in an iso-osmotic solution (9 ml) comprised of HEPES-buffered saline (155 mM NaCl, 5 mM glucose, 10 mM HEPES, pH 7.4) containing one or more of the quaternary ammonium compounds, each at a concentration of 10 mM. At predetermined intervals, aliquots (1.5 ml) of the suspension were transferred to microcentrifuge tubes in which the cells were then washed three times by repeated centrifugation and resuspension in an ice-cold 170 mM NaCl solution. Following the third wash the cells were lysed by the addition of H_2O (1 ml), then the tube was centrifuged (10 000 × g, 1 min) to precipitate cell debris. The supernatant solution, together with that from two further additions of H_2O to the cell residues, was made up to 10 ml with H_2O, filtered through a 10 000 kDa cut-off membrane in a stirred cell (Amicon) using 50 psi pressure (N_2 gas), then freeze-dried for later ^1H NMR analysis.

In later experiments the rate of influx of the quaternary ammonium compounds of interest, in all cases at a concentration of 10 mM, estimated from the amount of solute taken up within a fixed incubation time that fell within the initial linear portion of the uptake time course. For all solutes other than PhTMA the protocol was similar to that outlined above, with uptake being terminated at 10 min by repeated washing and centrifugation of the cells in an ice-cold 170 mM NaCl solution. In the case of PhTMA the flux into (and out of) infected cells was too fast to be measured using the repeated wash technique. For this solute, uptake was measured over a 1 min period and was terminated by centrifuging the cells through a layer of dibutylphthalate (10 000 × g, 30 s). The aqueous layer was removed, then residual solution removed by rinsing the tubes four times with water. The dibutylphthalate was aspirated then the cell pellet was lysed with H_2O and processed for ^1H NMR analysis as described above.

All experiments with the quaternary ammonium compounds were carried out in the presence and absence of furosemide (0.1 mM), an effective inhibitor of the NPP [4], thus enabling in each case an estimate of the furosemide-sensitive (parasite-induced) component of influx.

Immediately prior to the NMR analysis the freeze-dried ultrafiltrates were reconstituted in 0.6 ml ^2H_2O containing 2,2,3,3-tetradeuterotrimethylsilyl 1-propionic acid as an internal chemical shift and intensity reference. All ^1H NMR spectra were acquired at 400.12 MHz across 32 000 data points with a 20 s delay between successive acquisition pulses, using a Varian INOVA spectrometer (Varian Associates Ltd., Palo Alto, CA, USA).

2.4.3. Radio-isotope influx measurements

Estimates of the unidirectional influx of choline were made from the uptake of [^{14}C]choline using methods described previously [20], with cells suspended in a HEPES-buffered saline containing either Cl^- or Br^- as the principal anion.

In one series of experiments the quaternary ammonium compounds (together with a number of *n*-alkanols) were tested for their effect on the transport of [^{14}C]choline via the NPP. As the larger, more hydrophobic compounds of this class are haemolytic on prolonged incubation at high concentrations, the compounds of interest were combined with the cells at the time of addition of radiolabelled choline, thereby minimising their time of exposure to the cells.

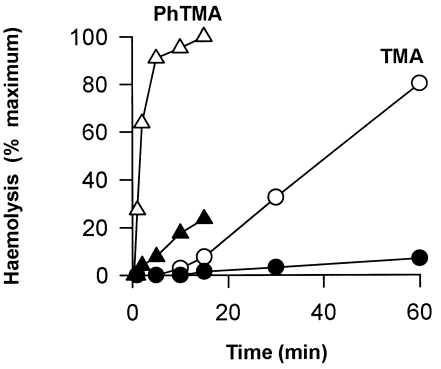

Fig. 2. Time courses for haemolysis of trophozoite-infected erythrocytes suspended in iso-osmotic solutions of TMA chloride (○,●) and PhTMA chloride (△,▲) in the presence (closed symbols) and absence (open symbols) of furosemide (0.2 mM). Uninfected erythrocytes exposed to the same conditions remained stable throughout the duration of the relevant time course, as did both infected and uninfected erythrocytes suspended in hypertonic media containing 150 mM NaCl together with 160 mM PhTMA chloride (not shown). The data are representative of those obtained in three experiments, each on erythrocytes from a different donor.

