Perturbation of the pump-leak balance for Na\(^+\) and K\(^+\) in malaria-infected erythrocytes

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Perturbation of the pump-leak balance for Na\(^+\) and K\(^+\) in malaria-infected erythrocytes. Am J Physiol Cell Physiol 280: C1576–C1587, 2001.—In human erythrocytes infected with the mature form of the malaria parasite Plasmodium falciparum, the cytosolic concentration of Na\(^+\) is increased and that of K\(^+\) is decreased. In this study, the membrane transport changes underlying this perturbation were investigated using a combination of \(^{86}\)Rb\(^+\), \(^{42}\)K\(^+\), and \(^{22}\)Na\(^+\) flux measurements and a semiquantitative hemolysis technique. From >15 h postinvasion, there appeared in the infected erythrocyte membrane new permeation pathways (NPP) that caused a significant increase in the basal ion permeability of the erythrocyte membrane and that were inhibited by furosemide (0.1 mM). The NPP showed the selectivity sequence erythrocyte membrane and that were inhibited by furosemide new permeation pathways (NPP) that caused.

15 h postinvasion, there appeared in the infected erythrocyte membrane vesicles prepared from parasitized erythrocytes from mature (trophozoite-stage) Plasmodium falciparum parasites. Subsequent characterization of the increased passive (ouabain-insensitive) K\(^+\)-to-Na\(^+\) flow through a well-characterized pump-leak mechanism (46). Na\(^+\) is pumped out of the cell, and K\(^+\) is pumped into the cell via the ouabain-sensitive Na\(^+\)/K\(^+\)/ATPase, which thereby generates substantial opposing concentration gradients for both ions. The pumping counterbalances the “leak” of the two ions down their respective concentration gradients via various cotransporters, exchangers, and channels. The net result, in normal human erythrocytes, is a steady-state cytoplasmic [Na\(^+\)]-to-[K\(^+\)] ratio of 0.12–0.16 (3, 34).

It has long been recognized that in mammalian erythrocytes infected with malaria parasites (Plasmodium species) there is a marked perturbation of the normal Na\(^+\)/K\(^+\) levels (9, 15, 34, 38). Using Sendai virus to permeabilize the plasma membrane of human erythrocytes infected with mature (trophozoite-stage) forms of Plasmodium falciparum and thereby release the ions in the host cell compartment for analysis, Ginsburg et al. (15) estimated the [Na\(^+\)]-to-[K\(^+\)] ratio in the erythrocyte cytosol to have increased 10-fold to ~1.25. Using X-ray microanalysis, Lee et al. (34) obtained evidence for an even greater perturbation, estimating the [Na\(^+\)]-to-[K\(^+\)] ratio in the cytosol of late-stage P. falciparum-infected human erythrocytes to be ~11.6. This value implies an almost complete loss of the normal transmembrane Na\(^+\) and K\(^+\) gradients across the parasitized erythrocyte membrane. The high [Na\(^+\)]-to-[K\(^+\)] ratio in the cytosol of the malaria-infected erythrocyte contrasts with that in the cytosol of the parasite itself, estimated in the same study to be 0.06–0.12 (34).

Neither the origin nor the possible physiological role(s) of the altered Na\(^+\)/K\(^+\) levels in the infected erythrocyte cytosol are well understood. In an early study of Na\(^+\) and K\(^+\) in erythrocytes from monkeys infected with P. knowlesi, Dunn (9) postulated that the increased [Na\(^+\)]-to-[K\(^+\)] ratio observed during malaria infection is due to both an impairment of the Na\(^+\)/K\(^+\) pump and a twofold increase in the Na\(^+\) leak. Using membrane vesicles prepared from parasitized erythrocytes from P. chabaudi-infected mice, Tanabe et al. (44) found no change in Na\(^+\)/K\(^+\) pump activity under optimal assay conditions. However, Bookchin et al. (4) and Kirk et al. (25) reported a somewhat variable increase in the pump-mediated (ouabain-sensitive) uptake of K\(^+\) (42)K\(^+\) or \(^{86}\)Rb\(^+\)) and an increase in passive K\(^+\) transport in human erythrocytes infected in vitro with mature P. falciparum parasites. Subsequent characterization of the increased passive (ouabain-insensitive) transport of K\(^+\)\(^{86}\)Rb\(^+\)) (26, 27) led to the proposal...
that it is due to the induction by the parasite in the host cell membrane of new permeation pathways (NPP) that have a broad selectivity and are permeable to a wide range of organic and inorganic solutes (16, 41). These pathways are anion selective (8, 27) but, nevertheless, cause a significant increase in the basal permeability of the erythrocyte membrane to both K\(^+\) (\(P_{K}\)) and Rh\(^+\) (\(P_{Rbi}\); see Ref. 26) as well as to a range of organic cations (43). There is semiquantitative evidence that the permeability of the NPP to K\(^+\) (\(P_{K}\)) is higher than that to Na\(^+\) (26). However, it remains to be demonstrated whether Na\(^+\) is actually transported via the NPP and, if so, whether this process plays a significant role in the altered Na\(^+\)/K\(^+\) balance in the parasitized erythrocyte.

In the present study, we have extended our earlier work on the altered transport of monovalent cations across the membrane of human erythrocytes infected with \(P. falciparum\), the most virulent of the malaria parasites infectious to humans. We have characterized the permeability of the NPP to a range of alkali metal cations, with the aim of investigating the relationship between the activities of the major Na\(^+\)/K\(^+\) transport pathways operating in the infected cell membrane and assessing their relative contributions to the net perturbation of the normal pump-leak balance. Incorporation of the data into a mathematical model of the human erythrocyte membrane indicates that the measured alterations in the transport of Na\(^+\) and K\(^+\) across the host erythrocyte membrane account for the observed perturbation of the Na\(^+\) and K\(^+\) levels in the infected erythrocyte cytosol and have significant consequences for the host cell volume.

