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Malaria parasites tolerate a broad range of ionic environments and do not require host cation remodeling

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Summary

Malaria parasites grow within erythrocytes, but are also free in host plasma between cycles of asexual replication. As a result, the parasite is exposed to fluctuating levels of Na^+ and K^+, ions assumed to serve important roles for the human pathogen, *Plasmodium falciparum*. We examined these assumptions and the parasite's ionic requirements by establishing continuous culture in novel sucrose-based media. With sucrose as the primary osmoticant and K^+ and Cl^- as the main extracellular ions, we obtained parasite growth and propagation at rates indistinguishable from those in physiological media. These conditions abolish long-known increases in intracellular Na^+ via parasite-induced channels, excluding a requirement for erythrocyte cation remodeling. We also dissected Na^+, K^+, and Cl^- requirements and found that unexpectedly low concentrations of each ion meet the parasite's demands. Surprisingly, growth was not adversely affected by up to 148 mM K^+, suggesting that low extracellular K^+ is not an essential trigger for erythrocyte invasion. At the same time, merozoite egress and invasion required a threshold ionic strength, suggesting critical electrostatic interactions between macromolecules at these stages. These findings provide insights into transmembrane signaling in malaria and reveal fundamental differences between host and parasite ionic requirements.

Keywords

Malaria parasites; invasion; transmembrane signaling; cation transport

Introduction

Malaria parasites are successful pathogens, infecting a broad range of vertebrates and causing disease in 200–500 million humans each year. This success depends, in part, on intracellular growth within host erythrocytes, which allows the parasite to evade host immune responses (Scherf, 2006) and access hemoglobin as a nutritive protein source (Gluzman *et al.*, 1994). The erythrocytic cycle has been extensively studied in *P. falciparum*, the most virulent human pathogen, because facile *in vitro* propagation using donor human erythrocytes has permitted the full array of modern research approaches.

Since the development of *in vitro* culture more than three decades ago (Trager and Jensen, 1976), the conditions used to propagate this parasite have not changed significantly. The

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universally accepted culture medium is a physiological NaCl-based saline supplemented with soluble nutrients and a lipid source. This medium is designed to simulate human serum, which contains approximately 140 mM Na^+, 4 mM K^+, and 100 mM Cl^-. Because serum and erythrocyte ionic compositions are conserved and tightly regulated in vertebrates susceptible to malaria, it has been tacitly assumed that the parasite has similar ionic needs. For example, merozoites encounters marked changes in external Na^+ and K^+ concentrations upon both invasion and egress from erythrocytes; the decrease in K^+ at the time of egress is thought to trigger merozoite maturation (Singh *et al.*, 2010), as also reported for other parasite stages and genera (Moudy *et al.*, 2001; Kumar *et al.*, 2007).

Intracellular parasite growth is also associated with erythrocyte ionic remodeling. While uninfected human erythrocytes maintain transmembrane Na^+ and K^+ gradients through the action of the Na^+/K^+ ATPase pump (Jorgensen *et al.*, 2003), infection produces a marked increase in erythrocyte Na^+ content and a parallel decrease in K^+ (Overman, 1948; Ginsburg *et al.*, 1986; Lee *et al.*, 1988). These changes result from increased ion permeabilities at the host membrane, as mediated by the plasmodial surface anion channel (PSAC) with possible contributions from altered host transporters (Desai *et al.*, 2000; Nguitragool *et al.*, 2011; Staines *et al.*, 2007). They are presumed to benefit the parasite through several mechanisms. First, increased Na^+ in host cytosol creates a gradient across the parasite plasma membrane that may be used by coupled transporters to drive uptake and efflux of other solutes. A Na^+/H^+ exchanger, proposed for the parasite plasma membrane (Bosia *et al.*, 1993; Bennett *et al.*, 2007), could facilitate extrusion of metabolic acid. A Na^+-phosphate cotransporter has also been proposed (Saliba *et al.*, 2006); coupling to Na^+ may allow phosphate import to levels greater than possible with uncoupled passive uptake. Second, the increasing intracellular Na^+ concentration may play a role in parasite egress from the erythrocyte by promoting osmotic lysis of infected cells (Staines *et al.*, 2001), though further modeling suggests that a lytic threshold is not reached within the required timeframe (Mauritz *et al.*, 2009). Finally, these changes in Na^+ and K^+ concentrations may contribute to the membrane potential of the parasite; although a V-type H^+ pump is thought to be the primary determinant of the parasite's membrane potential (Allen and Kirk, 2004), changes in Na^+ and K^+ may also influence the membrane potential through electrogenic transport on coupled transporters or other mechanisms. Consistent with these predictions, workers have, to date, been unable to cultivate *P. falciparum* in media having reduced Na^+ (Brand *et al.*, 2003), but have reported that accentuated changes in erythrocyte Na^+ and K^+ content do not interfere with parasite growth (Tanabe *et al.*, 1986).

We now report continuous *P. falciparum* cultivation in sucrose-based media. Our studies exclude a physiological role for cation remodeling in erythrocyte cytosol after infection. They also reveal a surprising parasite tolerance to variations in extracellular Na^+, K^+, and Cl^-, provide new insights into parasite ion utilization, and suggest targets for chemotherapeutic intervention.

Results

Continuous P. falciparum growth in sucrose-based media

Entry of Na^+ into infected cells is passive and therefore requires extracellular Na^+ concentrations above electrochemical equilibrium. The standard medium used for *P. falciparum* culture, RPMI 1640 supplemented with human serum, contains ~140 mM Na^+ and fulfills this requirement (Tables S1–S3). In this medium or under *in vivo* conditions, the large inward Na^+ gradient allows passive uptake and yields an increased erythrocyte Na^+ (Overman, 1948; Ginsburg *et al.*, 1986; Lee *et al.*, 1988). To examine whether Na^+ uptake serves an essential role for the parasite, we surveyed impermeant monovalent cations that could replace Na^+ and abolish the gradient. We designed and prepared media that follow

RPMI 1640, but substituted NaCl, $NaHCO_3$, and Na_2HPO_4 with the corresponding salts of other cations. Replacement with either Li^+ or N-methyl-d-glucamine$^+$ salts failed to support parasite growth, but these ions may be toxic in continuous culture. Because K^+ is the predominant cation in erythrocyte cytosol, we reasoned it cannot be directly toxic to the parasite. Nevertheless, equimolar substitution of Na^+ with K^+ also failed to support even short-term parasite growth (full-K^+ medium, Tables S2–S3). This may reflect either an essential role for elevated host cytosolic [Na^+] or an indirect toxic effect of high K^+ concentrations. One mechanism of indirect toxicity is through osmotic lysis of infected cells in KCl-based media because K^+ has a higher PSAC permeability than Na^+ (Cohn *et al.*, 2003). Consistent with this possibility, we found that trophozoite-infected cells undergo osmotic lysis in the full-K^+ medium (red trace, Figure 1D; halftime of 92 ± 4 min, $n = 3$), as predicted by theory and previous experimental studies of Donnan equilibria in cation-permeable erythrocytes (Jacobs and Stewart, 1947; Freedman and Hoffman, 1979). This lysis was prevented by addition of 50 mM sucrose, an impermeant disaccharide that offsets the lytic effects of K^+ uptake (Freedman and Hoffman, 1979).