3. Results

3.1. Haemolysis measurements

An initial comparison of the relative rates of influx into trophozoite-infected cells of the quaternary ammonium compounds shown in Fig. 1 was made using a simple haemolysis method [4,5]. Cells were suspended in iso-osmotic solutions of the Cl^- and Br^- salts and, in the case of choline, the I^- salt of the various cations. For TPA and larger compounds both infected and uninfected cells haemolysed rapidly. For iso-osmotic solutions of compounds smaller than TPA, uninfected cells remained stable over the relevant time periods whereas the trophozoite-infected cells underwent progressive haemolysis.

The phenomenon is illustrated in Fig. 2 which shows time courses for the haemolysis of parasitised cells suspended in iso-osmotic solutions of TMA chloride and PhTMA chloride. Although these two cations differ only in that the latter has a phenyl group in place of a methyl group in the former (Fig. 1) there is a marked difference in the haemolysis rates: haemolysis of infected cells in the PhTMA-containing media was substantially faster than that of cells in the TMA-containing media. In both cases the rate of haemolysis of parasitised cells was slowed markedly by furosemide (0.2 mM), an effective inhibitor of the NPP of *P. falciparum*-infected erythrocytes [4,21]. In an experiment in which infected and uninfected cells were suspended in a normal physiological saline made hyper-osmotic by the addition of a 160 mM concentration of PhTMA, both cell types remained stable for over 60 min (not shown). This rules out the possibility that the haemolysis of parasitised erythrocytes suspended in an iso-osmotic PhTMA solution was due to a direct lytic effect of high concentrations of this compound on the parasitised erythrocyte membrane.

Table 2 summarises the results of the iso-osmotic haemolysis experiments, listing the inverse of the half-times for haemolysis (i.e. the inverse of the time taken for the measured haemoglobin release to reach half its maximum value) of parasitised cells suspended in iso-osmotic solutions of the different halide salts of the various cations. This parameter ($1/T_{50}$) provides a measure of the rate of influx of the different cations into the infected cell [5].

For each quaternary ammonium compound the rate of haemolysis ($1/T_{50}$) of parasitised cells in the iso-osmotic Br^- salt solution was higher than that in the corresponding Cl^- salt solution ($P < 0.001$ for all solutes except PhTMA). In the case of choline (for which the I^- salt was available) the $1/T_{50}$ values in iso-osmotic solutions of the three halide salts was in the order $I^- > Br^- > Cl^-$. For all solutes tested furo-

semide (0.2 mM) protected the infected cells against haemolysis, consistent with haemolysis of the infected cells being due to the influx of these compounds via the NPP.

The relative rates of haemolysis of cells in iso-osmotic solutions of either the Br^- or Cl^- salts of the different compounds of interest are consistent with the following permeability sequence: PhTMA $\gg$ MTEA > TEA > TMA $\approx$ choline > TPA.

3.2. ^1H NMR and [^{14}C]choline influx measurements

The iso-osmotic haemolysis method suffers from a number of inherent limitations and the estimates of relative permeabilities made using this method are, at best, semiquantitative [1]. A second, more quantitative approach was therefore employed, using ^1H NMR spectroscopy of cell extracts to measure the uptake of a number of the quaternary ammonium compounds. A representative series of ^1H NMR spectra, illustrating the influx into malaria-infected cells of TMA, TEA and TPA (each at a concentration of 10 mM), is shown in Fig. 3A. The corresponding time course is also shown (Fig. 3B).

The ^1H NMR-derived influx rates, together with estimates of the relative permeation rates of PhTMA, TMA, TEA, MTEA, TPA and choline, made using both the haemolysis and ^1H NMR influx measurements are summarised in Table 3. Despite the limitations of the haemolysis technique, the relative permeabilities of the different quaternary ammonium compounds estimated using the different methods were similar. Table 3 also includes the influx rate for choline, estimated from the uptake of [^{14}C]-choline.

