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Why do malaria parasites increase host erythrocyte permeability?

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Abstract

Malaria parasites increase erythrocyte permeability to diverse solutes including anions, some cations, and organic solutes, as characterized with several independent methods. Over the last decade, patch-clamp studies have determined that the permeability results from one or more ion channels on the infected erythrocyte host membrane. However, the biological role(s) served by these channels, if any, remain controversial. Recent studies implicate the plasmodial surface anion channel (PSAC) and a role in parasite nutrient acquisition. A debated alternative role in remodeling host ion composition for the benefit of the parasite appears to be nonessential. Because both channel activity and the associated *clag3* genes are strictly conserved in malaria parasites, channel-mediated permeability is an attractive target for development of new therapies.

Keywords

host-pathogen interactions; erythrocyte remodeling; parasite ion channels; nutrient acquisition; intracellular parasitism; antimalarial drug discovery; *clag3*

Increased permeability of infected erythrocytes: background and mechanism

Ex vivo experiments using blood from monkeys first detected changes in erythrocyte ionic content after infection with malaria parasites some 65 years ago [1]; although that heroic study used outdated flame photometry methods, the findings were correctly interpreted as suggestive of increased erythrocyte permeability. This study also identified infection-associated changes in erythrocyte Na^+ and K^+ concentrations as discussed in detail below. After a hiatus of some 20 years, several groups took up next-level tracer flux and osmotic fragility measurements on cultured and *ex vivo* samples, confirming the above findings and adding to the evidence for increased permeability after infection [2–7]. These and other studies determined the range of solutes with parasite-induced uptake: sugars and sugar alcohols, amino acids, purines, organic cations, some vitamins, and inorganic monovalent

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ions to varying extents. Another advance was the identification of transport inhibitors of low to moderate affinity: when added at micromolar concentrations, phloridzin, furosemide, 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), and glybenclamide all blocked uptake of each of the above solutes [6,8,9]. Nevertheless, exactly how infected cells took up these solutes was unclear, with proposed mechanisms including pinocytosis, membranous ducts, nonspecific leaks at the site of merozoite invasion, and channels or transporters. Because the multiple membranes within infected cells could undergo dynamic changes, the precise site(s) of solute transport were also unclear [6].

At the turn of the last century, these uncertainties were addressed with a relatively new technology known as the patch-clamp method [10]. Patch-clamp actually refers to a collection of methods that capitalize on electrical currents associated with transmembrane ion movement to obtain unparalleled insights into the responsible transport mechanisms (Box 1). A consensus was quickly reached on two important issues as several groups used patch-clamp to determine that the transport activity localizes to the host erythrocyte membrane and that it is mediated by one or more ion channels [11–14]. At the same time, a number of new questions were raised: (i) how many distinct channel types are present on uninfected and infected cells; (ii) what are the responsible genes, and do they derive from the parasite, the host, or both; and (iii) what roles, if any, do the channels serve for the intracellular parasite? These questions led to divergent answers in various studies, as summarized by a National Institutes of Health (NIH) consensus workshop co-authored by the competing groups [15].

Box 1

Patch-clamp methods

Patch-clamp methods allow the dynamic tracking of capacitance and membrane resistance. Capacitance measurements are used to estimate membrane surface area and to follow membrane recycling events such as endo- and exocytosis in real time [82]. Membrane resistance quantifies how easily dissolved ions cross the membrane, as occurs typically through ion channels or carriers [83]. Both types of measurements utilize a pipette fabricated from borosilicate or quartz capillaries [84]. This pipette is filled with a solution containing the ions whose transport is being examined, connected to an electrical amplifier via a Ag:AgCl junction, and brought into contact with the biological membrane under study. With practice and luck, a poorly understood chemical reaction occurs between the pipette tip and the membrane to yield a “gigaohm seal”. With such a seal, the currents measured by the electrical amplifier correspond to the movement of ions across the patch of membrane delimited by the pipette tip.

There are several patch-clamp configurations [83], but infected erythrocytes have been primarily studied with the cell-attached and whole-cell configurations. In a cell-attached experiment, the currents flowing through one or more ion channel molecules in the membrane patch can be recorded with sub-millisecond resolution; the stochastic transitions between open and closed states of individual channel molecules can be studied with or without modulators or inhibitors, providing a wealth of molecular and biophysical information [81]. In the whole-cell configuration, the membrane patch

beneath the pipette is disrupted with electrical or mechanical pulses. When done properly, the gigaohm seal on the cell surface can remain intact; then, the amplifier records the total conductive permeability of the surface membrane, which may be the sum of currents flowing through channels, transporters, and leaks. Quantitative comparisons of single channel and whole-cell currents from infected erythrocytes suggest that PSAC is the primary channel on trophozoite-stage infected cells and that it is present at some 1000–2000 functional copies/cell [10]. Other groups, using somewhat different patch-clamp conditions, have observed various other channel types [15].