In light of this rescue from osmotic lysis, we partially replaced KCl in the full-K^+ medium with sucrose and found that various ratios of sucrose:KCl supported parasite growth (Fig. 1A). At a 4:6 mixture by calculated osmolarity, parasite growth was quantitatively identical to that in RPMI-based medium. In this medium (4suc:6KCl, Table S2), intracellular parasites were microscopically healthy, egressed from host cells, and invaded new erythrocytes with a developmental cycle indistinguishable from that in standard medium (Fig. 1B and C). This medium supported growth of each parasite line we tested (HB3, 3D7A, W2, Indo) without detectable delay upon transfer from standard medium. Expansion of naïve cultures without delay suggests that these parasites do not require adaptive changes to grow in 4suc:6KCl. There was also no loss of parasite viability after prolonged culture in 4suc:6KCl (> 10 weeks).

Figure 1A shows a strong dependence of parasite growth on mole fractions of sucrose and KCl, with growth failure when either constituent predominates. This bimodal response suggests a balance between opposing factors. Although osmotic lysis can account for growth failure in media with high K^+ mole fractions, supraphysiological K^+ concentrations may also be toxic to the parasite. We therefore supplemented the full-K^+ medium with 50 mM sucrose (Table S2). Although this medium is hypertonic (Table S3), parasite cultures are known to tolerate elevated osmolarities resulting from addition of sucrose (Ginsburg *et al.*, 1986). The supplemented medium supported continuous *in vitro* growth and expansion of parasite cultures, in contrast to complete sterilization within 3 days in the full-K^+ medium. Because sucrose is impermeant and cannot be utilized by the parasite, restored growth upon its addition strongly supports PSAC-mediated osmotic lysis as the primary mechanism of growth failure in full-K^+ medium. Successful propagation also indicates that extracellular K^+ levels up to 148 mM are tolerated by the parasite under these *in vitro* culture conditions.

Figure 1A reveals that media containing predominantly sucrose fail to support parasite growth. The possible mechanisms are examined in later sections.

Erythrocyte cation remodeling is unnecessary

The Na^+ present in 4suc:6KCl (measured at 6.8 mM, Fig. 2A) was primarily contributed by serum, added as a lipid source for parasite growth. This markedly reduced value is below electrochemical equilibrium relative to the intracellular value of ~ 11 mM reported for uninfected erythrocytes (Beilin *et al.*, 1966). This outward gradient should prevent Na^+ uptake and may produce net efflux from infected cells.

We therefore measured Na^+ and K^+ contents in uninfected erythrocytes and in the host cytosol of infected cells. Host cell cytosol was selectively released with saponin, a detergent that permeabilizes the erythrocyte but not the intracellular parasite (Hsiao *et al.*, 1991). Saponin also releases the contents of the parasitophorous vacuole surrounding the parasite; the effect on measured ion concentrations will be small because this vacuole has a negligible volume and an ionic composition similar to that of host erythrocyte cytosol due to nonselective ion channels on the vacuolar membrane (Desai *et al.*, 1993). After correction for the parasite volume, these measurements confirmed the dramatic increase in intracellular Na^+ reported for infected cells cultured in standard medium and revealed that cultivation in 4suc:6KCl abolishes these changes (Fig. 2B), consistent with elimination of the inward Na^+ gradient.

Host erythrocyte K^+ content was also significantly determined by extracellular availability. While growth in standard medium produced a precipitous reduction in cytosolic K^+, we found that increasing extracellular K^+ concentration preserved or even augmented the high erythrocyte K^+ content (Fig. 2C). Notably, our measurements of erythrocyte Na^+ and K^+ concentrations in standard medium are in quantitative agreement with recent estimates obtained using x-ray microanalysis (Mauritz *et al.*, 2011), allaying concerns of significant contamination by parasite contents.

These measurements establish the relationship between extracellular and cytosolic cation concentrations, as predicted by electrochemical gradient considerations. Thus, passive uptake at the host membrane accounts for the long-known changes in infected erythrocyte $[Na^+]$ and $[K^+]$ occurring under physiological conditions. Unabated growth in 4suc:6KCl reveals that this cation remodeling is not essential for parasite development.

A requirement for low external Na^+

We next explored whether the parasite can tolerate further decreases in external Na^+ concentration. Dialysis of human serum removed Na^+, allowing preparation of a supplemented 4suc:6KCl medium with a measured $[Na^+]_o$ of 0.57 mM. This medium could not support parasite growth (Fig. 3A, $n > 6$ attempts). Addition of NaCl restored parasite growth with an EC_{50} of 2.9 ± 0.5 mM $[Na^+]_o$, excluding loss of other essential factors during dialysis and implicating a parasite requirement for low millimolar Na^+ concentrations.

In vitro culture without Na^+ supplementation for only 24 h revealed reproducible morphological changes in Giemsa-stained smears (Fig. 3B). These parasites exhibited central clearing that resembled engorgement of the parasite digestive vacuole (DV) seen after exposure to protease inhibitors (Rosenthal *et al.*, 1991). To explore a possible link between Na^+ depletion and defective DV function, we examined formation of hemozoin, a byproduct of hemoglobin digestion within the DV (Hempelmann, 2007), and found a marked reduction upon culture without Na^+ (Fig. 3C and D, EC_{50} = 1.2 mM $[Na^+]_o$).

To examine ultrastructural changes associated with Na^+ depletion, we next performed transmission electron microscopy on trophozoite-stage parasites. Parasite morphology and hemozoin formation appeared normal for cells cultivated in 4suc:6KCl with dialyzed serum when the medium was supplemented with 7 mM Na^+ (Fig. 3E, left panel). Cultivation without Na^+ supplementation revealed abnormalities that extended the above findings. After only 24 h, these cells exhibited not only engorged digestive vacuoles and reduced hemozoin production (Fig. 3E, right panel), but also appeared to have compromised digestive vacuolar integrity. The parasite nuclei also contained numerous punctate electron densities suggestive of chromatin condensation (black arrows); these densities were never seen in the Na^+-supplemented cells. This pattern of observations resembles those seen in apoptotic cells

from higher organisms (Zhivotovsky and Kroemer, 2004), and now also recognized in some protozoan parasites (Reece *et al.*, 2011).

Physiological increases in erythrocyte [Na^+] do not facilitate phosphate acquisition and utilization

The increased erythrocyte $[Na^+]_i$ observed under physiological conditions may benefit the parasite by creating an inward Na^+ gradient at the parasite plasma membrane. Such a gradient could be used to transport required solutes on Na^+-coupled transporters. We tested the proposal of facilitated uptake of inorganic phosphate (P_i) on a 2:1 Na^+:P_i cotransporter known as PfPiT (Saliba *et al.*, 2006). With such a coupled transporter, cultivation in 4suc:6KCl would reduce parasite P_i uptake because the Na^+ gradient at the parasite plasma membrane has been abolished. Based on the measured reduction in host cytosolic $[Na^+]$ (Fig. 2B) and requirements for coupled transport (Turner, 1985), steady-state P_i accumulation would be reduced by 28-fold if the proposed cotransporter is the primary uptake mechanism for this nutrient. This reduction is based on thermodynamic considerations and does not depend on the relative rates of uptake and subsequent P_i utilization. Reduced P_i accumulation may adequately sustain parasite growth in media containing high P_i concentrations (5.6 mM, Table S1). However, when the external P_i concentration is reduced to threshold levels required for parasite survival, its acquisition via a Na^+-coupled cotransporter would depend critically on an inward Na^+ gradient at the parasite plasma membrane. We therefore compared the dose response for extracellular P_i requirement in 4suc:6KCl medium and in RPMI (Fig. 4). These experiments confirmed a need for external phosphate and identified limiting concentrations. In contrast to the prediction of an increased requirement for external P_i when erythrocyte $[Na^+]_i$ is reduced, the dose responses were identical in these two media (EC_{50} = 0.6 ± 0.2 mM, P = 0.3 for no measurable difference). Because the intracellular sites and rates of P_i utilization are unknown and may be affected by changes in P_i availability, our studies cannot exclude the proposed cotransporter. However, they do indicate that the parasite's demands for P_i are fulfilled equally well without increases in erythrocyte $[Na^+]_i$.