**MATERIALS AND METHODS**

**Chemicals**

\(^{86}\)Rb\(^+\) and \(^{22}\)Na\(^+\) were obtained from DuPont New England Nuclear. \(^{43}\)K\(^+\) was from the Medical Research Council Cyclotron Unit (Hammersmith Hospital, London). Ouabain, bumetanide, furosemide, DIDS, and DMSO were from Sigma Chemical (Poole, Dorset, UK). Alkali metal salts were from Aldridge Chemical (Gillingham, Dorset, UK).

**Parasite Culture**

Human erythrocytes (type O) infected with the ITO4 line of \(P. falciparum\) (2) were cultured under 1% O\(_2\)-3% CO\(_2\)-96% N\(_2\) in RPMI 1640 culture medium at pH 7.4 (GIBCO) and supplemented with D-glucose (10 mM), glutamine (2 mM), HEPES (40 mM), gentamicin sulfate (25 mg/l), and human serum (8.5% vol/vol, pooled from different blood donors; National Blood Services, South West, Bristol). The low-O\(_2\) gas environment mimics conditions in the venous blood and is an important factor in the growth of \(P. falciparum\) in culture (47). With the exception of the experiments giving rise to Figs. 2 and 6 (see below), all experiments were carried out using mature, trophozoite-stage infected cells (~36–44 h postinvasion) synchronized by a combination of sorbitol hemolysis (32) and gelatin flotation (39). Parasitized cells were harvested from culture immediately before experimentation, either by gelatin flotation or by centrifugation on Percoll as described elsewhere (28). The former method yielded suspensions of 40–70% parasitemia and the latter method suspensions of 80–96% parasitemia.

Two experiments (see Figs. 2 and 6) required that flux measurements be made at specific time points over the 48-h period of occupancy of the erythrocyte by the malaria parasite. For these experiments, the parasite cultures were synchronized to within a few hours of one another by repeated sorbitol lysis and gelatin flotation steps throughout the week before the experiment was carried out. The experiment commenced with the addition of late “schizont-stage” parasitized cells (~48 h postinvasion) to fresh blood cells. Approximately 5 h later, the cells were suspended transiently in an isosmotic sorbitol solution to remove any contaminating mature-stage parasitized cells (32) before the culture was continued. Immediately before each flux measurement was carried out, parasitized cells were harvested from the tightly synchronized culture by centrifugation on Percoll (80–65% vol/vol, with the osmolality adjusted to ~320 mosmol/kgH\(_2\)O and the pH 7.4 by the addition of 10× PBS + H\(_2\)O). The Percoll dilution used varied with the time postinvasion as the density of the parasitized erythrocytes decreased with increasing parasite maturity. Parasitized cells up to 30 h postinvasion were harvested by centrifugation on 75% vol/vol Percoll layered over 80% vol/vol Percoll. Parasitized cells from 30–44 h postinvasion were harvested by centrifugation on 65% vol/vol Percoll.

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 before the experiment. 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) before experimentation.

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

\(^{86}\)Rb\(^+\), \(^{43}\)K\(^+\), and \(^{22}\)Na\(^+\) Influx Measurements

Estimates of unidirectional influx rates were made from the uptake of \(^{86}\)Rb\(^+\), \(^{43}\)K\(^+\), and \(^{22}\)Na\(^+\) using methods similar to those described previously (26). Cells harvested from culture were washed (4 times) and then resuspended in HEPES-buffered saline that, unless specified otherwise, contained 150 mM NaCl, 5 mM KCl, 10 mM HEPES, and 5 mM glucose (pH 7.4; 300 ± 3 mosmol/kgH\(_2\)O). The resulting suspension was dispensed into microcentrifuge tubes and allowed to equilibrate to temperature (37°C unless stated otherwise) for at least 10 min before the influx was begun by the addition of either \(^{86}\)Rb\(^+\), \(^{43}\)K\(^+\), or \(^{22}\)Na\(^+\) to give an activity in each case of ~1 μCi/ml, a cell concentration of 1–4 × 10\(^6\) cells/ml, and a total sample volume of 1 ml. After an appropriate incubation period, aliquots of the suspension (200 μl) were transferred to microcentrifuge tubes containing 0.3 ml of dibutyl phthalate oil. The tubes were centrifuged immediately (10,000 g, 20 s) to sediment the cells below the oil. The aqueous supernatant solution was removed by aspiration, and the radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water. The oil was aspirated, and then the cell pellet was lysed by 0.1% vol/vol Triton X-100 (0.5 ml), deproteinized by the addition of 5% w/vol TCA (0.5 ml), and centrifuged (10,000 g, 10 min). Radioactivity was measured using a β-scintillation counter.

Throughout this study, influx rates were estimated from the amount of radiolabel taken up within a fixed incubation period.
The relative permeability of trophozoite-stage *P. falciparum*-infected erythrocytes to different alkali metal cations was investigated using a semiquantitative hemolysis method. Isosmotic solutions of both the chloride and nitrate salts of the different cations of interest were prepared by dissolving the compounds to a concentration of 160 mM in a solution containing 10 mM HEPES and 5 mM glucose (pH 7.4). The osmolality was then adjusted (by the addition of either the salt or the hyposmotic HEPES + glucose solution) to 300 ± 3 mosmol/kg H₂O measured using a freezing-point osmometer (Roebing). The isosmotic solutions were, in all cases, supplemented with ouabain (0.1 mM), bumetanide (0.01 mM), and nitrendipine (0.01 mM) to inhibit the endogenous erythrocyte Na⁺/K⁺ pump, Na⁺-K⁺-Cl⁻ cotransporter, and Ca²⁺-activated K⁺ channel, respectively (11).

Time course measurements commenced with the addition of a 0.2-ml aliquot of cell suspension to 3.3 ml of the isosmotic solutions of interest to give a cell concentration of ~0.5 × 10⁸ cells/ml. All such experiments were carried out at 37°C. At predetermined intervals, 0.5-ml aliquots of the suspension were transferred to microcentrifuge tubes containing 0.5 ml of an ice-cold “stopping solution” (400 mM sucrose in H₂O). The tubes were centrifuged for 30 s (10,000 g), and then 0.9 ml of the supernatant solution was transferred to another tube for the subsequent spectrophotometric absorbance measurements at 540 nm (ε₅₄₀) estimation of Hb concentration.