Critical assessment of these studies requires consideration of several experimental parameters. The quality of the seal between the pipette tip and the membrane, the “seal resistance”, is one of the most important because it is inversely proportional to the magnitude of leak currents that contaminate the biological signal; higher seal resistances are always better and can be routinely achieved through optimization of pipette tip geometry and use of fresh pipettes, healthy cells, and protein-free solutions. Other parameters that are important to assess and report include mechanical agitation of the cell (e.g. via perfusion, [85]), sources of stray currents (e.g. through improper grounding of equipment), the data acquisition rate and filter frequency, and reproducibility of channel events [84].

Recent findings that fall into two broad categories support a primary role for the plasmodial surface anion channel (PSAC), an unusual parasite-derived channel on the host membrane [10,16]. In the first category, mutant parasites with altered transport properties were generated by selections with blasticidin S and/or leupeptin, toxins that require channel-mediated uptake to reach their intracellular targets and kill parasite cultures (Figure 1A). These resistant mutants have significantly reduced permeability not only to these toxins, but to many other solutes [17,18]. Single channel patch-clamp of infected red cells implicated PSAC specifically as channels from these mutant parasites exhibited altered gating and conductance. *In vitro* growth inhibition determined that PSAC inhibitors reduce the antimalarial activities of both leupeptin and blasticidin S [19]. These findings provided strong evidence for uptake of both toxins and unrelated nutrient solutes via a single, shared channel.

In the second line of evidence, pharmacological studies have found that uptake of each solute is inhibited by any PSAC inhibitor, regardless of the chemical structures of either the permeating solute or the inhibitor [11,20–23]. One concern with this approach, that some inhibitors can act against multiple channel types [24], is partially mitigated when multiple inhibitors from distinct classes are available. With Ca^{++} and K^{+} channels, such pharmacological studies have provided important molecular insights [25,26]. To more definitively address this concern in the case of parasite-induced transport, chemical screens subsequently identified isolate-specific PSAC antagonists (ISPA) [16]. One such compound, ISPA-28 (Figure 1B), potently inhibits transport into cells infected with the Dd2 parasite line ($K_{0.5} = 56 \text{ nM}$), but is largely inactive against other laboratory lines such as HB3 ($K_{0.5} = 43 \text{ }\mu\text{M}$). This difference in activity suggests that ISPA-28 inhibits channels by interacting with a parasite protein that differs between these lines; many parasite proteins have slightly

different sequences in Dd2 and HB3 because these lines were obtained from patients in different parts of the world. Specific action against a single parasite addresses concerns of action against unrelated channels because erythrocytes infected with other parasite lines provide a negative control and confirm specificity of ISPA-28. Because differential ISPA-28 activity holds up with permeant solutes from multiple classes [16], these findings support a single shared transport mechanism. Studies with less specific inhibitors have also produced similar correlations suggestive of a shared route [9,22,27].

Both transport mutants and specific inhibitors have provided the molecular handles necessary to address the genetic basis of PSAC activity; both have implicated the *clag* genes of *Plasmodium* species with a special role served by two *clag3* paralogs on chromosome 3 [16,28,29]. These genes were initially thought to encode antigens involved in cytoadherence [30,31], but more recent studies suggest the encoded proteins localize to the host membrane and function in solute transport. In the first of these studies, ISPA-28 was used to track inheritance of PSAC block in the Dd2 x HB3 genetic cross. Linkage analysis using measurements of ISPA-28 channel block and the progeny of the cross identified the 5' end of the parasite chromosome 3 with high confidence. DNA transfection studies that included *piggyBac* complementation and allelic exchange along with independent gene switching studies provided convincing evidence for a role of the *clag3* genes.

Most parasite lines have two *clag3* genes (*clag3.1* and *clag3.2*); these two genes are under epigenetic control so that individual parasites in a culture express only one of the two genes [32]. The encoded proteins are almost identical, even amongst geographically divergent *P. falciparum* lines. However, there is a short polymorphic domain on the protein that differs between the two paralogs and amongst parasite lines (Figure 1C). Maybe, the most conclusive evidence for a single shared ion channel is that this variable domain appears to be the ISPA-28 binding site, based on DNA transfection experiments [16]; the longer, divergent sequence of this domain on the Dd2 CLAG3.1 protein likely accounts for the remarkable specificity of ISPA-28 (Figure 1C). Biochemical studies have determined that CLAG3 is integral to the host membrane and that the above polymorphic domain is exposed at the surface of the infected erythrocyte [16].