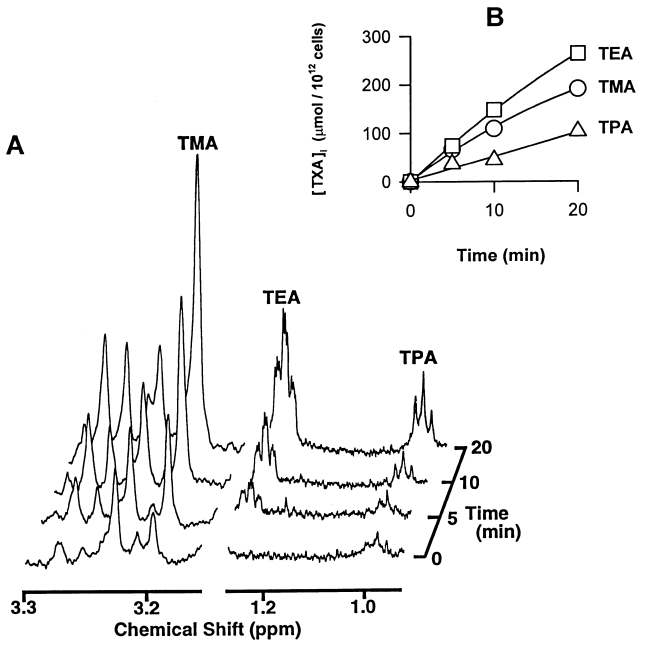

Fig. 3. (A) ^1H NMR spectra showing the time-dependent uptake of three quaternary ammonium compounds TMA, TEA and TPA into parasitised erythrocytes (40% parasitaemia). (B) Influx time courses derived from the ^1H NMR data. $[TXA]_i$ denotes the intracellular concentration of quaternary ammonium compound.

Table 2
Rates of haemolysis of trophozoite-infected cells suspended in iso-osmotic solutions of the halide salts of the various cations of interest

Cation	Anion	$1/T_{50}$ (min^{-1})	n	P
TMA	Cl^-	0.022 ± 0.001	8	–
	Br^-	0.048 ± 0.002	5	< 0.001
TEA	Cl^-	0.028 ± 0.001	6	–
	Br^-	0.056 ± 0.003	7	< 0.001
MTEA	Br^-	0.079 ± 0.003	3	–
PhTMA	Cl^-	0.55 ± 0.07	3	–
	Br^-	0.69 ± 0.05	3	0.24
Choline	Cl^-	0.022 ± 0.001	3	–
	Br^-	0.042 ± 0.003	3	< 0.001
	I^-	0.053 ± 0.002	3	< 0.001 < 0.05 (I^- vs Br^-)

The parameter $1/T_{50}$ denotes the inverse of the time taken for the measured haemoglobin release to reach half its maximum value and provides a semi-quantitative measure of the influx rate for the different cations. The errors are S.E.M. The P values are from paired t-tests and, except where specified otherwise, relate to the differences between the $1/T_{50}$ values obtained for each cation in Cl^- and Br^- media.

3.3. Blockade of the NPP by quaternary ammonium ions and n-alkanols

Preliminary ^1H NMR experiments in which the uptake of TMA, TEA and/or TPA was monitored in the presence of larger quaternary ammonium compounds indicated that the larger compounds slowed the rate of entry of the smaller ions, consistent with their blocking the NPP. To investigate this phenomenon in more detail we tested the effect of a range of concentrations of each of the tetraalkylammonium compounds of interest on the transport of [^{14}C]choline into infected cells. The dose-response

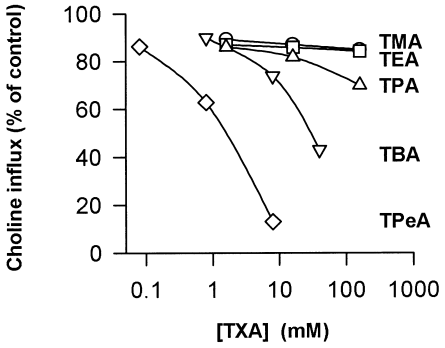

Fig. 4. Dose-response curves for the effect of tetraalkylammonium compounds on the influx of [^{14}C]choline into parasitised erythrocytes. The cells were suspended in a HEPES-buffered saline (155 mM NaBr, 5 mM glucose, 10 mM HEPES, pH 7.4) with the quaternary ammonium compounds added as the Br^- salts. [TXA] denotes the concentration of tetraalkylammonium compound. The data are averaged from three experiments, each on erythrocytes from a different donor. Error bars (denoting ± S.E.M.) fall within the symbols.

curves are shown in Fig. 4. TMA and TEA had little effect on malaria-induced choline influx up to a concentration of 160 mM. TPA caused a slight inhibition at this concentration. The tetrabutylammonium ion (TBA) inhibited induced choline transport with an IC_{50} (i.e. the concentration at which it reduced choline influx to 50% of its value in the absence of inhibitor) of approximately 30 mM. The largest and most hydrophobic compound tested, the tetrapentylammonium ion (TPeA) exerted the strongest inhibition, with an IC_{50} of approximately 1.8 mM.