In all such experiments, the A₅₄₀ value corresponding to full hemolysis of trophozoite stage-infected erythrocytes was estimated from the final A₅₄₀ value achieved in the supernatant solution from infected cells suspended in an isosmotic CsNO₃ solution (see Fig. 4C).

Mathematical Model

The consequences for the host erythrocyte of the transport changes revealed in this study were investigated using the integrated mathematical model of the erythrocyte developed by Lew and Bookchin (35). The model, together with instructions for its use, is available from http://www.physiol.cam.ac.uk/staff/kew/index.htm. In running the model, the parameters defining the cells and extracellular solution were set to the normal default values, except where specified otherwise.

RESULTS

**K⁺(⁸⁶Rb⁺) Transport in Uninfected and Malaria-Infected Erythrocytes**

As has been reported previously (25, 27), human erythrocytes infected with mature, trophozoite-stage *P. falciparum* parasites (36–44 h postinvasion) showed a marked elevation in unidirectional ⁸⁶Rb⁺ influx rates. Figure 1 shows the influx of ⁸⁶Rb⁺ into infected and uninfected cells, measured in the absence of inhibitors and in the presence of ouabain (0.1 mM) to block the Na⁺/K⁺ pump, bumetanide (0.01 mM) to block the Na⁺-K⁺-Cl⁻ cotransporter, and furosemide to inhibit the parasite-induced NPP (27). The flux is expressed in terms of K⁺ influx and is referred to as K⁺(⁸⁶Rb⁺) influx; i.e., the K⁺ influx calculated from the measured uptake of ⁸⁶Rb⁺, assuming that Rb⁺ and K⁺ are transported via the pathways of interest at the same rate and that ⁸⁶Rb⁺ is therefore an accurate tracer for K⁺. This assumption is approximately valid for both the Na⁺/K⁺ pump (37) and the Na⁺-K⁺-Cl⁻ cotransporter (6) but results in an overestimate of the flux of K⁺ via the NPP induced by the parasite (see below).

The relative contributions of each of the different transport pathways to the estimated K⁺(⁸⁶Rb⁺) influx are shown in Fig. 1, inset. In normal, uninfected erythrocytes, the majority of the measured K⁺(⁸⁶Rb⁺) influx was via the ouabain-sensitive Na⁺/K⁺ pump, with most of the remainder being via the bumetanide-sensitive Na⁺-K⁺-Cl⁻ cotransporter. In parasitized erythrocytes, 36–44 h postinvasion, there was, on average, a 1.4-fold increase in the measured ouabain-sensitive K⁺(⁸⁶Rb⁺) influx (Fig. 1, inset). However, there was significant variability between experiments, and the increase did not reach statistical significance (P = 0.059, paired t-test, n = 6).

The bumetanide-sensitive influx of K⁺(⁸⁶Rb⁺), attributed primarily to the Na⁺-K⁺-Cl⁻ cotransporter, showed a small (1.2-fold) increase in infected, compared with uninfected, erythrocytes. However, this was minor compared with the very substantial flux of K⁺(⁸⁶Rb⁺) via the furosemide-sensitive (ouabain- and bumetanide-insensitive) NPP that was present in infected but not uninfected cells.

The furosemide-sensitive K⁺-Cl⁻ cotransporter is unlikely to contribute significantly to the observed
furosemide-sensitive influx of $^{86}\text{Rb}^+$ into parasitized cells. Although present in reticulocytes and young erythrocytes, this transporter is lost rapidly from cells on incubation in vitro at $37^\circ\text{C}$ (as occurs in culturing parasitized erythrocytes; see Ref. 19) and is, in any case, only weakly sensitive to furosemide, with an IC$_{50}$ (i.e., the concentration required to bring about a 50% inhibition) of 1–2 mM (22, 33), much higher than the 0.1 mM used here.

**Stage Dependence of the Activity of the Na$^+$/K$^+$ Pump and the NPP in Parasitized-Erythrocytes**

To understand better the relationship between the induction of the NPP and the observed (variable) increase in flux via the Na$^+$/K$^+$ pump in erythrocytes housing mature, trophozoite-stage parasites, the activity of both pathways was measured as a function of time after parasite invasion. The results of this experiment, carried out using highly synchronized cultures, are shown in Fig. 2.

In uninfected erythrocytes, cultured in parallel with the parasitized erythrocytes, there was no induction of NPP, and the Na$^+$/K$^+$ pump activity remained constant over 44 h. In parasitized erythrocytes up to 12–15 h postinvasion, the $^{86}\text{Rb}^+$ fluxes remained normal; Na$^+$/K$^+$ pump activity was the same as that in uninfected cells, and there was no evidence for NPP. From ~15 h, however, there was a significant flux via the furosemide-sensitive NPP, and this increased progressively as the parasite matured, showing some tendency to level off to a maximum value in the final hours before the bursting of the parasitized cell and release of the new generation of parasites at 48 h.

From the time that flux via the NPP was first evident (~15 h), there was an increase in Na$^+$/K$^+$ pump activity. This reached a maximal value (of approximately double the normal activity) at 28–36 h postinvasion and then underwent a decline, reaching a value similar to that seen in normal cells by 44 h postinvasion.

The marked decrease in the activity of the Na$^+$/K$^+$ pump from >36 h accounts for the significant variability noted in the pump activity measured in cells during the period 36–44 h postinvasion (Fig. 1).

From 28 h postinvasion, the flux of $^{86}\text{Rb}^+$ via the NPP exceeded that via the Na$^+$/K$^+$ pump, and by 44 h the flux via the NPP was more than threefold higher than that via the pump (at an external [K$^+$] of 5 mM).

**Temperature Dependence of $^{86}\text{Rb}^+$ Transport Via the NPP**

The temperature dependence of the influx of $^{86}\text{Rb}^+$ via the NPP was investigated to provide an estimate of the energy of activation ($E_a$). Figure 3 shows an Arrhenius plot for the furosemide-sensitive influx of K$^+$($^{86}\text{Rb}^+$) into parasitized erythrocytes. $E_a$ was estimated as 47 $\pm$ 8 kJ/mol [equivalent to 11 $\pm$ 2 (SE) kcal/mol].