Molecular studies with PSAC mutants have also implicated the *clag* genes. A previously identified leupeptin-resistant PSAC mutant carries a non-synonymous mutation in a predicted transmembrane domain near the protein's C-terminus [16]. Although the precise effect of this mutation is presently unknown, its position is tantalizing because mutations within transmembrane domains can produce altered solute selectivity as already documented through functional studies of this mutant. A separate mutant, generated by selection with blasticidin S, did not have mutations in either the *clag* genes or other genomic loci [33]. Instead, this mutant uses epigenetic mechanisms to suppress both *clag3* genes. *Clag2*, a paralog on chromosome 2, was also silenced, providing the first evidence for a role of this gene in solute transport. This novel epigenetic mechanism of antimalarial resistance is consistent with rapid reversal of blasticidin S resistance upon removal of drug pressure, as reported previously [29,33].

Together, these molecular studies implicate a central role of the parasite-derived PSAC in transport of most solutes at the infected cell surface. The associated CLAG proteins, found using chemical screens and unbiased genetic approaches, have several unexpected features. They lack homology to ion channel proteins from other organisms and have fewer predicted transmembrane domains than usually seen in transporters. They also lack a motif termed PEXEL (plasmodium export element) or VTS (vacuolar transport signal), as implicated in the export of parasite proteins to host cytosol [34–36]. The absence of this motif is, however, not without precedent as several PEXEL-negative exported proteins have been characterized in recent years [37]. Finally, CLAG proteins already had proposed roles in cytoadherence, erythrocyte invasion, and protein trafficking [31,38,39], none of which are related to solute transport. These alternate roles have been examined mainly for the CLAG9 paralog encoded from chromosome 9 [30], but the similar gene transcription and protein trafficking profiles for members of this gene family had suggested that all CLAGs might serve related roles [40–44]. Hints to the contrary came from differences in selective pressures on individual genes, expansion of subgroups in certain plasmodial species, and differing patterns of transcriptional regulation for family members [16,29,32,33,45–47]. In addition to roles in transport for the *clag3* and *clag2* products, these alternate roles deserve continued study.

To further complicate molecular understanding, ion channels often have multiple protein subunits that come together to create a functional pore. Because these subunits may or may not have sequence homology with one another, we must consider unrelated proteins in formation of PSAC; these other subunits may be of either parasite or host origin [48]. Finally, host-derived channels with distinct functional properties have also been proposed [12–15,49]. Although transport inhibition studies with ISPA-28 suggest PSAC is the predominant channel activity induced after infection, contributions from unrelated ion channels cannot be formally excluded (Box 2).

Box 2

Host-derived channels

In contrast to the molecular evidence for PSAC and parasite *clag* genes, other patch-clamp studies have reported multiple distinct channels on both infected and uninfected cells. Based on similarities between channels detected on uninfected and infected cells, these studies have suggested that the parasite-induced permeability results from upregulation of human channels. CFTR, CLC-2, stretch-activated channels, benzodiazepine receptors, and aquaporin-9 have all been proposed as upregulated channels based on functional properties in patch-clamp, parasite culture in erythrocytes from donors with known channel gene polymorphisms, biochemical or pharmacological studies, or knock-out mice infected with *P. berghei* [12–14,49]. Although none of these proposed channels is normally permeant to all the organic solutes having infection-associated uptake, the parasite might alter the range of solutes that can be transported when it activates the putative host channel.

Host-derived channels have appealed to some workers because these channels do not require trafficking of parasite integral membrane proteins to the host membrane. Such

host channels may still be targets for antimalarial drug development because these channels appear to be quiescent on uninfected erythrocytes; transport inhibitors would therefore not interfere with normal host physiology. There are several experimental approaches that could yield more compelling evidence: (i) functional studies should include approaches that do not depend on patch-clamp technologies. Despite the power of patch-clamp, its use with human erythrocytes may be especially prone to selection bias and other artifacts. To address this concern, some studies have used a transmittance-based osmotic lysis assay to seek quantitative correlations with patch-clamp and execute high-throughput screens for specific inhibitors [21]. (ii) Unbiased molecular techniques should be used to support each proposed channel type. These may include DNA transfection of the parasite or erythroid precursor cells to alter the transport, or heterologous expression and demonstration of transport activities matching those seen on infected erythrocytes. (iii) Account for differences in transport observed with different parasite lines. Known transport mutants (e.g., the blasticidin S and leupeptin resistant mutants) and isolate-specific inhibitors (e.g., ISPA-28) can be used to examine changes in host channels. Such studies may reconcile the observations of various labs, provide important insights into host-parasite interactions, and unveil additional targets for antimalarial drug development.