External K^+ is also required

Having determined that the parasite tolerates both reduced Na^+ and increased K^+, we next examined *in vitro* cultivation with reduced extracellular $[K^+]$. Because the erythrocyte contains K^+ levels greater than those in either human plasma or standard medium (Fig. 2B, Tables S2–S3), we initially predicted parasites could obtain required K^+ from erythrocyte stores and be propagated in K^+-free medium. Nevertheless, an NaCl-based medium prepared without use of K^+ salts and supplemented with dialyzed serum was unable to support parasite propagation (Fig. 5A; zeroK medium, Tables S2–S3; measured extracellular $[K^+]$ < 60 μM). Addition of 5 mM KCl restored growth, excluding loss of other factors upon dialysis of serum. Dose response experiments revealed an EC_{50} of 0.17 mM external K^+ for parasite growth over 72 h (Fig. 5B). This estimate was, however, confounded by the large K^+ stores within erythrocytes: extracellular K^+ concentrations may be raised by either efflux from cells or by low-level hemolysis in cultures.

A nontoxic organic anion quantifies external Cl^- requirement

In light of the malaria parasite's remarkable tolerance to changes in monovalent cation concentrations, we wondered whether Cl^-, the predominant physiological anion, serves an essential role in parasite biology. Unabated growth in the 4suc:6KCl medium indicates that significant reductions in extracellular Cl^- concentration are tolerated (measured $[Cl^-]$ after serum supplementation, 73 mM), but compromised growth with KCl mole fractions less than 0.5 may reflect a Cl^- requirement (Fig. 1A). To obtain more direct evidence, we sought to reduce the Cl^- concentration in 4suc:6KCl without changing the K^+ or sucrose

concentrations. This can be achieved by substitution of Cl^- with a nontoxic, nonphysiological anion. We therefore surveyed toxicity of candidate anions through partial replacement of NaCl in standard media with salts of candidate anions. Because these media provide sufficient Na^+, K^+, and Cl^- to support continuous *in vitro* propagation, growth in these substitution experiments would indicate that the candidate anion is nontoxic. These surveys revealed that gluconate$^-$ is nontoxic at concentrations up to 65 mM (NaCl:KGluc in Table S2, Fig. 6A; $P = 0.16$ for a growth difference relative to standard media over 5 days, paired Student's *t* test using 4 trials).

We then examined parasite growth at a range of Cl^- concentrations below those in the 4suc:6KCl medium using isomolar substitution of KCl with K-gluconate; this approach maintains constant solution osmolarity, fixed concentrations of all other constituents, and is not confounded by toxicity. These experiments revealed a parasite requirement for external Cl^- with an EC_{50} of 31 ± 3.3 mM (Fig. 6B, $n = 6$). The graded nearly linear dependence we observed suggests Cl^- serves multiple roles in parasite propagation. Consistent with this, studies using synchronous ring- or trophozoite-stage cultures in Cl^- deficient media suggested detrimental effects at each stage (not shown).

An ionic strength requirement for merozoite egress and invasion

Although an inadequate supply of Cl^- partially accounts for growth failure in sucrose:KCl mixtures with high sucrose mole fractions (Fig. 1A), the relatively low Cl^- EC_{50} value suggested that there may be additional determinants of parasite survival and replication. For example, a medium containing a KCl mole fraction of 0.3 provides Cl^- at a level near the EC_{50} (7suc:3KCl, 32.6 mM in Table S3), but does not support parasite growth (Fig. 1A, blue circle). We therefore compared expansion of cultures in this medium to one with sucrose partially replaced with the nonphysiological salt, K-gluconate (4suc:3KCl:3Kgluc, Table S2). These two media have identical Na^+ and Cl^- concentrations, physiological osmolarities, K^+ at levels within parasite tolerances, and the full complement of essential organic solutes (Table S3). Nevertheless, we found that 4suc:3KCl:3Kgluc allowed expansion of parasite cultures whereas 7suc:3KCl could not support growth (Fig. 7A).

This result suggests that, in addition to the defined requirements for individual ions, *in vitro* parasite growth also depends on a minimum ionic strength. Ionic strength, calculated as the weighted sum of the concentrations of ions in a solution, is important in biology because the functions of many proteins and other macromolecules depend on solvation and electrostatic interactions with other cellular components (Spitzer and Poolman, 2009). Notably, 4suc:3KCl:3Kgluc has an ionic strength identical to that of 4suc:6KCl, but the value for 7suc:3KCl medium is significantly lower (Table S3).

We therefore examined ionic strength effects on growth of synchronous parasite cultures with the 4suc:3KCl:3Kgluc and 7suc:3KCl media. Ring-stage infected erythrocytes matured to schizonts equally well in these media when examined over a single parasite cycle (not shown), consistent with the expectation that reduced ionic strength in the extracellular space alone should not affect electrostatic interactions between macromolecules within parasite compartments. Instead, developmental events that require direct exposure to the external medium, such as merozoite egress and host cell invasion, may account for better propagation of cultures in 4suc:3KCl:3Kgluc. Similar reasoning has motivated studies showing that direct parasite exposure to ions such as K^+ influence merozoite activation in plasmodia and toxoplasma (Singh *et al.*, 2010; Moudy *et al.*, 2001).

To explore possible effects of K^+ and solution ionic strength on extraerythrocytic parasite forms, we first harvested late-stage schizont infected cells from standard medium and examined progression into the next host cell cycle after transfer to engineered media (Fig.

7B). After an 18 h incubation, measured ring-stage parasitemias revealed that cycle progression is not significantly affected by changes in external [K^+] varying over the range of undetectably low to 148 mM (zeroK and full-K + 50 mM sucrose media, respectively; $P = 0.27$). Progression was however abolished in an isotonic sucrose-based medium with a physiological K^+ concentration and the lowest achievable ionic strength without compromising HCO_3^- and other nutritive ions (sucrose medium + 5.4 mM K, Tables S2 and S3; $P = 10^{-4}$). Because reduced progression may result from effects on either merozoite egress and activation or the subsequent invasion of a new erythrocyte, we next examined these individual steps. Merozoite egress decreased monotonically with ionic strength (Fig. 7C). In contrast, changes in external K^+ concentrations over the range achievable with our media did not adversely affect egress, as long as a physiological ionic strength was preserved (Fig. 7C, zeroK media and full-K + 50 mM sucrose). Erythrocyte invasion, examined using mechanically released invasive merozoites (Srinivasan *et al.*, 2011), was also not affected by supraphysiological K^+ concentrations, but decreased as ionic strength was reduced (Fig. 7D). Confocal video microscopy revealed aborted interactions between merozoites and erythrocytes in the 7suc:3KCl medium (Videos S1 and S2, showing comparison to standard RPMI 1640 medium), further supporting reduced invasion efficiency. Reduced ionic strength therefore interferes with parasite growth via compounded effects on merozoite egress and invasion of new erythrocytes.