**Ion Selectivity Properties of the NPP**

Isosmotic hemolysis measurements. The relative permeability of the NPP to different alkali metal ions was investigated using a semiquantitative hemolysis...
method that has been used previously to monitor the net influx of solutes into malaria-infected erythrocytes (18, 27, 43). Normal and parasitized cells (36–44 h postinvasion) were suspended in isosmotic solutions of either the chloride or nitrate salts of Li⁺, Na⁺, K⁺, Rb⁺, or Cs⁺, and the hemolysis (that occurred as a result of the net influx of the extracellular cation, together with the anion present, exceeding the net efflux of intracellular solutes) was monitored spectrophotometrically from the measured Hb release. Normal (uninfected) erythrocytes remained stable for >3 h in each of the solutions tested (data not shown).

Parasitized erythrocytes suspended in isosmotic Cs⁺, Rb⁺, and K⁺ chloride solutions underwent progressive hemolysis, the rate of which was in the order Cs⁺ > Rb⁺ > K⁺ (Fig. 4A). Hemolysis in the isosmotic Na⁺ and Li⁺ chloride solutions was substantially slower, with no difference between the rates of hemolysis of cells in these two media. Furosemide (0.2 mM) protected the cells in Cs⁺, Rb⁺, and K⁺ chloride against hemolysis, reducing the rate of hemolysis to a level similar to that observed in the isosmotic Na⁺ and Li⁺ chloride solutions (Fig. 4B).

The rates of hemolysis of parasitized cells suspended in the nitrate salt solutions were significantly higher than those in the corresponding chloride salt solutions; with parasitized erythrocytes suspended in isosmotic Cs⁺, Rb⁺, and K⁺ nitrate solutions all undergoing full hemolysis within 3 h (Fig. 4C). As in the chloride solutions, cells suspended in isosmotic Na⁺ and Li⁺ nitrate solutions underwent hemolysis at a much lower rate, and furosemide (0.2 mM) reduced the rate of hemolysis of cells in Cs⁺, Rb⁺, and K⁺ nitrate solutions to a similar level to that observed in the Na⁺ and Li⁺ nitrate solutions (Fig. 4D).

The data are consistent with the hemolysis of cells in Cs⁺, Rb⁺, and K⁺ solutions being due predominantly to the influx of the salts via the furosemide-sensitive NPP. The much lower rates of hemolysis of cells in the Na⁺ and Li⁺ solutions indicates that the net entry of solute into the parasitized cells in these media was insufficient to cause the majority of parasitized cells to reach a hemolytic volume within the time scale of the experiment.

In the case of the Cs⁺, Rb⁺, and K⁺ solutions, the inverse of the half-times for hemolysis (i.e., the inverse of the time taken for the measured Hb release to reach half its maximum value; 1/T₅₀) provides a semiquantitative measure of the rate of influx of the different cations into the infected cell (18, 43). The estimated 1/T₅₀ values are given in Table 1 together with the corresponding estimates of the permeability of the NPP-induced permeability for Cs⁺ and Rb⁺, relative to that for K⁺ (P₉/R₉, P₉/K, and P₉/K, respectively). For each cation, the 1/T₅₀ for hemolysis in the chloride medium was significantly less than that in the corresponding nitrate medium (P ≤ 0.037; paired t-test, n = 3). For both the chloride and nitrate salts, the 1/T₅₀ value for K⁺ was significantly lower than that for Rb⁺ (P ≤ 0.035), which was, in turn, significantly lower than that for Cs⁺ (P ≤ 0.037).

Radioisotope flux measurements. Although the data of Fig. 4 indicate that K⁺ is transported via the NPP significantly faster than Na⁺, it provides no information as to whether, and how rapidly, Na⁺ might permeate the NPP nor the relative rates of permeation of Na⁺ and K⁺. Quantitative estimates of the rates of
transport of Na\(^+\) and K\(^+\) via the NPP were therefore made from the uptake of \(^{22}\text{Na}\) and \(^{43}\text{K}\), respectively.

Figure 5 shows time courses for the influx of Na\(^+\) into infected and uninfected cells, measured in the presence of bumetanide (0.01 mM, to block the Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter) and DIDS [0.01 mM, to block Na\(^+\)-\(\text{CO}_3\)\(^-\) cotransporter via the band 3 anion exchanger (1)]. The suspending solution also contained 5 mM K\(^+\), which inhibits Na\(^+\)/Na\(^+\) exchange via the Na\(^+\)/K\(^+\) pump (14).

Under these conditions, there was minimal influx of Na\(^+\) into uninfected erythrocytes. By contrast, there was a substantial flux of Na\(^+\) into parasitized cells. This flux was inhibited by furosemide (0.1 mM), consistent with its being via the parasite-induced NPP. However, an alternative possibility is that a component of this flux was via the endogenous erythrocyte Na\(^+\)/H\(^+\) exchanger, which can be stimulated in human erythrocytes by a reduction in intracellular pH (12). Attempts to use inhibitors of the Na\(^+\)/H\(^+\) exchanger to determine the possible contribution of this system to the measured Na\(^+\) transport proved unsuccessful as both amiloride and its analog dimethylamiloride were found to inhibit the NPP (data not shown). In a separate study, however, the Na\(^+\)/H\(^+\) exchanger of uninfected erythrocytes was shown to be inhibited by dimethylamiloride (0.02 mM) but to be largely unaffected by 0.1 mM furosemide (data not shown). The furosemide-sensitive influx of \(^{22}\text{Na}\) into infected erythrocytes may therefore be attributed to the parasite-induced NPP.

A characteristic feature of the transport of cations via the NPP is a marked dependence on the nature of the anion present, with cation flux increasing substantially on replacement of Cl\(^-\) with NO\(_3\)\(^-\) in the suspending medium (Refs. 26, 42, and 43 and Table 1). The same phenomenon was observed for Na\(^+\), with the furosemide-sensitive influx of \(^{22}\text{Na}\) increasing approximately threefold on replacement of Cl\(^-\) with NO\(_3\)\(^-\) (Fig. 5, inset; \(P = 0.012\), paired \(t\)-test, \(n = 3\)).