Proposed roles for increased permeability

A second important question in this field has been whether increased erythrocyte permeability benefits the intracellular parasite and, if so, how? An unambiguous answer to this question is critical to determining whether the responsible channel(s) can be targeted for therapeutic intervention. A number of possible roles have been proposed and discussed at length, but experimental evidence has been difficult to obtain. Conservation of PSAC activity and the associated *clag3* genes in all examined *Plasmodium* spp. suggests that the channel is required for intracellular growth of malaria parasites [40,50]. A correlation between channel block and *in vitro* parasite growth inhibition, as found with a large family of PSAC inhibitors, also implies an essential role [21].

Nutrient acquisition

Many groups have suggested that increased permeability may benefit the intracellular parasite by facilitating uptake of essential nutrients at the host membrane [2,6,8,10,51]. This is an attractive hypothesis because host plasma has higher concentrations of many nutritive solutes than erythrocyte cytosol. Moreover, PSAC permeability has been documented for nearly all of the nutrients deemed essential for *Plasmodium falciparum* cultivation by studies that removed individual solutes from the standard culture medium [52–55].

For this proposed role, the host membrane channel would function as a first step in nutrient acquisition (Figure 2A). For each uncharged nutrient, Fick's law of diffusion dictates that PSAC-mediated uptake will be proportional to the difference in concentration between host plasma and erythrocyte cytosol. These nutrients would then encounter the parasitophorous vacuolar membrane (PVM) and cross that barrier through giant non-selective channels distinct from PSAC [56,57]. The distantly related *Toxoplasma* parasite appears to have

channels with similar pore size on their PVM [58], but mechanistic confirmation with patch-clamp is missing. In *P. falciparum*, membranous extensions from the PVM, known as the tubulo-vesicular network (TVN) [59], may function to increase the area for uptake via these PVM channels. Once nutrients enter the vacuolar space, they need only to cross the parasite plasma membrane to become available for use. Carrier-type transporters for various nutrients have been identified through homology searches and localized to this membrane [60–63].

One problem with this hypothesis has been that some nutrients have significant permeability in uninfected cells, casting doubts on whether the increased uptake is really necessary. For instance, hypoxanthine, the preferred purine source for parasite nucleic acid biosynthesis, can be taken up by human nucleobase transporters present on erythrocytes [22,64]. Whether parasite demands can be met by these endogenous transporters is not well-established. For other solutes, quantitative arguments about requirement from external sources have suggested that the parasite channels are indeed needed. Isoleucine, an essential amino acid, cannot be obtained through hemoglobin digestion because human hemoglobin is completely devoid of this amino acid; it must therefore be obtained from extracellular sources [65]. Its measured permeability into uninfected erythrocytes may be too low to sustain parasite demands [3].

Experimental evidence for nutrient uptake was lacking until only recently, in part because the number of distinct transporters or channels for each solute was unclear. A breakthrough came with the discovery of isolate-specific inhibitors such as ISPA-28, which could confidently distinguish uptake via PSAC from that mediated by host transporters [28]. If PSAC activity is essential, then ISPA-28 should inhibit growth of Dd2 parasites in culture, but be inactive against the HB3 line, whose channels are not blocked by this compound. Although *in vitro* growth inhibition studies are often confounded by ‘off-target effects’, a term that describes killing due to action on unrelated parasite targets, the marked difference in ISPA-28 potency against transport on these two lines should overcome this concern. Such off-target activity has been a major problem in determining the biological roles of some parasite enzymes; it has also frustrated drug discovery efforts against otherwise attractive targets [66].

Disappointingly, initial growth inhibition studies using ISPA-28 and standard culture conditions revealed little or no effect on propagation of either Dd2 or HB3. However, RPMI 1640, the general use culture medium used for *in vitro* propagation, has supraphysiological levels of many nutrients [67]. For example, the isoleucine concentration in this medium is 381 μM , some six fold higher than estimated for plasma from healthy American donors [68]. These high extracellular nutrient concentrations may reduce the effectiveness of PSAC inhibitors in growth inhibition studies because the resulting large inward gradient can increase passive uptake via incompletely blocked channels; channel block, like enzyme inhibition, is invariably not 100% complete when reversible small molecule inhibitors are used. The ensuing passive uptake may meet parasite demands and sustain intracellular survival.