Discussion

Although Na^+ and K^+ share many physicochemical properties, most cells readily distinguish these cations. Animals and some other eukaryotes use a conserved Na^+/K^+ ATPase pump to establish opposing transmembrane gradients for these ions (Jorgensen *et al.*, 2003); these gradients store energy that can be used to generate transmembrane potentials, drive co- and countertransport of solutes, and fine-tune enzymatic activities in controlled ionic environments. Although the *P. falciparum* genome lacks a clear ortholog of known Na^+/K^+ ATPases, it has many genes for P-type ATPases that could function as Na^+ and/or K^+ pumps (Thever and Saier, Jr., 2009; Martin *et al.*, 2009). Unfortunately, the precise function of these putative transporters cannot be determined through computational analysis alone because the determinants of solute selectivity within this superfamily are poorly understood (Sanchez and Blanco, 2004). For this and other reasons, it has been tacitly assumed that malaria parasites recognize and use these ions in ways resembling those of their vertebrate hosts (Ginsburg *et al.*, 1986; Lee *et al.*, 1988; Staines *et al.*, 2001; Brand *et al.*, 2003; Singh *et al.*, 2010). In contrast to these expectations, our studies reveal that the parasite tolerates a surprisingly broad range of Na^+, K^+, and Cl^- concentrations. Broad parasite tolerance to changes in extracellular H^+ has also been recently demonstrated (Lyko *et al.*, 2012).

Malaria parasite infection is associated with increased Na^+ and decreased K^+ concentrations in erythrocyte cytosol (Fig. 2; Overman, 1948; Ginsburg *et al.*, 1986; Lee *et al.*, 1988). These changes result primarily from passive cation flux through PSAC (Desai *et al.*, 2000), but there may also be contributions from activated host channels and reduced Na^+/K^+ pump activity (Staines *et al.*, 2001; Staines *et al.*, 2007). Stable PSAC mutants, linkage analysis, and DNA transfection studies support an unusual ion channel determined by parasite *clag3* genes (Hill *et al.*, 2007; Lisk *et al.*, 2008; Nguitragool *et al.*, 2011). Parasite cultivation studies using reduced concentrations of key nutrients have also implicated the *clag3* genes and determined that PSAC serves an essential role in parasite nutrient acquisition (Pillai *et al.*, 2012).

Increased Na^+ and decreased K^+ in erythrocyte cytosol, though well-established, are dispensable because *in vitro* propagation is undeterred by conditions that abolish both of these changes. These findings conflict with the conclusions reached by another study that

also used modified culture conditions (Brand *et al.*, 2003). There, the effects of 8–16 hour exposures to buffered salt and sugar solutions on short-term parasite growth were examined. Because replacement of Na^+ with either N-methyl-D-glucamine ($NMDG^+$) or K^+ adversely affected expansion of cultures at the 48 h timepoint, the authors concluded that Na^+ uptake at the host membrane is required for parasite survival. Comparison to our conditions for successful cultivation reveals several differences that may account for decreased viability in their study. Maybe most importantly, osmotic lysis of infected cells in K^+ solutions, as occurs with media lacking impermeant solutes such as Na^+ or sucrose (Fig. 1D), was apparently not considered; such lysis would contribute to overestimation of parasite Na^+ requirement. Na^+ replacement with $NMDG^+$ may also produce osmotic lysis of infected cells, depending on this substitute cation's PSAC permeability. Another experimental difference—their use of buffered solutions prepared without amino acids, vitamins, or a lipid source (Table S1 and dialyzed human serum in our media)—may also have contributed to decreased viability because these constituents are essential for parasite cultivation (Divo *et al.*, 1985; Istvan *et al.*, 2011). Finally, removal of HCO_3^-, also absent from their solutions, may have yielded acute pH changes in parasite compartments due to CO_2 redistribution across membranes.

Parasite growth without erythrocyte cation remodeling challenges presumed roles for coupled transporters at the parasite plasma membrane. Increased host erythrocyte Na^+, as observed under physiological *in vitro* and *in vivo* conditions, creates an inward Na^+ gradient at the parasite plasma membrane because the intervening parasitophorous vacuolar membrane is freely permeable to ions (Desai *et al.*, 1993; Desai and Rosenberg, 1997). The Na^+ gradient at the parasite plasma membrane can, in principle, be used to facilitate transport of other solutes on coupled transporters. In addition to the proposed Na^+-phosphate cotransporter we considered (Fig. 4), a Na^+/H^+ exchanger has been proposed to utilize the increased erythrocyte $[Na^+]_i$ for metabolic acid extrusion (Bosia *et al.*, 1993; Wunsch *et al.*, 1998; Bennett *et al.*, 2007). Because 4suc:6KCl aborts formation of a large Na^+ gradient at the parasite plasma membrane, our studies provide evidence against such facilitated acid extrusion. Instead, an H^+ ATPase pump seems to be more likely (Mikkelsen *et al.*, 1986; Spillman *et al.*, 2008).

Our findings also have implications for other actively-studied aspects of host-parasite interactions, especially those related to mechanisms for parasite egress and invasion of erythrocytes. It has been unclear whether parasite egress depends on an osmotic component of host erythrocyte rupture. The ongoing Na^+ uptake by infected cells under physiological conditions creates an osmotic load, producing cell swelling and eventual host cell lysis. Mathematical modeling has suggested that this osmotic lysis may be the final trigger for host cell rupture and parasite egress (Staines *et al.*, 2001). However, a more advanced model that incorporates the effects of hemoglobin digestion predicts that a lytic threshold would not be reached by the end of the intracellular cycle (Mauritz *et al.*, 2009). Our study resolves this uncertainty with direct experimental evidence. Normal progression of the parasite cycle and unaltered growth rates in the 4suc:6KCl medium excludes an osmotic trigger for host cell rupture: this medium abolishes the osmotic load by preventing Na^+ uptake and further prevents cell swelling through inclusion of sucrose, which remains impermeant at the host membrane (Ginsburg *et al.*, 1985). An explosive membrane rupture event driven by specific parasite enzymes seems to be more likely (Glushakova *et al.*, 2005; Yeoh *et al.*, 2007; Arastu-Kapur *et al.*, 2008; Dvorin *et al.*, 2010).

Shortly after rupture, the released merozoites must expose specific protein ligands that then interact with erythrocyte surface receptors to mediate invasion of new host cells. This ligand exposure is thought to be triggered by a decrease in external K^+ concentration at the merozoite surface upon host cell rupture (Singh *et al.*, 2010). Merozoites subjected to

decreased external K^+ exhibit both phospholipase C activation and Ca^{2+} mobilization, consistent with the proposed trigger. One caveat is that the threshold K^+ concentration for merozoite activation would need to be below the reduced K^+ concentration in infected erythrocyte cytosol (Fig. 2C, estimated at < 30 mM in Mauritz *et al.*, 2011; Lee *et al.*, 1988) to avoid merozoite activation prior to egress; it would also need to be higher than normal human plasma K^+ concentrations to ensure faithful triggering. The threshold K^+ concentration estimated for activation of *Toxoplasma gondii* merozoites, 80 mM (Moudy *et al.*, 2001), is too high to be suitable for plasmodia. The precise contribution of K^+ in plasmodial merozoite maturation and invasion should be reevaluated in light of our studies, which demonstrate continuous cultivation and the requisite completion of merozoite invasion with a broad range of external K^+ concentrations (1 to 148 mM). One possibility is that a drop in external K^+ may be only one of several triggers for merozoite activation, a scenario that would parallel known redundancies in invasion ligands (Jiang *et al.*, 2011). Abscisic acid may be one such alternative trigger because it has been shown to mobilize Ca^{++} in *T. gondii* (Nagamune *et al.*, 2008). Use of multiple triggers and ligands during invasion is appealing because it may maximize the likelihood of successful parasite replication.