The observed correlation between the size and/or hydrophobicity of the tetraalkylammonium ions and the potency with which they inhibited [^{14}C]choline influx prompted us to carry out similar experiments with a series of *n*-alkanols. These compounds were again found to inhibit malaria-induced choline transport, with their efficacy increasing with hydrocarbon chain length: IC_{50} values for butan-1-ol, hexan-1-ol and octan-1-ol were approximately 43 mM, 7 mM and 1.1 mM, respectively.

4. Discussion

4.1. Permeation of the NPP by quaternary ammonium compounds

In this study a range of quaternary ammonium compounds that vary systematically in their structural features were shown to enter malaria-infected erythrocytes via the furosemide-sensitive NPP induced by the parasite in the host cell membrane. Previous demonstrations of an increased permeability of parasitised erythrocytes to organic cations have been limited to choline. These data therefore extend the range (and size) of solutes known to permeate the NPP and lend further support to the view that the increased

Table 3
Rates of permeation of six quaternary ammonium compounds via the furosemide-sensitive pathway of malaria-infected erythrocytes

Solute	Furosemide-sensitive influx (mmol/(10^{12} cells·h))	P_X/P_{PhTMA}	
		Direct influx measurement	Haemolysis measurement
TMA	1.40 ± 0.12 (n = 4)	0.067	0.070
TEA	2.15 ± 0.10 (n = 3)	0.102	0.081
TPA	0.54 ± 0.12 (n = 2)	0.026	–
MTEA	3.20 ± 0.11 (n = 2)	0.152	0.114
PhTMA	21.0 ± 0.9 (n = 2)	1	1
Choline	2.25 ± 0.25 (n = 9)	0.107	0.061

The direct influx measurements were made on cells suspended in a HEPES-buffered saline containing Cl^- as the predominant anion, whereas the haemolysis data are from cells suspended in iso-osmotic solutions of the Br^- salts of the compounds of interest. For all solutes other than choline, influx measurements were made using ^{1}H NMR to quantify solute uptake. Choline influx was calculated from the uptake of [^{14}C]choline. The furosemide-sensitive influx rates were calculated by subtracting the flux measured in the presence of 0.1 mM furosemide from that measured in its absence, and were corrected to 100% parasitaemia. The extracellular concentration of each of the quaternary ammonium compounds was 10 mM. P_X/P_{PhTMA} denotes the permeability of each compound relative to that of the most rapidly permeating solute, PhTMA, estimated using either the haemolysis method or by direct (^{1}H NMR or [^{14}C]choline) influx measurements.