To explore whether supraphysiological nutrient concentrations aggravate these growth inhibition experiments, a recent study designed a medium that follows the RPMI 1640 formulation but with reduced, more physiological levels of three key nutrients: isoleucine, glutamine, and hypoxanthine [28]. These nutrients were identified by surveying solutes with known permeability through the parasite channels. Dose response experiments were used to optimize each nutrient's concentration, yielding a modified medium termed PGIM (PSAC Growth Inhibition Medium). Because parasite culture requires a lipid source, PGIM was supplemented with human serum that had been dialyzed to remove soluble nutrients. The resulting medium supported continuous growth of both Dd2 and HB3 parasite lines, albeit at rates somewhat slower than seen with RPMI 1640 formulations. Notably, control experiments showed that the growth rate was comparably reduced when parasites were grown in human serum without addition of synthetic media formulations, suggesting that PGIM may be more representative of *in vivo* growth conditions than RPMI 1640.

When PGIM was used in growth inhibition studies, a marked difference in ISPA-28 activity against Dd2 and HB3 parasites was uncovered [28]. Dd2 parasites are killed at > 75-fold lower concentrations (IC_{50} value of 0.66 μ M vs. > 50 μ M for HB3 parasites), paralleling the difference in ISPA-28 efficacy against channels from these parasites. PGIM and ISPA-28 were then used in growth experiments with the progeny of the Dd2 x HB3 cross to examine the genetic basis of differential killing. These experiments mapped the *clag3* locus on parasite chromosome 3, as had previously been done with ISPA-28 transport block. The identical mapping results with these two independent phenotypes (growth inhibition and transport inhibition) exclude significant off-target effects for ISPA-28; they provide genetic validation of PSAC as a drug target. Additional evidence for an essential role was obtained with *clag3*-specific transfections and *in vitro* selections that yielded a genomic recombination event at this locus [28].

ISPA-28 is not likely to be a good starting point for antimalarial drug development because it is active against only Dd2 and closely related parasite lines. Fortunately, high-throughput screens have identified PSAC inhibitors with potent and uniform activity against *P. falciparum* lines representing all three continents endemic with human malaria [21]. Some compounds are also active against PSAC activity on the phylogenetically divergent primate malaria parasite, *P. knowlesi*, consistent with action at highly conserved sites on the channel pore [50]. Importantly, growth inhibition studies with these broad-spectrum PSAC inhibitors revealed that each has significantly greater efficacy in PGIM than in RPMI 1640 (often by 100-fold or more), confirming that the effect of reducing nutrient concentrations is not restricted to ISPA-28 [28]. Finally, control experiments found that antimalarial drugs that act on unrelated targets—artemisinin, chloroquine, and mefloquine—do not exhibit improved killing in PGIM, excluding a nonspecific effect of this engineered medium. These findings validate PSAC as a new and distinct antimalarial target. Because they required optimization of nutrient concentrations, these experiments also provide direct experimental evidence for a role of PSAC in nutrient acquisition.

Host erythrocyte cation remodeling

A second proposed role, cation remodeling in the host cytosol, is based on changes in erythrocyte Na^+ and K^+ concentrations observed with parasite maturation [1,69–71]. Healthy uninfected erythrocytes maintain large transmembrane Na^+ and K^+ gradients through the action of the Na^+/K^+ ATPase pump [72]. After infection, however, the erythrocyte cytosolic Na^+ increases from ~ 10 mM to >100 mM; there is a commensurate decrease in K^+ from ~ 120 mM to < 40 mM (Figure 2B). These changes result from modest, but nonzero transport of these ions through PSAC with possible contributions from inhibition of the erythrocyte Na^+/K^+ ATPase pump. Notably, PSAC permeability to Na^+ is very low, estimated at $10^{-3.5}$ to 10^{-5} relative to Cl^- [73]. This level of exclusion of any small monovalent ion is unprecedented amongst other broad-selectivity ion channels, which are known to achieve 10^{-1} selectivity at best. From an evolutionary standpoint, PSAC permeability to Na^+ must be kept low to avoid osmotic lysis of infected cells in host plasma because it is the low permeability of this cation that balances impermeant intracellular solutes such as hemoglobin. Nevertheless, this low permeability is sufficient to remodel erythrocyte cation composition over the course of the many hours required for intracellular parasite development.