Our studies reveal previously unknown effects of extracellular ionic strength on both merozoite egress and host cell invasion. Ionic strength is a derived parameter that quantifies electrostatic contributions to intermolecular interactions. Because egress and invasion were adversely affected by reductions in ionic strength (7suc:3KCl and sucrose medium + 5.4 mM K, Fig. 7; Table S3), we propose charged domains on proteins or lipids that are critical for these events under physiological conditions. Intermolecular interactions that occur in direct contact with the extracellular medium will be most susceptible. Multiple protein-protein interactions are required for invasion, including RON2-AMA1 (Lamarque *et al.*, 2011; Srinivasan *et al.*, 2011), the MSP1 complex and its processing by subtilisin proteases (Holder, 2009), and basigin-PfRH5 (Crosnier *et al.*, 2011). Compromised interactions between these or other macromolecular complexes may conservatively account for the observed ionic strength effect; a better understanding of these interactions may lead to the development of merozoite-specific therapies.

We propose that Na^+ uptake at the host membrane is a nonessential by-product of channels required for nutrient acquisition and possibly other purposes (Pillai *et al.*, 2012). Then, PSAC's broad selectivity profile may have been acquired through strong pressure for uptake of nutrients of varying size and charge. There must also have been selection for sufficiently low Na^+ permeability to prevent osmotic lysis of infected cells in host plasma. When combined with possible salutary effects of excess hemoglobin digestion (Mauritz *et al.*, 2009), this requirement is adequately met by the observed Na^+ permeability, estimated at $10^{-3.5}$–10^{-5} relative to that of Cl^-. Consistent with these unique evolutionary pressures, we are unaware of another well-characterized ion channel that combines broad solute selectivity with similarly stringent exclusion for a single small monovalent ion. PSAC apparently achieves its unparalleled selectivity profile through complex, incompletely understood mechanisms that recognize and distinguish between permeating solutes (Cohn *et al.*, 2003). Greater exclusion of Na^+ would prevent changes in erythrocyte ionic composition under physiological conditions, but our studies indicate that the resulting benefit to the parasite would be negligible.

Parasite growth was steeply dependent on the relative amounts of sucrose and KCl (Fig. 1A), with only intermediate ratios capable of supporting continuous growth. Growth failure at KCl mole fractions greater than 0.7 resulted from osmotic lysis of infected cells in these media (Fig. 1D); consistent with a primary effect of osmotic lysis, continuous parasite growth was restored in the full-K^+ medium upon isolated addition of sucrose at

concentrations known to prevent lysis due to the Donnan effect (Freedman and Hoffman, 1979; full-K^+ medium with 50 mM sucrose, Table S2). Under physiological conditions, infected erythrocytes avoid this lytic effect by maintaining a lower PSAC permeability to Na^+ than K^+ (Cohn *et al.*, 2003). Whereas increasing Na^+ permeability accelerates osmotic lysis, a higher K^+ permeability would yield infected cell shrinkage in host plasma due to an outward K^+ gradient at the erythrocyte membrane. Channel design considerations therefore favor greater K^+ permeability to avoid premature cell lysis, but also avoid excessive cell shrinkage by optimizing the relative permeabilities of these two cations. The multiple design constraints on PSAC may be partially relaxed by the parasite's tolerance to large changes in Na^+ and K^+ concentrations.

Although parasite cultures tolerate marked changes in external Na^+ and K^+ concentrations, we also found that *P. falciparum* requires both ions, albeit at levels well below those present in normal human plasma. Our experiments implicate distinct sites of requirement for these two ions, but additional studies will be needed to reveal the molecular targets and the corresponding roles of the ions in parasite physiology. Because uninfected erythrocytes have a high intracellular [K^+], we were surprised to find that parasite propagation requires extracellular K^+ (Fig. 5). Our attempts to examine the precise role and stage-specificity were complicated by the erythrocyte K^+ stores. Growth failure without external K^+ may therefore reflect a gradual depletion of erythrocyte and parasite stores, a K^+ requirement for extracellular merozoite forms that we could not discern with short-term egress and invasion studies, and possibly other mechanisms.

Growth failure in sucrose-based media with KCl mole fractions below 0.5 (Fig. 1A) resulted from a combination of at least two factors: reduced [Cl^-] and an effect of low ionic strength on merozoite egress and invasion (Figs. 6 and 7). By identifying gluconate$^-$ as a nontoxic substitute anion, we were able to isolate and quantify growth inhibition resulting from reduced [Cl^-]. In addition to a specific parasite need for Cl^-, reducing Cl^- may lead to indirect detrimental effects on *in vitro* cultures. One such mechanism may involve reversal of Cl^-/HCO_3^- exchange on the erythrocyte membrane anion exchanger. Cl^- efflux, resulting from the imposed outward gradient, may significantly raise erythrocyte cytosolic pH via coupled HCO_3^- uptake (Alper, 2009). Removal of external HCO_3^- and cultivation in a CO_2-free atmosphere should alleviate this pH change and determine if this mechanism contributes to parasite killing in reduced [Cl^-]. Unfortunately, our attempts to adapt parasite cultures to HCO_3^--free media have been unsuccessful.

The broad range of Na^+, K^+, Cl^-, and H^+ concentrations tolerated by malaria parasites is consistent with their close phylogenetic relationship to dinoflagellates (Keeling *et al.*, 2005). These free-living eukaryotes tolerate drastic changes in external ion composition, growing well in both fresh and sea water environments (Pistocchi *et al.*, 2011). Retaining their ancestors' tolerance to variations in ion concentrations is clearly beneficial to malaria parasites as they encounter large shifts in Na^+ and K^+ during their life cycle within vertebrate hosts. This tolerance should also assist the parasite in navigating diverse environments within the mosquito vector, where changes in fluid osmolarity, pH, and ion concentration are well-known (Kang'ethe *et al.*, 2007).

Despite this broad tolerance to changes in external composition, several new directions for therapeutic intervention against malaria are suggested by our findings. Rapid killing of the parasite when Na^+ is removed suggests mechanisms to fine-tune this ion's concentration with the intracellular parasite, possibly via regulated transporters on its plasma membrane. K^+ and Cl^-, also required at low concentrations, may be similarly controlled in intracellular and extracellular stages of bloodstream parasites. The novel media reported here may assist in identifying and characterizing specific inhibitors of these regulatory mechanisms.

Experimental Procedures

Parasite cultivation and design of new media

P. falciparum laboratory lines were cultivated by standard methods and maintained under 5% O_2, 5% CO_2, 90% N_2 at 37 °C. Transfer to engineered media utilized naïve parasite cultures after washing to prevent carryover.

To prepare low Na^+ media, we followed RPMI 1640 composition (Tables S1–S2), but replaced $NaHCO_3$ and Na_2HPO_4 with corresponding K^+ salts and substituted NaCl with identical concentrations of LiCl, NMDG-Cl, or indicated mixtures of KCl and sucrose. The pH of each medium was adjusted to 7.4 with KOH prior to addition of 5% human serum, which contributed nearly all of the measured Na^+. Exhaustive dialysis of serum, where used, was performed against 4suc:6KCl or distilled water as required; the molecular weight cutoff of the dialysis tubing was 3500 Da. Successful removal of cations after dialysis was confirmed with ion sensitive electrodes. Albumax II, a lipid-rich bovine serum albumin formulation, was found to have a significant residual Na^+ content and was therefore not used.