Quantitative estimates of the relative rates of permeation of \(^{22}\text{Na}\) and \(^{86}\text{Rb}\) via the NPP were made in a series of paired experiments, in which the influx of \(^{22}\text{Na}\) and \(^{86}\text{Rb}\) was measured in parasitized cells suspended in HEPES-buffered saline (containing 150 mM NaCl, 5 mM KCl, 10 mM HEPES, and 5 mM glucose), supplemented with 0.1 mM ouabain, 0.01 mM bumetanide, 0.01 mM nitrendipine, and 0.01 mM bumetanide, 0.01 mM nitrendipine, and 0.01 mM nitrendipine.

Table 1. Relative rates of permeation of Na\(^+\), K\(^+\), Rb\(^+\) and Cs\(^+\) via the NPP induced by the intracellular malaria parasite in the host erythrocyte membrane

<table>
<thead>
<tr>
<th>Cation</th>
<th>(1/T_{50}) min(^{-1})</th>
<th>(P_X/P_K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Na}^+)</td>
<td>ND</td>
<td>0.44</td>
</tr>
<tr>
<td>(K^+)</td>
<td>0.0257 ± 0.0023</td>
<td>1</td>
</tr>
<tr>
<td>(Rb^+)</td>
<td>0.0389 ± 0.0015</td>
<td>1.51</td>
</tr>
<tr>
<td>(Cs^+)</td>
<td>0.0655 ± 0.0016</td>
<td>2.55</td>
</tr>
</tbody>
</table>

Data for the inverse of the time taken for the measured Hb release to reach its maximum value (\(1/T_{50}\); means ± SE) are from isosmotic hemolysis experiments such as those represented in Fig. 4 A and C. \(1/T_{50}\) provides a semiquantitative measure of the influx rate for the different cations. The \(1/T_{50}\) values are averaged from those estimated in 3 separate experiments, each on cells from a different donor. In the 3 experiments, the cells suspended in isosmotic KCl solution underwent between 33 and 49% hemolysis at 180 min (i.e., the final time point). In each case, \(1/T_{50}\) was estimated by linear extrapolation of the hemolysis time course data. \(P_X/P_K\) rate of permeation via the NPP of cation \(X\), relative to that of K\(^+\) estimated either from the hemolysis (\(1/T_{50}\)) data or from the measured influx of \(^{22}\text{Na}\), \(^{43}\text{K}\), and \(^{86}\text{Rb}\). ND, not determined.
DIDS [which, as well as blocking Na\(^+\)-CO\(_3\)\(^-\) transport via band 3 will inhibit any residual KCl cotransporter activity (7)]. Under these conditions, the furosemide-sensitive influx of \(^{22}\text{Na}\) was 0.31 ± 0.02 times that of \(^{86}\text{Rb}\)\(^+\) \((P = 0.006,\) paired \(t\)-test, \(n = 4)\).

In another series of paired experiments, the influx of \(^{43}\text{K}\)\(^+\) and \(^{86}\text{Rb}\)\(^+\) was measured in parasitized cells suspended under identical conditions to those used for the paired \(^{22}\text{Na}/^{86}\text{Rb}\) influx measurements. The furosemide-sensitive influx of \(^{43}\text{K}\) was 0.70 ± 0.10 times that of \(^{86}\text{Rb}\)\(^+\) \((P = 0.026,\) paired \(t\)-test, \(n = 3)\).

If it is assumed that, as for other solutes (e.g., Refs. 8, 27, 30), the transport of Na\(^+\), K\(^+\), and Rb\(^+\) via the NPP is nonsaturable, the \(^{43}\text{K}/^{86}\text{Rb}\) and \(^{22}\text{Na}/^{86}\text{Rb}\) influx data may be used to calculate relative permeabilities of these three ions. These are listed in Table 1.

**Origin of the Increased Na\(^+\)/K\(^+\) Pump Activity in Trophozoite-Infected Erythrocytes**

The observation (Fig. 2) that the activity of the Na\(^+/\)K\(^+\) pump increased after the activation of the NPP is consistent with the hypothesis that the increased pump activity was a secondary consequence of the leakage of Na\(^+\) into and K\(^+\) out of the cell via the NPP. To test this, trophozoite-infected cells (\(\sim 30\) h postinvasion) were exposed to an Na\(^+\)-free medium (see the legend for Fig. 6) for 1 h to deplete them of intracellular Na\(^+\) and then were incubated for a further 2 h in Na\(^+\)-containing medium (supplemented RPMI 1640 without human serum) in the presence and absence of furosemide (0.2 mM) before the ouabain-sensitive K\(^+\)/Rb\(^+\) influx was measured in the same medium. As shown in Fig. 6, for infected cells in which the NPP were blocked by furosemide throughout the 2-h preincubation period, the ouabain-sensitive K\(^+\)/Rb\(^+\) influx was not significantly different from that in normal uninfected cells. It was, however, significantly less than that in cells preincubated in the same solutions in the absence of furosemide \((P = 0.04,\) paired \(t\)-test, \(n = 4)\) and significantly less than that in cells suspended in Na\(^+\)-containing medium in the absence of furosemide throughout the preincubation period \((P = 0.015,\) paired \(t\)-test, \(n = 4)\).

Preincubation of trophozoite-infected cells with 0.01 mM bumetanide, which, like furosemide, blocks the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransporter but which, unlike 0.2 mM furosemide, has little effect on the furosemide-sensitive NPP (Fig. 1A), had no effect on ouabain-sensitive K\(^+\)/Rb\(^+\) influx (data not shown). This rules out any involvement of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransporter in the stimulation of the Na\(^+/\)K\(^+\) pump. The data in Fig. 6 therefore implicate the leakage of ions via the NPP as being the primary cause of the stimulation of the Na\(^+/\)K\(^+\) pump in the trophozoite-infected erythrocyte.