How could changes in host erythrocyte cation concentrations benefit the parasite? It has been tacitly assumed that the growing intracellular parasite prefers a high Na^+ , low K^+ environment because this is the typical extracellular environment for most eukaryotic cells, which generally cannot be cultivated in media with significantly altered Na^+ or K^+ concentrations. To be useful to the parasite, changes in Na^+ and K^+ in the erythrocyte cytosol should be transmitted across the PVM. In contrast to PSAC, the PVM channel is highly permeable to both cations; although it has not been directly measured, the ionic composition of the vacuolar space likely matches that of erythrocyte cytosol because of this channel's large, nonselective pores [57]. Then, the parasite may theoretically utilize the increased Na^+ to drive coupled transporters on its plasma membrane (Figure 2B). With this in mind, a Na^+/H^+ counter-transporter and a Na^+ -phosphate co-transporter have been proposed [74–76]; both of these transporters could benefit the parasite by coupling inward Na^+ movement to more efficiently extrude metabolic acid or acquire nutritive phosphate. However, there is evidence against both of these transporters [77]. The higher Na^+ at the parasite surface may also benefit the parasite if Na^+ serves essential roles within the parasite such as promoting essential enzyme activities or modulating membrane potentials at various parasite membranes. It is also possible that the decrease in erythrocyte K^+ levels is beneficial to the intracellular parasite [78].

To explore the physiological significance of host cation remodeling, a recent study sought to prevent remodeling in viable infected erythrocytes [77]. This could be achieved by successfully cultivating parasites in media with a low Na^+ concentration: host cell remodeling is only possible with a high external Na^+ because channel-mediated uptake is passive. The standard medium, RPMI 1640 supplemented with human serum or Albumax, has ~ 140 mM Na^+ in the form of Cl^- , HCO_3^- , and HPO_4^- salts. Simple replacement of these salts with the corresponding Li^+ or N-methyl-D-glucamine $^+$ salts did not support growth, but these cations may be toxic to the intracellular parasite. Replacement of Na^+ with

K^+ also did not support parasite growth, but this cannot reflect a direct toxic effect as K^+ is normally present at high concentrations within healthy uninfected erythrocytes. Although this result might reflect an essential role for erythrocyte Na^+ uptake, another possibility is that the high K^+ medium produces osmotic lysis of infected cells; high extracellular K^+ , but not high Na^+ , could produce cell lysis because of PSAC's significantly greater permeability to K^+ than Na^+ [73]. To test this explanation, the KCl in the high K^+ medium was partially replaced with sucrose, an impermeant disaccharide. With 84 mM sucrose, 65 mM KCl, and all nutrients at levels that match those in standard media, continuous parasite propagation was achieved [77]. Growth rates matched those in RPMI 1640-based medium and did not require parasite adaptation to the nonphysiological sucrose-KCl medium. This medium has a low measured Na concentration of 6.8 mM, derived mainly from human serum added as a lipid source; its total K^+ concentration is 105 mM. Because these values are similar to the cation concentrations within uninfected erythrocytes, host cation remodeling should be largely prevented throughout parasite cultivation in this medium. Measurement with ion-sensitive electrodes confirmed this prediction. Thus, host cation remodeling is not essential for intracellular growth of the malaria parasite. Because growth is not measurably affected in the sucrose-KCl medium, the parasite does not appear to benefit from either Na^+ nor K^+ gradients created by PSAC activity.

Sucrose-based media were also used to dissect the ionic requirements for parasite growth [77]. Remarkably low Na^+ concentrations could support parasite growth (EC_{50} of 2.9 mM). Surprisingly, although the erythrocyte has large K^+ stores, parasite propagation also required low, but nonzero amounts of extracellular K^+ (EC_{50} of 0.17 mM); K^+ could be raised to 148 mM without clear detriment to development, egress, and reinvasion. By identifying gluconate as a nontoxic substitute anion, this study also found that parasites tolerate significant reductions in external Cl^- (EC_{50} of 31 mM). Combined with another study showing that the parasite tolerates a broad range of extracellular H^+ concentrations [79], these studies indicate that the parasite has a significantly greater tolerance of changes in monovalent ion concentrations than cultured mammalian cells. This tolerance parallels that seen with dinoflagellates, to which apicomplexan parasites are closely related [80].

Concluding remarks and future perspectives

There have been some recent breakthroughs in our understanding of increased erythrocyte permeability linked to malaria parasite infection. An unusual ion channel, PSAC, serves a primary role, as now demonstrated through pharmacological studies with highly specific inhibitors [16]. The parasite-specific *clag* gene family is critical to this activity [16,29,33]. Direct experimental evidence is also now available for an essential role of PSAC in nutrient uptake [28]; erythrocyte cation remodeling, a long-recognized consequence of increased permeability [1,70], is an unnecessary byproduct of PSAC activity because conditions that selectively prevent net Na^+ and K^+ transport do not adversely affect parasite development or replication [77]. There appears to have been evolutionary pressure on the parasite to design channels that meet two competing requirements. There must be sufficient permeability to nutrients to meet the demands of intracellular parasite development. At the same time, Na^+ uptake must be kept low enough to prevent premature osmotic lysis of infected cells. The balance between these competing demands presumably accounts for PSAC's many unusual

functional properties [10,55,73,81]. It may also explain why the *clag3* genes lack homology to known ion channels in other organisms.