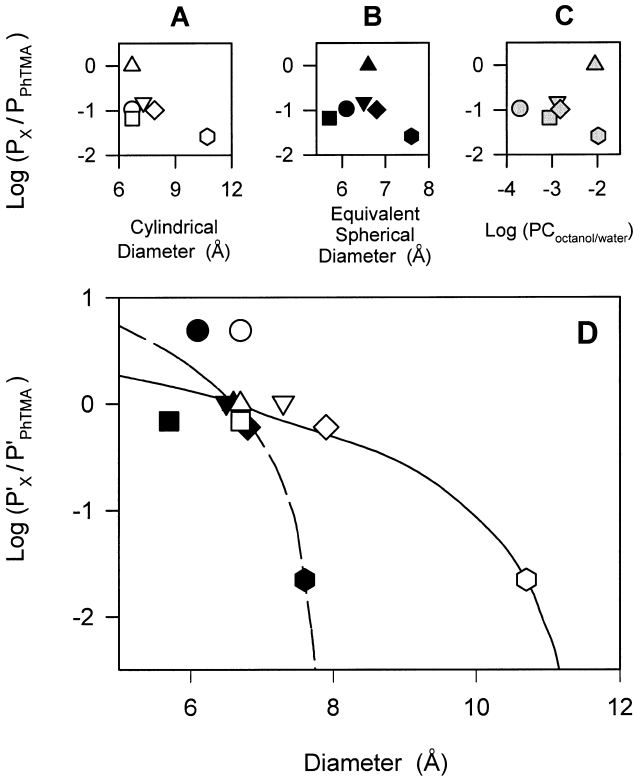

Fig. 5. Relationship between solute permeation rate and the physical properties of the solute. (A–C) Relative rates of permeation of monovalent organic cations via the NPP (P_X/P_{PhTMA}) are plotted as a function of: (A) the estimated minimum cylindrical diameter; (B) the equivalent spherical diameter; and (C) the log of the octanol/water partition coefficient for each solute. (D) Relative 'hydrophobicity-corrected permeabilities' (P'_X/P'_{PhTMA}) of quaternary ammonium compounds that permeate the NPP, plotted as a function of the estimated minimum cylindrical diameter (open symbols) and equivalent spherical diameter (closed symbols) for each solute. The relative permeation rates shown in A–C were estimated from the furosemide-sensitive influx rates listed in Table 3. For each solute P'_X was calculated by dividing the furosemide-sensitive influx rate by the octanol/water partition coefficient. The curves were derived by fitting Eq. 1 to the two data sets, with $d=7.8$ Å (broken line) and $d=11.4$ Å (closed line), where d is the pore diameter. Symbols: upright triangles, PhTMA; squares, TMA; diamonds, TEA; inverted triangles, MTEA; hexagons, TPA; circles, choline.

permeability of the malaria-infected erythrocyte is via broad-specificity pathways rather than substrate-specific transporters.

The factors governing the rate of permeation of solutes through the NPP are not well understood. In Fig. 5 the relative permeation rates of the quaternary ammonium ions used in this study are plotted as a function of two different measures of their size: their minimum cylindrical diameter (Fig. 5A) and their equivalent spherical diameter (Fig. 5B). Also shown (Fig. 5C) is a plot of the relative permeation rate of each solute as a function of its octanol/water partition coefficient (a measure of hydrophobicity).

The rate of permeation showed no systematic dependence on either measure of solute size (Fig. 5A,B). Nor was there any systematic relationship between the relative permeation rate and solute hydrophobicity (Fig. 5C). However, the finding that PhTMA, a compound which combines a relatively high hydrophobicity with a relatively small size (Table 1), permeates the NPP at a much higher rate than any of the other solutes tested raises the possibility that these two factors together influence the permeation rate and that it is this particular combination that is responsible for the relatively high rate of permeation of PhTMA.

In an attempt to test whether the selectivity properties of the NPP might be accounted for on the basis of the combined effects of solute size and hydrophobicity we calculated for each solute ('X') a relative 'hydrophobicity-corrected permeability' (denoted by P'_X). This was done by dividing the measured transport rate by the octanol/water partition coefficient. This value was then expressed relative to that for the most rapidly permeating solute, PhTMA, to give a relative hydrophobicity-corrected permeability (denoted by P'_X/P'_{PhTMA}). The approach of applying a correction factor to the measured permeabilities is similar to that taken by Ginsburg and Stein in their analyses of a number of different solute permeation pathways induced in erythrocytes by a range of different perturbations [6], and the term P'_X used here is actually proportional to the term D_{mem} used by Stein for the coefficient for diffusion within the membrane [22].

In Fig. 5D the relative hydrophobicity-corrected permeation rates are plotted as a function both of the cylindrical diameter of each solute (open symbols) and the diameter of a sphere having the same molecular volume as the compounds of interest (closed symbols). The two sets of data were fitted to a simple model of the permeation of solutes through a cylindrical pore. The model is based on the premise that the ease with which a spherical or

cylindrical solute can enter a cylindrical pore of fixed diameter (d) decreases as the diameter of the solute (s_X) increases, because the cross-sectional area in which the solute is free to diffuse within the pore decreases in proportion to $(1-(s_X/d))^2$ through a simple size exclusion effect [23,24]. This is represented by the expression:

$$\log(P'_X/P'_{PhTMA}) = \log((1-(s_X/d))^2/(1-(s_{PhTMA}/d))^2) \quad (1)$$

where s_{PhTMA} is the estimated cylindrical or equivalent spherical diameter of PhTMA (6.7 and 6.6 Å, respectively).