**Mathematical Modeling**

The consequences of the ion transport changes measured here for the physiological properties of the host erythrocyte were investigated using the integrated erythrocyte model developed by Lew and Bookchin (35). Although the model makes no allowance for the presence of the parasite within the erythrocyte, or for the (unknown) effects of the parasite on such parameters as the protein concentration in the host cell cytosol, it does allow at least a semiquantitative assessment of the effect of Na\(^+\) and K\(^+\) transport perturbations on the physiological properties of the host erythrocyte.

Figure 7A shows the time-dependent increase in \(P_{\text{Na}}\) and \(P_{\text{K}}\) arising from the induction of the NPP. \(P_{\text{Na}}\) and \(P_{\text{K}}\) were estimated at different times throughout the period of occupancy of the erythrocyte by the parasite, from the data of Fig. 2, assuming that \(P_{\text{Na}}/P_{\text{Rb}} = 0.31\) and \(P_{\text{K}}/P_{\text{Rb}} = 0.70\) (Table 1).

The estimation of \(P_{\text{Na}}\) and \(P_{\text{K}}\) from the influx of Na\(^+\) and K\(^+\) measured at extracellular concentrations of 150 and 5 mM, respectively, rests on the assumptions that 1) the flux of Na\(^+\) and K\(^+\) via the NPP is linear with concentration (i.e., nonsaturable) within this concentration range and 2) that the membrane potential of the erythrocyte membrane is zero [the potential of normal erythrocytes is only \(-10\) mV (35) and/or that the permeation of Na\(^+\) and K\(^+\) via the NPP is in the form of electroneutral cation-anion pairs (as has been postulated to account for the anion dependence of cation transport (26, 43)] and therefore unaffected by the membrane potential. For the purpose of modeling, it
was also assumed that the NPP confers upon the erythrocyte membrane an additional Cl⁻ permeability ($P_{\text{Cl}}$) with $P_{\text{Cl}} = P_{\text{K}} \times 10^3$ on the basis of earlier observations that the flux of $^{36}\text{Cl}^-$ via the NPP was at least three orders of magnitude higher than that of $^{86}\text{Rb}^+$ (27).

Setting $P_{\text{Cl}}$ to $P_{\text{K}} \times 10^3$ ensures that the rate of anion transport is not rate limiting for the movement of cations either into or out of the cell.

Incorporation of the time-dependent increases in $P_{\text{Na}}$ and $P_{\text{K}}$ shown in Fig. 7A into the model (together with a time-dependent increase in $P_{\text{Cl}}$) predicts a time-dependent increase in the activity of the Na⁺/K⁺ pump shown in Fig. 7B as a percentage of that in uninfected cells. Flux via the Na⁺/K⁺ pump is predicted to reach a maximum at ~32 h postinvasion and then decreases slightly over the remainder of the time course. Figure 7B also shows the observed Na⁺/K⁺ pump activity (taken from Fig. 2). The discrepancy between the predicted and observed activities is indicative of there being significant inhibition of the erythrocyte Na⁺/K⁺ pump activity from as early as 12 h postinvasion, increasing to a maximum inhibition of as much as 50% by 44 h postinvasion.

The extent of inhibition of the Na⁺/K⁺ pump at each time point was estimated (to a first approximation) from the discrepancy between the predicted and observed Na⁺/K⁺ pump activities. This was incorporated into the model and then further refined using an iterative procedure to find what degree of pump inhibition was required at each time point for the pump flux predicted by the model to match that actually observed. The final outcome of this process (i.e., the pump flux predicted by the model, having incorporated a suitable level of pump inhibition at each time point) is also shown in Fig. 7B.

Figure 7, C and D, shows the predicted cytosolic [Na⁺] and [K⁺] (Fig. 7C) and the predicted relative cell volume (Fig. 7D) for a human erythrocyte that undergoes the time-dependent increase in $P_{\text{Na}}$ and $P_{\text{K}}$ shown in Fig. 7A, together with sufficient inhibition of the Na⁺/K⁺ pump (0–50%) to produce the pump activity actually observed. The model predicts that from 12 h there is a progressive decrease in the intracellular [K⁺] and a corresponding increase in the intracellular [Na⁺], with both approaching their respective extracellular concentrations (5 and 145.5 mM, respectively; Fig. 7C). From 12 to 24 h, there is also a progressive decrease in the cell volume (i.e., shrinkage; Fig. 7D) arising from the fact that, for the 12 h after the induction of the NPP, K⁺ efflux via the NPP exceeds Na⁺ influx. However, from 24 h the relative cell volume increases, reaching a predicted value of almost 1.8 at 46 h postinvasion. Also shown in Fig. 7D is the relative cell volume at which a normal human erythrocyte would lyse [1.7 (35)]. The model therefore predicts that an erythrocyte undergoing Na⁺ and K⁺ transport changes of the sort induced by the intracellular malaria parasite in the infected cell membrane would lyse at ~44 h postinvasion.

As well as allowing modeling of the changes in host cell ionic composition and water volume occurring as a result of the progressive perturbation of the membrane transport properties of the parasitized cell, the Lew-Bookchin model may be used to assess the effect of suspending the cells (at any given time postinvasion) in the isosmotic NaCl or KCl solutions used in the
experiments, giving rise to the hemolysis time courses of Fig. 4A. As shown in Fig. 8, normal (uninfected) erythrocytes that have undergone the progressive induction of the NPP and inhibition of the Na+/K+ pump shown in Fig. 7 and that are suspended (at 36 h postinvasion) in isosmotic KCl solution are predicted to undergo a progressive increase in cell water volume, reaching the lytic volume after ~1 h. By contrast, the same erythrocytes suspended in the isosmotic NaCl solution are predicted to remain below the lytic volume for >4 h. The 1 h predicted by the model as being required for hemolysis of cells in isosmotic KCl solution is somewhat shorter than the time taken for the majority of parasitized cells to hemolyze under these conditions (Fig. 4A), showing the shortcomings of the model as applied to infected cells. Nevertheless, the marked discrepancy between the predicted behavior of cells in KCl vs. NaCl media is consistent with the finding that parasitized cells suspended in the isosmotic KCl medium underwent progressive, furosemide-sensitive hemolysis, whereas those suspended in NaCl did not (Fig. 4A).