Despite these advances, the work in this field is still in an early stage, with many fundamental questions still largely unexplored (Box 3). Some of these questions relate to the precise composition of the channel and how it mediates solute transport; others relate to how increased permeability serves the parasite and contributes to the complex interactions between pathogen and host cell. Continued study by groups with differing perspectives and areas of expertise will be needed to address these questions. Driving this research is the excellent potential for development of novel antimalarial drugs that either inhibit PSAC or interfere with its finely regulated function.

Box 3

Questions for future research

- How exactly do CLAG proteins contribute to increased permeability? CLAG3 may participate in channel formation directly or it may activate or modulate other proteins that form the pore [16]. Are other CLAG proteins also involved in erythrocyte permeability? CLAG2 has recently implicated in PSAC activity [33], but what this paralog does and whether its structural contribution differs from that of CLAG3 is presently unknown.
- Are unrelated proteins, of either parasite or host origin, also required to increase erythrocyte permeability? How are parasite components trafficked out and inserted at the erythrocyte plasma membrane? How do these putative subunits assemble into a functional channel?
- How are solutes recognized and transported through this unusual channel? While PSAC transports a diverse collection of charged and uncharged solutes, it has an exceedingly low Na⁺ permeability [73]. Understanding the structural basis of this selectivity profile is an important frontier because no other broad selectivity channel achieves a comparable exclusion of an individual small solute.
- Which nutrients are essential for the parasite? Isoleucine seems to be critical, but if this were the only required nutrient in host plasma, a parasite-derived isoleucine transporter might have been a more-straightforward solution. Other candidate nutrients with known PSAC permeability require examination. Do antimalarial drugs or drug leads use this channel to reach their targets, as could be tested with either tracer flux or growth inhibition experiments in combination with PSAC blockers [19]?
- Are there other roles for the channel? One possibility is removal of metabolic waste products generated in the parasite. Another, erythrocyte volume regulation, is suggested by mathematical modeling [86]; this role is not supported in experiments using altered osmolarities and sucrose as an extracellular impermeant solute [77], but more direct experiments are required. Other roles should also be sought.

- How are the channels regulated? Because *P. falciparum* frequently affects malnourished children, feedback mechanisms that sense host nutritional status and respond by changing erythrocyte permeability require consideration. Possible mechanisms include epigenetic transcriptional regulation, altered trafficking of channel protein to the host membrane, or soluble modulators that regulate channel opening. Interestingly, both functional PSAC copy number and conductance rates are conserved across species [50]; it is hard to imagine that this is a coincidence.

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Glossary

Blasticidin S	an antimalarial toxin that requires PSAC-mediated uptake; it kills the parasite by inhibiting protein translation on ribosomes
Carrier	a distinct group of solute transport proteins that mediate transport at rates that are usually slower than through ion channels. Carriers require protein conformational changes for each transport cycle. Also known as solute transporters, they may couple the movement of two or more solutes (e.g. co- and counter-transporters)
Clag genes	a multigene family specific to <i>Plasmodium</i> spp, named for early evidence linking these genes to cytoadherence. Family members on chromosomes 2 and 3 have more recently been associated with PSAC activity
Conductance	an electrical measure of the rate of ion transport through an ion channel
EC₅₀	the concentration of a nutrient or essential solute that supports half-maximal parasite growth under <i>in vitro</i> conditions
Flame photometry	a method for measuring metal ion concentrations based on the color and intensity of flames in a controlled vaporization
Gating	the kinetic transitions between open and closed states of a functioning channel
IC₅₀	the concentration of an antimalarial compound that inhibits parasite growth by 50%, typically estimated through <i>in vitro</i> growth inhibition dose responses
Ion channel	a class of solute transport proteins that facilitates rapid transmembrane flux of solutes. Channels typically have an