Phosphate dose responses were performed using media prepared with HPO^{-}_{4} salts at indicated concentrations. The K^+-free medium, zeroK, was prepared by replacement of KCl in RPMI 1640 with NaCl. Parasite cultivation with reduced extracellular Cl^- required a non-toxic replacement anion. To find a suitable anion, we surveyed $acetate^-$, $citrate^-$, $glutamate^-$, $gluconate^-$, F^-, $SO4^{-2}$, SCN^- and $NO3^-$ by equimolar substitution of 65 mM NaCl with K^+ or Na^+ salts; this concentration was chosen because it approximates the Cl^- concentration of 4suc:6KCl medium. Because 4suc:6KCl medium produces unabated parasite growth, lack of measurable toxicity at this concentration is a minimum requirement for anion substitution experiments designed to examine parasite Cl^- requirement. By this criterion, $gluconate^-$ was the only nontoxic anion; it was therefore also used in ionic strength experiments.

Growth assays

Expansion of synchronous parasite cultures was evaluated with Giemsa stained smears and SYBR Green I detection of parasite DNA; both methods producing similar results in all experiments. SYBR Green I measurements were carried out as described previously (Lisk *et al.*, 2008) with modifications. Sorbitol synchronized ring-stage parasites were seeded in 96-well plates at 0.5–1.0% parasitemia and 2% hematocrit in indicated media. Cultures were maintained at 37 °C for 72 h prior to addition of lysis buffer (20 mM Tris, 10 mM EDTA, 0.016% saponin, and 1.6% triton X100, pH 7.5) and SYBR Green I nucleic acid gel stain at a 5,000× dilution (Invitrogen). After incubation for 45 min in the dark, parasite nucleic acid production was quantified with fluorescence measurements (excitation/emission wavelengths of 485/528 nm).

Flow cytometry was used for quantifying merozoite invasion efficiency and daily changes in parasitemia on cultivation using engineered media; results were consistent with counts obtained by examination of smears. The method used incubation of cultures simultaneously with SYBR Green I nucleic acid stain (8000× dilution, Invitrogen; excitation/emission of 497/520 nm) and MitoTracker Deep Red (84 nM, Molecular Probes; excitation/emission of 644/665 nm) to identify viable intracellular parasites, as described previously (Ekland *et al.*, 2011). After a 30 min incubation at 37 °C in 96-well flat bottom plates at a 0.2% hematocrit, the cells were washed to remove unbound dye and resuspended in PBS before counting on an Accuri C6 flow cytometer (BD Biosciences).

Osmotic lysis experiments

Trophozoite-infected cells were harvested from cultures in standard media by the percoll/sorbitol method, washed, and resuspended at 0.1% hematocrit in sorbitol lysis solution (280 mM sorbitol, 20 mM Na-HEPES, 0.1 mg/mL BSA, pH 7.4) or in indicated parasite culture media. Transmittance of 700 nm light through these cell suspensions was then continuously recorded to track cell lysis, as described (Wagner *et al.*, 2003).

Ion content measurements

Na^+, K^+, and Cl^- sensitive electrodes (Orion) were used to measure final ion concentrations in engineered media, exclude contaminants in commercial reagents, and estimate erythrocyte cytosolic ion composition. Synchronous ring-stage cultures were cultivated in indicated media for 24 h, enriched by percoll-sorbitol, washed and returned to culture for 2 h. Cells were then counted with a hemocytometer and washed in 50 mM sucrose with either 150 mM NaCl or 150 mM KCl to remove extracellular K^+ or Na^+, respectively. Erythrocyte cytosol was selectively released into the same buffer with 0.3% saponin (Sigma Aldrich), a detergent that does not lyse the intracellular parasite. Na^+ and K^+ contaminants in saponin, as detected in control ion sensitive electrode measurements, were removed by dialysis against distilled water (MWCO of 3500 Da); the lowest saponin concentration required for host membrane permeabilization with minimal leakage from parasite compartments was determined using the dialyzed detergent. After a brief incubation (15–30 s), erythrocyte cytosol was harvested by centrifugation to remove the intracellular parasite (2300 × *g*, 4 min); ionic strength adjustor was added to the cytosol as recommended by the manufacturer. Standards were also prepared using ionic strength adjustor and revealed Nernstian responses for each electrode (52–56 mV/decade), indicating excellent sensitivity and specificity.

Ion content measurements were converted to concentrations based on the independently measured volume of erythrocyte cytosol within trophozoite-infected cells. Cultures were harvested and percoll-enriched 24 h after sorbitol synchronization to match conditions used in the above ion-sensitive electrode experiments. Hemoglobin released by saponin treatment was quantified using oxidation of 3, 3', 5, 5'-tetramethylbenzidine and used to estimate the volume of the host compartment of infected cells as described previously (Wagner *et al.*, 2003). The average volume of erythrocyte cytosol, 41 ± 3 fL/cell, was used for the uniform conversion of all content measurements to concentrations.

Hemozoin measurements

Insoluble hemozoin, a byproduct of parasite hemoglobin digestion, was measured as described previously (Lisk *et al.*, 2008). Briefly, sorbitol synchronized parasites were cultivated in indicated media for 30 h, harvested, lysed in ice-cold 5 mM Na_2HPO_4, pH 7.6. Matched cultures in RPMI 1640 medium without or with 20 μM chloroquine served as positive and negative controls for hemozoin production. Hemozoin was pelleted by centrifugation (27,000 × *g*, 30min), washed, and digested for 16 h at room temperature in 2 ml of 2.5% sodium dodecyl sulfate, 25 mM Tris, pH 7.8. The insoluble pellet was incubated in the same solution after addition of 0.1 N NaOH to release the ferriprotoporphyrin IX incorporated in beta-hematin. The pigment released was detected with absorbance wavelength scans and was quantified at 405 nm.

Transmission electron microscopy

Sorbitol synchronized cultures were cultivated in 4suc:6KCl medium supplemented with 10% dialyzed serum and either 0 or 7 mM NaCl for 30 h. The cells were washed in PBS with 0.1 g/L BSA prior to fixation at 4 °C for 1 h in the same medium with 2% paraformaldehyde and 2.5% glutaraldehyde. The cells were then postfixed for 1 h with 0.5%

osmium tetroxide-0.8% potassium ferricyanide, stained overnight with 1% uranyl acetate at 4°C, dehydrated with a graded ethanol series, and embedded in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome (Ventana), stained with 1% uranyl acetate and Reynold's lead citrate, and visualized at 80 kV on a Philips CM-10 transmission electron microscope (FEI Company). Images were acquired with a digital camera system (Advanced Microscopy Techniques).

Merozoite egress and erythrocyte invasion studies

Mature schizont-infected cells were grown in standard RPMI 1640 medium, purified by percollsorbitol centrifugation, and allowed to recover in RPMI 1640 for 30 min at 37 °C. To examine the effects of medium composition or ionic strength on merozoite egress, the harvested infected cells were suspended in engineered media for 4 h at 37 °C; free merozoites were then counted using a hemocytometer. RPMI 1640 supplemented with serum at the same concentration was used a positive control in each experiment. Control experiments revealed that 2 μM E64 abolished egress in the engineered media, confirming active egress of merozoites (not shown).

Invasive merozoites for erythrocyte invasion studies were prepared using a parasite line selected for prolonged merozoite viability, as previously described (Srinivasan *et al.*, 2011). Free merozoites were centrifuged, washed once in the respective media and resuspended. A matched control of freed merozoites that were identically washed with standard medium was included in each experiment. Pre-warmed human erythrocytes were added to these merozoites prior to incubation at 37 °C for 4 h. The efficiency of invasion was measured by flow cytometry of SYBR Green I-stained parasites and is presented after normalization relative to the control RPMI 1640 medium.