A fit of Eq. 1 to the data in which the molecular diameters were taken as those of spheres having the same molecular volume of the solutes of interest gave an estimated pore diameter of 7.8 Å, with a correlation coefficient of 0.91 (Fig. 5D, closed symbols, broken line). A fit of Eq. 1 to the data in which the molecular radii were taken as those of the cylinders enclosing the solutes of interest (in their longest, narrowest conformation) gave an estimated pore diameter of 11.4 Å, with a correlation coefficient of 0.95 (Fig. 5D, open symbols, solid line). More complex models, incorporating terms to account for the frictional drag between permeating solutes and the insides of the pore (as used in [6,23,25]), gave similar estimates of the pore diameter, with no improvement in the correlation.

The analyses represented in Fig. 5D are based on various assumptions and are perhaps best viewed as providing evidence for an empirical relationship between permeation rate and the combination of solute size and hydrophobicity, rather than for a particular model of solute permeation. Nevertheless, the finding of a systematic relationship between the hydrophobicity-corrected permeability coefficients and the size of the solute, and the reasonable fit of the simple size-exclusion model to the data is at least consistent with the selectivity properties of the pathway being those of a pore with a hydrophobic interior. For solutes of similar hydrophobicity, smaller solutes permeate faster than larger solutes (e.g. PhTMA > TPA), reflecting the size-selectivity properties of the pathway. For solutes of similar size, the rate of permeation increases with solute hydrophobicity (e.g. PhTMA > TMA), reflecting the hydrophobic nature of the pathway.

The hypothesis that solute hydrophobicity and size together influence the rate of permeation via the NPP is consistent with the previous observations that amongst neutral amino acids of a similar size the rate of permeation increases with increasing hydrophobicity [4,5]. It is also consistent with the observation by Cabantchik that for a series of electroneutral polyols (of broadly similar hydrophobicities) there is an inverse correlation between permeation rate and solute size [1]. In this latter study, a fit of the graph of the relative permeation rates against the equivalent spherical radii of the solutes to the Renkin equation ([25]; similar to Eq. 1) was consistent with permeation being via a cylindrical pore with a diameter of around 7.3 Å. This is similar to the value of 7.8 Å obtained here using the equivalent spherical diameters of the different cations used in this study. Both values are substantially lower than the pore diameter of 11.4 Å estimated here on the basis of the estimated minimum cylindrical diameters of the solutes of interest. The latter value is likely to be the more accurate as these diameters represent those of the narrowest conformations that the molecules can realistically adopt.

Although the present work deals exclusively with organic cations, it is relevant to compare the size of the larger solutes tested here with those of other solutes for which there is evidence of their permeating the NPP. The largest molecules for which there is direct evidence of NPP permeation are the oxidised form of glutathione (GSSG, two tripeptides linked by a disulphide bond and having a M_r of 613) [26] and pepstatin A, a peptidomimetic protease inhibitor, with a M_r of 686 [27]. Although the molecular weights of these compounds are substantially higher than those of the compounds used in this study, construction of molecular models such as those shown in Fig. 1 reveals that both molecules can adopt stable conformations that can be accommodated within a pore of 11–12 Å diameter (not shown).

4.2. Anion dependence of the flux of cations via the NPP

For those cations for which the rate of influx was measured in cells suspended both in Cl^- and in Br^-

media the rate of influx was higher for cells in a Br^--containing medium than for those in a Cl^- medium. In the case of TMA, TEA and choline the estimated influx rate for cells in a Br^- medium was 1.9–2.2 times that for cells in Cl^- ($P < 0.001$), whereas for PhTMA the effect was smaller (and not statistically significant), with the flux into cells in Br^- only 1.25 times that in cells in Cl^-. The anion dependence of the rate of cation permeation has been reported previously, for choline and for the inorganic cation Rb^+ [7]. In this earlier study it was shown that substitution of Cl^- for NO_3^- in the suspending medium caused a 4–6-fold increase in the influx of Rb^+, compared to a 2–3-fold increase in the influx of choline via the NPP of parasitised cells.

Two different models have been proposed to account for the anion dependence of cation permeation via the NPP [7]. In one, the anion interacts with a positively charged site within the pathway, thereby shielding permeant cations from exposure to the positive charge as they move across the membrane. In the other the anion interacts with the cation, with the cations permeating the pathway in the form of cation-anion pairs. The latter model in particular provides several possible explanations for the observation that the relative increase in the rate of cation transport resulting from the replacement of Cl^- with a more lyotropic anion such as Br^- or NO_3^- varies with the nature of the cation.