DISCUSSION

Perturbation of Na+/K+ Pump-Leak Balance in the Parasitized Erythrocyte

In the hours immediately after the invasion of the human erythrocyte by the malaria parasite, *P. falciparum*, the Na+/K+ levels in the infected cell cytosol remain similar to those of uninfected cells (34). As was shown in Fig. 2, for the first 12–15 h postinvasion, the activity of the Na+/K+ pump remains normal, and the furosemide-sensitive NPP that dominates the flux of K+(86Rb+) at the later stages of infection is not yet activated (Fig. 2). Thus invasion of the erythrocyte by the parasite is achieved without any significant alteration of the Na+/K+ transport properties of the host cell membrane.

Between 15 and 20 h postinvasion, the parasite makes the transition from the largely inert “ring-stage” form to the metabolically and biosynthetically active trophozoite stage. As is again evident from Fig. 2, it is at approximately this time that there begins to occur a significant alteration in the ion transport properties of the parasitized erythrocyte membrane. In particular, the induction of NPP enhances the leak of ions across the membrane. The NPP are permeable to both K+ (Fig. 4) and Na+ (Fig. 5) and are bidirectional (transporting 86Rb+ both into and out of the cell (26)). They therefore provide a route for the net leakage of K+ out of and Na+ into the parasitized cell, down their respective concentration gradients.

As the flux of ions via the NPP increases, so too does the activity of the Na+/K+ pump (Fig. 2). The data of Fig. 6 provide evidence for the former being responsible for the latter; inhibition of the NPP with furosemide eliminated the increase in Na+/K+ pump activity observed in trophozoite-infected cells. The affinities for Na+ and K+ at the internal sites of the Na+/K+ pump are such that the pump operates at less than half-maximal velocity (Vmax) under normal physiological conditions, increasing in response to an increase in [Na+] and/or a decrease in [K+] in the cell cytosol (13). The increased activity of the Na+/K+ pump may therefore be attributed to NPP-induced changes in the cytosolic [Na+] and [K+]. As is evident from Fig. 7B, however, the increase in Na+/K+ pump activity predicted to occur (on the basis of the kinetic properties of the Na+/K+ pump) in response to the induction of the NPP is actually somewhat greater than was observed. This is consistent with the Na+/K+ pump of *P. falciparum*-infected erythrocytes undergoing partial inhibition, which increases to a maximum inhibition of as much as 50% by 44 h postinvasion. This contrasts with the recent finding that the activity of the erythrocyte Ca2+ pump (measured under Vmax conditions) is affected little by *P. falciparum* infection (45). The mechanism(s) underlying the inhibition of the Na+/K+ pump is unclear but may relate to any of the plethora of changes that occur in the parasitized erythrocyte (24).

As shown in Fig. 7C, incorporation of the induction of the NPP and the progressive inhibition of the Na+/K+ pump into an integrated model of the erythrocyte predicts that from 12 h there is a progressive decrease in the intracellular [K+] and a corresponding increase in the intracellular [Na+]. The cytosolic [Na+]-to-[K+] ratio predicted by the model soars from 0.07 before the induction of the transport changes to 6.7 by 30 h postinvasion. By 36 h, the [Na+]-to-[K+] ratio is predicted to be 12.6. This is close to the value of 11.6 measured by Lee et al. (34) for the [Na+]-to-[K+] ratio in the cytosol of the erythrocytes infected with “late-stage parasites” using X-ray microanalysis. The Na+/K+ transport changes reported here therefore account fully for the reported perturbation of [Na+] and [K+] levels in the *P. falciparum*-infected human erythrocyte.
Characteristics of the NPP

The furosemide-sensitive NPP are postulated to be anion-selective channels, with a high permeability to a range of monovalent anions and neutral molecules and a much lower (but nonetheless significant) permeability to monovalent cations (reviewed in Ref. 24). The $E_a$ for the flux of $^{86}\text{Rb}^+$ via the NPP (11 kcal/mol) is the same as that estimated previously for the parasite-induced transport of sorbitol [$E_a = 10$ kcal/mol (17)] and NBD-taurine [$E_a = 11$ kcal/mol (31)] and is consistent with the passage of solutes via the NPP being diffusive in nature rather than carrier mediated (10, 17, 31).

The estimates of relative rates of transport of the different alkali metal cations via the NPP (Table 1) are consistent with the pathways having a selectivity based on “Eisenman sequence I”: $\text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{Na}^+$, $\text{Li}^+$ (although note that this study provides no quantitative information on the relative rates of permeation of $\text{Na}^+$ and $\text{Li}^+$). This sequence is the same as that for the rates of diffusion of these ions in aqueous solution, although the relative diffusion coefficients for $\text{Na}^+$, $\text{K}^+$, and $^{86}\text{Rb}^+$ [0.67:1.06, respectively (21)] are somewhat closer to one another than are the relative rates of permeation of these ions via the NPP [0.44:1.43, respectively; Table 1]. The sequence is characteristic of a permeation pathway that has a low electric field strength and that is consequently unable to interact with the cations sufficiently strongly to remove their water of hydration (21).

Two alternative explanations have been put forward for the observed anion dependence of the rate of cation permeation via the NPP (24, 26, 43). One is that permeant anions interact with positively charged sites within the NPP, thereby shielding permeant cations from exposure to the positive charge as they move across the membrane. The other is that the anion interacts directly with the cation, with the cations permeating the pathway either wholly or partially in the form of cation-anion pairs. The possibility that the effect of anion substitution on the rate of cation influx is due to changes in erythrocyte membrane potential is ruled out by the finding that replacement of $\text{Cl}^-$ with $\text{NO}_3^-$ in the medium increases both the influx and efflux of $^{86}\text{Rb}^+$ via the NPP to the same extent while having little effect on the rate of permeation of the nonelectrolyte sorbitol or the anion lactate (26). The order for the effect of anions on the rate of cation permeation [$\text{SCN}^-, \text{NO}_3^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$ (26, 43)] corresponds to Eisenman sequence I for monovalent inorganic anions (48), again consistent with a pathway that interacts weakly, if at all, with permeating ions.