Experiments measuring the combined effects on egress and invasion used enriched schizont-infected cells incubated with prewarmed erythrocytes at 1% hematocrit and 2% parasitemia for 18 h in indicated media. Efficiency of rupture and invasion was measurement by counting the number of ring-stage infected cells by flow cytometry, as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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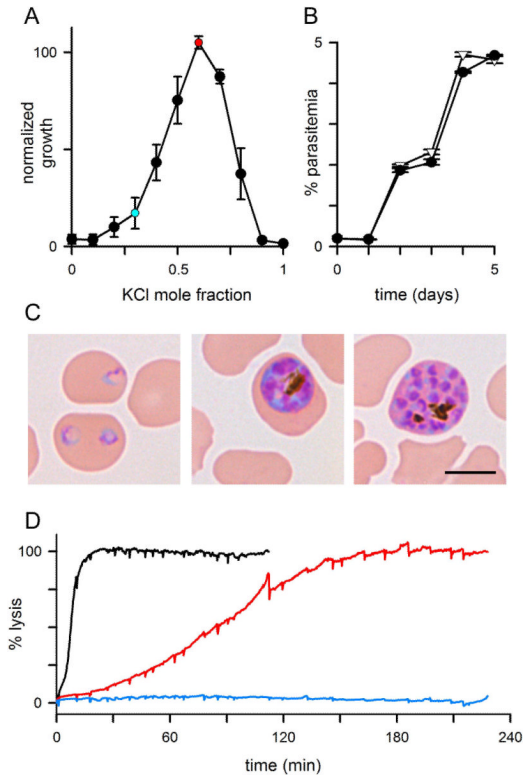

Fig. 1.

Parasite growth in low Na^+-media. (A) *P. falciparum* growth over 5 days in media prepared with sucrose and KCl. Abscissa shows increasing mole fractions of KCl and decreasing fractions of sucrose to achieve a constant total osmolarity. Each medium was supplemented with 5% serum; growth is normalized to 100% for a control culture in RPMI 1640 medium with serum. Symbols represent mean ± S.E.M. from 4 experiments. Red and blue circles represent 4suc:6KCl and 7suc:3KCl media, respectively. (B) Identical rates of growth and expansion in 4suc:6KCl and RPMI (circles and triangles, respectively), determined by flow cytometry ($n = 3$ replicates each). (C) Photomicrographs of Giemsa-stained ring-, trophozoite-, and schizont-stage parasites in sucrose:KCl medium reveal unchanged parasite morphology (left to right, respectively). Scale bar, 5 μm. (D) Osmotic lysis kinetics for trophozoite-infected cells resuspended in sorbitol lysis solution (black trace), full-K^+ medium with 5% serum (red), or full-K^+ with 5% serum and 50 mM sucrose (blue). Infected cells undergo osmotic lysis in the full-K^+ medium; lysis is prevented by addition of 50 mM sucrose. Lysis rates are inversely proportional to the PSAC permeabilities of sorbitol and K^+ (black and red traces, respectively).

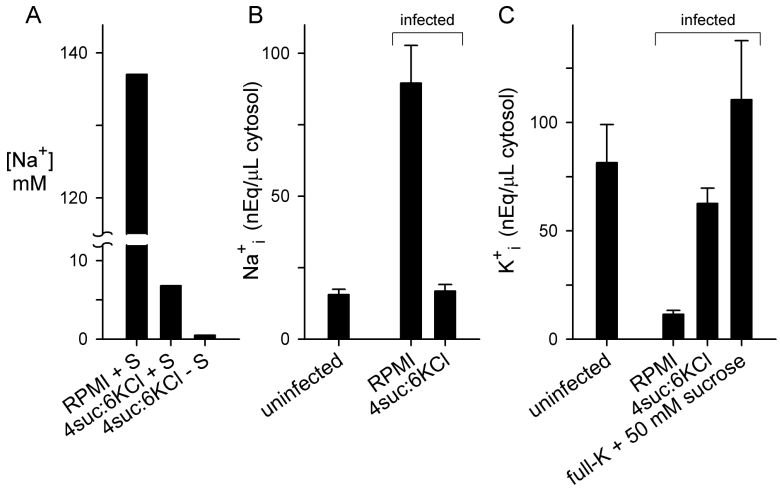

Fig. 2.

Effects on erythrocyte cytosolic ion contents. (A) Measured Na^+ concentrations in indicated media with or without 5% serum ("+ S" and "− S", respectively). (B) Erythrocyte cytosolic Na^+ content in uninfected erythrocytes or in enriched trophozoite-infected cells after culturing for 24 h in indicated media with 5% serum. (C) Erythrocyte cytosolic K^+ content in uninfected erythrocytes or in enriched trophozoite-infected cells after culturing for 24 h in indicated media with 5% serum.

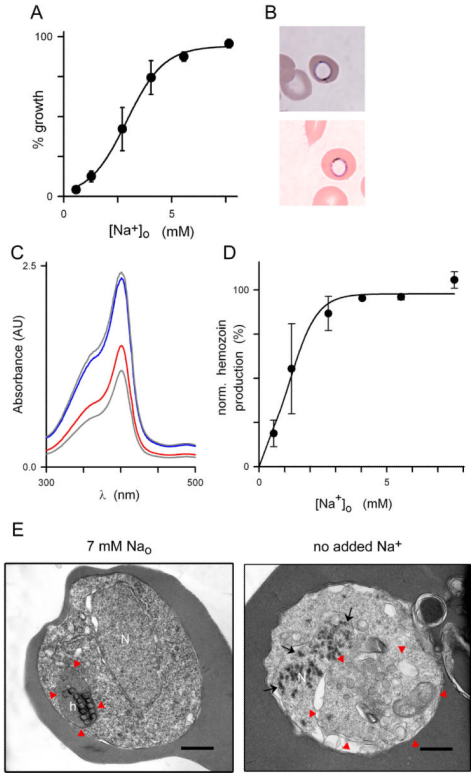

Fig. 3.

Low Na^+ concentrations are required. (A) Normalized parasite growth over 72 h in 4suc:6KCl with 10% dialyzed serum and indicated Na^+ concentrations, revealing a sigmoidal dose response for Na^+ requirement. Mean ± S.E.M. of 9 replicates from 3 experiments. (B) Photomicrograph of Giemsa-stained trophozoite-infected cells after 24 h cultivation in 4suc:6KCl with 10% dialyzed serum without Na^+ supplementation. Two parasites are shown with a rim of blue stain and central clearing. (C) Absorbance scan showing reduced β-hematin production in trophozoites cultivated in Na^+-deficient medium (red trace); supplementation with 7 mM Na^+ restores β-hematin production (blue trace), yielding levels similar to those observed with standard RPMI-based medium (upper gray trace). Positive control, 20 μM chloroquine (lower gray trace). AU, arbitrary units. (D) Mean ± S.E.M. β-hematin production vs. external Na^+ concentration, normalized to zero for control culture with 20 μM chloroquine. (E) Transmission electron micrographs showing trophozoite-infected erythrocytes cultivated in 4suc:6KCl with 10% dialyzed serum with or without Na^+ supplementation to a final 7 mM concentration (left and right panels, respectively). Red arrowheads demarcate the parasite digestive vacuole; black arrows show electron dense spots in nucleus when Na^+ is not added. N, nucleus; h, normal hemozoin crystals; scale bars, 0.5 μm. Images are representative of 89 cells from two separate experiments.