One possible explanation relates to the effect of the anion substitution on the hydrophobicity of the cation-anion pair, and to the postulated effect of solute hydrophobicity on the permeation rate. For a highly hydrophilic cation such as Rb^+, the replacement of Cl^- with a more lyotropic anion will result in a significant increase in the hydrophobicity of the permeating cation-anion pair, resulting in an increased permeation rate. For a more hydrophobic cation such as PhTMA the same manoeuvre will cause a much smaller relative increase in the hydrophobicity of the cation-anion pair, and the relative increase in the permeation rate will be correspondingly lower.

An alternative explanation might apply if cations were able to permeate the NPP both paired with an anion and unpaired, with the relative rates of permeation of the two species varying between cations. Hydrophilic cations to which the NPP have a relatively low permeability (such as Rb^+ [7]) may permeate predominantly in the form of cation-anion pairs, whereas for more hydrophobic cations (such as PhTMA), a substantial proportion of the flux may be in the form of the unpaired cation. If the rate of permeation of the cation-anion pair increased with the lyotropicity of the anion (whereas the rate of permeation of unpaired cations was unaffected by the nature of the anion in the suspending medium) then the degree of anion dependence of the total measured cation flux would vary between cations in the manner observed.

4.3. Blockade of the NPP

The smaller quaternary ammonium cations permeated the NPP freely and had no effect on the permeability of the NPP to other solutes. However, as the size (and with it, the hydrophobicity) of compounds of this class increased there was increasing tendency to block the pathway (Fig. 4). The same trend was observed with uncharged *n*-alkanols.

A similar pattern has been reported previously for a series of analogues of the widely used anion transport inhibitor, 5-nitro-2-(3-phenylpropylamino)benzoic acid [20]. This latter group of compounds are anionic, with a negatively charged 'headgroup' and a hydrophobic tail. They are more potent inhibitors of malaria-induced transport than the compounds tested here, with IC_{50}s in the micromolar to submicromolar range [20]. However, they show the same general trend of inhibitory potency increasing with the size and hydrophobicity of the compound. The inhibitor data, like those relating to relative permeation rates, are consistent with the NPP being hydrophobic in character.

Ancelin et al. [28] have demonstrated that monovalent, hydrophobic quaternary ammonium compounds inhibit the growth of *P. falciparum* in vitro. The effect was attributed to inhibition of uptake of choline via a choline carrier in the erythrocyte membrane. The demonstration here of an inhibitory effect of quaternary ammonium compounds on the NPP raises the possibility that the antiplasmodial effect of the compounds used by Ancelin et al. may have been due, at least in part, to inhibition of the NPP.

4.4. Conclusions

The NPP induced by the intracellular parasite confer upon the parasitised erythrocyte a substantial permeability to a range of monovalent organic cations, the largest having an estimated minimum cross-sectional diameter of 11–12 Å. Larger cations block the pathways. The data obtained here are consistent with the rate of permeation of solutes via the NPP being influenced by the combination of solute size and hydrophobicity, with the pathways showing a particularly high permeability to the phenyltrimethylammonium ion, a compound that combines a relatively small size with a relatively high hydrophobicity. The rate of influx of the different cations varied (to different extents) with the nature of the anion present, consistent with anions playing a role in the cation permeation process.

The identity of the NPP remains unknown. Nevertheless the finding that they are permeable to non-physiological and relatively large organic cations highlights the potential of these pathways as a route for targeting cytotoxic reagents into the malaria-infected erythrocyte. It remains to be demonstrated whether the NPP are induced to the same extent in erythrocytes from patients infected with field isolates of *P. falciparum*. However, the recent report [29] that parasitised (but not non-parasitised) erythrocytes from mice infected with *Plasmodium vinckei vinckei* show enhanced uptake of choline via pathways with similar characteristics to those described here is consistent with the induction of NPP being a general feature of *Plasmodium*-infected erythrocytes.

Acknowledgements

This work was supported by the Australian NHMRC (971008), the Wellcome Trust (041182/Z/94/Z) and the Lister Institute of Preventive Medicine. H.M.S. held an MRC studentship and was supported by the Oxford-ANU student exchange scheme. K.K. was a Lister Institute Research Fellow and C.R. an Oxford Nuffield Medical Fellow during part of the period over which the experimental work was carried out.

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