	aqueous pore designed to recognize and allow passage of specific solutes; they may also be ligand-gated or voltage-dependent
ISPA-28	an isolate-specific PSAC inhibitor found by high-throughput screening. This compound has enabled studies to identify PSAC's molecular basis and physiological role
$K_{0.5}$	the concentration of an inhibitor required to reduce enzyme function by 50%. For PSAC, concordant $K_{0.5}$ estimates for key inhibitors have been obtained with tracer flux, osmotic fragility, and patch-clamp methods
Laboratory parasite lines (e.g. Dd2, HB3, 3D7, 7G8, FVO)	genetically distinct cultures of <i>P. falciparum</i> , obtained from human patients around the world. These lines are clonal and provide an important resource for examining the molecular basis of PSAC activity and other phenotypes
Leupeptin	an antimalarial toxin that requires PSAC-mediated uptake; it kills the parasite by inhibiting multiple cysteine proteases within infected cells
Osmotic fragility	cell lysis in a solution of a permeant solute. The kinetics of cell lysis have been used to quantify the permeability of the solute
Paralog	closely related genes in a single species, typically arising through gene duplication events. These genes may serve related or highly divergent functions
Parasitophorous vacuolar membrane (PVM)	a membrane surrounding the intracellular parasite, derived originally from the erythrocyte membrane at the time of invasion. With parasite development, the lipids and proteins that make up this membrane change dramatically
Patch-clamp	a technique used to study single or multiple ion channels in cell membranes. Patch-clamp is based on measuring currents that result from net ion movement across membranes
Permeability (P)	a measure of how easily a solute can cross a biological membrane. For uncharged solutes, the rate of transport across a membrane is proportional to P , the transmembrane concentration gradient, and the membrane's surface area
Plasmodial surface anion channel (PSAC)	an ion channel on the host membrane of infected erythrocytes. PSAC is primarily responsible for increased permeability after infection; this channel and the associated <i>clag</i> genes are restricted to malaria parasites
Selectivity	the ability of ion channels to recognize and transport only certain solutes. Complex structure- and charge-based interactions between the channel, solutes, and associated water molecules may

allow remarkable discrimination and selective transport, as is critical for cellular functions

Tracer flux

a method that estimates membrane permeability of a radiolabeled solute by quantifying the rate movement across a membrane

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Highlights

- Malaria parasites increase their host erythrocyte's permeability to diverse solutes.
- The plasmodial surface anion channel, as determined by *clag* genes, plays a key role.
- These channels function to allow nutrient uptake by the intracellular parasite.
- Host cell cation remodeling is an unnecessary byproduct of the channels.

Figure 1.

Molecular insights into increased solute permeability after infection. **(A)** Structures of blasticidin S and leupeptin, antimalarial toxins that require channel-mediated uptake; notice that these toxins are large and polar. The schematic shows an infected erythrocyte with PSAC at the erythrocyte surface. Red, yellow, and blue compartments represent erythrocyte cytosol, parasitophorous vacuole, and parasite cytoplasm, respectively; Maurer's clefts (MC) are shown in the host cytosol. After channel-mediated uptake, leupeptin inhibits proteases in the parasite digestive vacuole (DV) and other sites [87]; blasticidin S inhibits translation on parasite ribosomes (R) [88]. Resistance to these toxins is most easily acquired by modifications to PSAC that reduce toxin uptake. **(B)** Structure of ISPA-28, an inhibitor specific for channels from Dd2 and genetically related parasite lines. **(C)** Alignment of CLAG3 sequences from the indicated parasites around a highly variable motif exposed at the host cell surface. This motif may be 10 to 30 aa in length (grey shading in consensus sequence); it is especially long in the Dd2 CLAG3.1 protein, possibly accounting for specific PSAC block by ISPA-28 in this parasite. Hydrophobic residues, marked with green shading, are enriched in the conserved flanking sequences but are underrepresented in the variable motif, consistent with a soluble loop exposed at the erythrocyte surface.

Figure 2.

Possible roles served by increased erythrocyte permeability. **(A)** Nutrient acquisition. In infected cells, nutrients can enter erythrocyte cytosol (red) by passive diffusion through PSAC at the erythrocyte membrane (RBC). These nutrients as well as those present in host cytosol cross the parasitophorous vacuolar membrane (PVM) through the PVM channel. From the vacuolar space (yellow), they are acquired at the parasite plasma membrane (PPM) through specific nutrient transporters. Maurer's clefts (MC) may function in trafficking parasite proteins such as CLAG3 to the host cell surface [89]. A tubulovesicular network (TVN) may increase the surface area of the PVM for nutrient uptake. **(B)** Host cation remodeling. A Na/K pump with a 3:2 stoichiometry maintains low Na^+ and high K^+ concentrations in erythrocyte cytosol [90]. Slow, but steady Na^+ uptake and K^+ efflux through PSAC abolishes these gradients. These changes are transmitted to the parasite via the PVM channel. A Na^+/H^+ exchanger and a $\text{Na}^+/\text{phosphate}$ co-transporter have been proposed at the PPM [74,76]; these transporters could take advantage of the changes in host cation concentrations. Coupled K^+ transporters might also utilize the gradients resulting from PSAC-mediated remodeling, but have not been proposed. Physiologically important coupling to these cation gradients is not supported by recent studies [77].