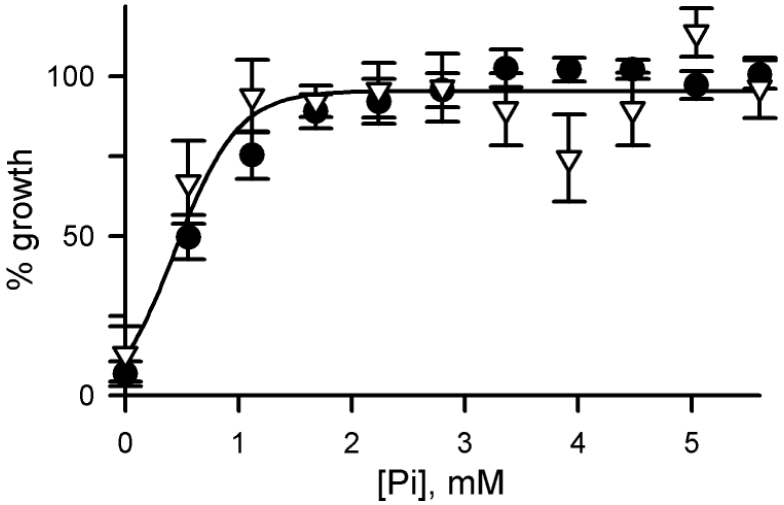

Fig. 4.

Increased cytosolic Na^+ does not promote phosphate acquisition. Phosphate dose responses for parasite growth over 5 days in RPMI vs. 4:6 sucrose:KCl with 5% serum (circles and triangles, respectively; mean of 3 measurements each). In contrast to the prediction for Na^+-coupled uptake, the phosphate EC_{50} is not increased when erythrocyte Na^+_i is reduced.

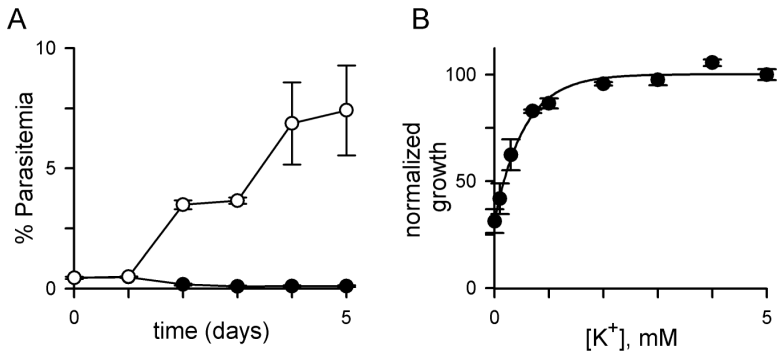

Fig. 5.

A requirement for low levels of external K^+. (A) Parasite growth in zeroK medium with 5% dialyzed serum with or without 5 mM KCl supplementation (white and black symbols, respectively; mean ± S.E.M of 4 trials), evaluated by microscopy of Giemsa-stained smears. (B) 72 h growth rate vs. external $[K^+]$ in the same medium, measured using SYBR Green I. Mean ± S.E.M. of replicates from 4 experiments.

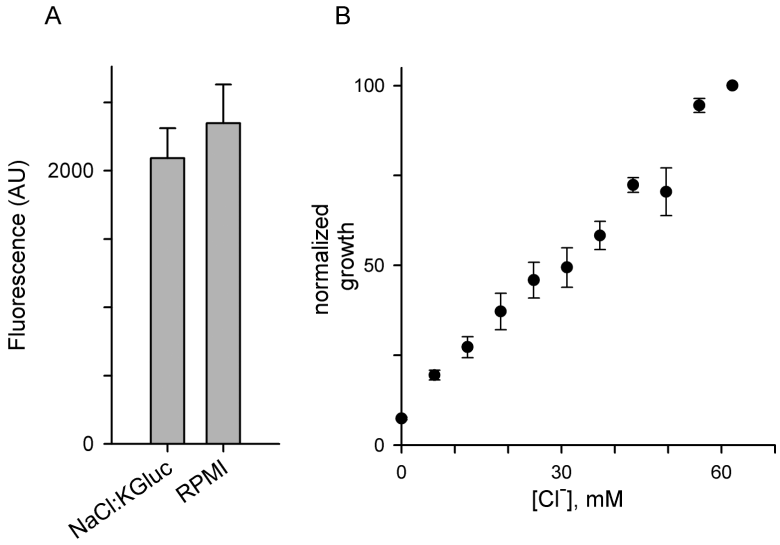

Fig. 6.

Gluconate^{-} substitution and a Cl^{-} requirement. (A) Matched parasite growth over 5 days in NaCl:KGluc in comparison to standard RPMI medium, each supplemented with 10% serum. Bars represent mean ± S.E.M. SYBR Green I fluorescence in arbitrary units. (B) 72 h parasite growth as a function of external [Cl^{-}] in 4suc:6KCl with 10% dialyzed serum. Reduced external Cl^{-} concentrations were achieved through isomolar substitution of KCl with K-gluconate. Mean ± S.E.M. of SYBR Green I fluorescence normalized to 100% for 4suc:6KCl (n = 6 trials).

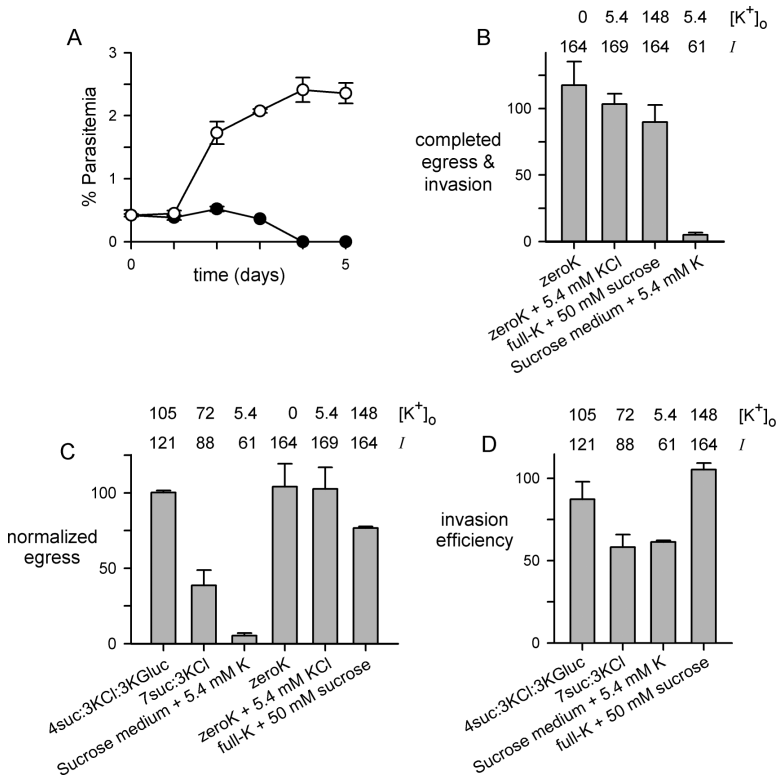

Fig. 7.

An ionic strength requirement for parasite cultivation. (A) Parasite growth in 4suc:3KCl: 3Kgluc and 7suc:3KCl media (white and black symbols, respectively; mean ± S.E.M. of 5 replicates from two trials). Both media were supplemented with 7% human serum, which provides sufficient Na^+ for cultivation. (B) Progression from late-stage schizonts to ring-stage infected erythrocytes in indicated media. (C) Number of merozoites released from late stage-schizont cultures over a 4 h incubation in each medium. (D) Erythrocyte invasion by mechanically freed merozoites resuspended in indicated media. In panels (B), (C), and (D), each bar represents the mean ± S.E.M. of up to 8 trials after normalization to 100% in matched controls using RPMI 1640 medium. The nominal K^+ concentration and calculated ionic strength (*I*) of each medium prior to addition of serum is listed above the bars. Two media, zeroK with or without added K^+, were supplemented with dialyzed serum to minimize contaminating K^+, but all other media used undialyzed serum to provide Na^+ at required levels.