Physiological Role(s) and Consequences of the Perturbation of $\text{Na}^+/\text{K}^+$ Transport

The NPP induced by the intracellular malaria parasite in the host erythrocyte membrane facilitate the uptake of a number of key nutrients (29). Although strongly anion selective (8, 27), they do mediate the flux of monovalent cations across the infected cell membrane and, as shown here, are primarily responsible for the dissipation of the normal [$\text{Na}^+$] and [$\text{K}^+$] gradients across the infected erythrocyte membrane.

Whether the profound alteration to the $\text{Na}^+/\text{K}^+$ levels in the infected erythrocyte cytosol is of physiological significance for the intracellular parasite is yet to be established. It has been reported that the parasite has at its surface an $\text{Na}^+/\text{H}^+$ exchanger that plays a central role in the extrusion of $\text{H}^+$ produced by the high glycolytic activity of the parasite in the later stages of infection (5). The ability of a system of this type to mediate the net efflux of $\text{H}^+$ is dependent on there being a significant inward $\text{Na}^+$ gradient across the parasite plasma membrane, and the parasite may require a raised [$\text{Na}^+$] in the host cell compartment for this reason. However, two recent studies providing evidence that the extrusion of $\text{H}^+$ from the parasite is mediated primarily by a (Na$^+$-independent) V-type H$^+$/ATPase cast some doubt on this (20, 40). It is unclear to what extent, if any, the parasite actually uses other Na$^+$-dependent transporters to energize the flux of solutes across its plasma membrane, but, if present, these would rely on there being an increased [Na$^+$] in the host erythrocyte.

The altered concentration of K$^+$ in the host cell compartment may also have a role to play. In many cell types, K$^+$ plays a key role in the maintenance of the membrane potential. The magnitude, origin, and role(s) of the membrane potential in P. falciparum are yet to be elucidated. It is possible, however, that the intraerythrocytic [K$^+$] exerts a significant influence on the electrical potential across the parasite plasma membrane and that the lowering of the [K$^+$] in the host cell compartment and its consequent effects on the parasite membrane potential are of physiological significance for the parasite.

The changes in Na$^+$ and K$^+$ transport across the parasitized erythrocyte membrane have implications not only for the levels of Na$^+$ and K$^+$ in the erythrocyte cytosol but for the volume of the parasitized cell. The selectivity of the NPP for K$^+$ over Na$^+$ ensures that for the first 12 h after induction of the NPP (i.e., 12–24 h postinvasion), the efflux of ions (predominantly KCl) from the cells exceeds the influx of ions (predominantly NaCl) from the extracellular medium. Under these conditions, a normal human erythrocyte is predicted to undergo significant shrinkage (Fig. 7D). The net influx of monovalent ions, together with the ingestion and digestion of portions of the host cell cytosol with the free amino acids resulting from protein digestion effluxing from the cell via the NPP (49), provides a means for the parasitized cell to counter the swelling induced by the physical presence of the parasite as it switches from the relatively inert ring stage to the fast-growing trophozoite stage over this period. The NPP may therefore play an important role in host cell volume control throughout the initial phase of parasite growth, with its function being somewhat similar to that of swelling-activated osmolyte channels in other cell types (23).
From ∼24 h postinvasion, there is a net influx of monovalent cations, and the cell volume is predicted to increase as a result, reaching a hemolytic volume at a time corresponding to ∼44 h postinfection. This is close to the time (48 h postinfection) at which the \( P. falciparum \)-infected erythrocyte undergoes lysis, releasing 20–30 new parasites. It is tempting to speculate that the flux of cations via the NPP might contribute to the hemolysis of parasitized cells at 48 h postinfection and that NPP inhibitors might therefore prevent the release of the new generation of parasites. This is yet to be tested. It should be emphasized, however, that the model does not take full account of the effects of the parasite on the infected cell. A more quantitative assessment of the effects of the ion transport changes reported here on the cell volume would require a more elaborate model incorporating the volume of the growing intracellular parasite and the consumption by the parasite of the host cell cytoplasm as well as the (largely uncharacterized) changes brought about by the parasite on the physicochemical properties of the erythrocyte cytosol. Lew and Hockaday (36) have described a mathematical model that incorporates the volume of the growing intracellular parasite and the progressive consumption of the cytosol of the parasitized cell. This model predicts that the consumption of the erythrocyte cytosol results in a progressive decrease in the water volume of the infected cell. This would counteract the cell swelling predicted to occur as a result of the membrane transport changes described here (Fig. 7D).

The results of the present study provide an explanation for the dramatic elevation in [\( \text{Na}^+ \)] and decrease in [\( \text{K}^+ \)] shown previously to occur in the host cell compartment of the parasitized erythrocyte (15, 34). The induction in the host cell plasma membrane of NPP, which are permeable to both \( \text{Na}^+ \) and \( \text{K}^+ \), combined with impairment of the host cell Na+/K+ pump account for the observed reversal of the normal transmembrane [\( \text{Na}^+ \)] and [\( \text{K}^+ \)] gradients.

The NPP play an important role in the parasitized cell by facilitating the uptake of essential nutrients (29). They have been shown previously to be strongly anion selective, with a \( P_{\text{Cl}} \) some three orders of magnitude higher than their permeability to monovalent cations (8, 27). The results of the present study show that the cation permeabilities of the NPP are such that their induction causes a significant perturbation of the normal Na+ and K+ levels in the infected erythrocyte while not threatening the osmotic stability of the host cell until the last few hours of the parasite’s occupancy, if at all. Whether the altered [\( \text{Na}^+ \)] and [\( \text{K}^+ \)] composition of the erythrocyte compartment plays a physiological role in the parasitized cell is yet to be established.

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NA+/K+ TRANSPORT IN MALARIA-INFECTED ERYTHROCYTES