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RESEARCH ARTICLE

MALARIA PARASITE PROTEINS INVOLVED IN NUTRIENT CHANNELS AT THE HOST ERYTHROCYTE MEMBRANE: ADVANCES AND QUESTIONS FOR FUTURE RESEARCH

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ABSTRACT

Erythrocytes infected with malaria parasites have increased permeability to nutrients and other solutes, as mediated by an unusual ion channel known as the plasmodial surface anion channel (PSAC). Although the increased permeability of infected erythrocytes was identified more than 70 years ago and subsequently characterized with tracer studies, its mechanism and role in parasite biology remained unclear until the introduction of patch-clamp methods and high-throughput screening technologies. These methods discovered and implicated PSAC as the primary mechanism, determined that this channel is essential for parasite development, led to identification of the channel's genes, and stimulated antimalarial drug discovery against this target. Despite theseadvances, many questions remain about this unusual parasite channel. Our review highlights some recent advances and describes important questions for future research.

INTRODUCTION

Malaria remains a leading infectious cause of morbidity and mortality worldwide, with some 500,000 deaths still occurring annually (Nkumama *et al*., 2017). This disease is caused by single-celled eukaryotic parasites in the *Plasmodium* genus; these parasites evade host immune attack by invading and replicating within circulating erythrocytes in the bloodstream. The most virulent human pathogen, *P. falciparum*, remodels its host cell to facilitate its own development. This parasite exports many proteins into the host cell (Goldberg and Cowman, 2010), establishes a membraneous network in erythrocyte cytosol (Tamez *et al*., 2008), and increases erythrocyte permeability to many ions and organic solutes (Desai, 2014). Recent studies have determined that this increased host cell permeability is mediated by a broad selectivity ion channel known as the plasmodial surface anion channel, PSAC (Desai *et al*., 2000; Alkhalil *et al*., 2004). Ion channels consist of proteins integral to the cell membrane and serve to facilitate movement of solutes across the membrane (Hille, 2001). Because this flux enables cells to acquire needed solutes (e.g. ions and nutrients) and to interact with their environment, nearly all cells have channels in their membranes.

REVIEW OF CURRENT KNOWLEDGE

Background and mechanistic basis of increased permeability in malaria

The human erythrocyte is largely devoid of ion channels because it is terminally differentiated and lacks metabolic activities present in most other cells of our bodies. Thus, early studies in basic malaria research readily detected the increased permeability associated with infection by *Plasmodium* parasites (Overman, 1948; Kutner *et al*., 1982). Numerous reports used tracer flux and osmotic fragility to characterize these permeability changes, determine the range of permeating solutes, and identify some low-affinity inhibitors (Ginsburg *et al*., 1985; Gero and Wood, 1991; Kirk *et al*., 1993). Nevertheless, the mechanisms responsible for increased permeability were unclear with proposals including a membrane defect in the host membrane from invasion by the merozoite form of the pathogen, a membranous duct that connects the parasite to the host membrane, upregulation of erythrocyte transporters, fluid phase endocytosis, and one or more ion channels. This uncertainty reflected a key limitation of macroscopic tracer flux studies.

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To overcome this limitation, patch-clamp methods were first applied to the host membrane of infected cells in 2000 (Desai *et al*., 2000). These methods use high-gain electrical amplifiers and a small capillary sealed on the membrane of interest to record transmembrane ionic currents in real time; various patch-clamp configurations permit measurements on single ion channel molecules and on single cells (Hamill *et al*., 1981). Cell-attached patch-clamp identified PSAC activity as the only ion channel on infected cells under our recording conditions and confirmed that uninfected erythrocytes are devoid of channel activity under normal conditions. The channel on infected cells exhibited relatively slow ion uptake, as indicated by a small single channel conductance of 20 picosiemens in molar salt. It also showed fast flickering transitions between open and closed states; gating analyses revealed that the mean open duration was only ~200 microseconds (Desai, 2005). By changing the imposed membrane potential, this initial study also found that the channels open more frequently and for longer durations at negative potentials (Desai *et al*., 2000). Total currents from single cells were then measured with the whole-cell configuration. This approach confirmed the channel's voltage dependence, revealed that PSAC is the primary conductive pathway in the infected cell membrane, and implicated 1000-2000 functional copies of the channel per erythrocyte infected with mature trophozoite parasites. These features were conserved in phylogenetically distant malaria parasites (Lisk and Desai, 2005), but this channel is absent from erythrocytes infected with *Babesia* parasites (Alkhalil *et al*., 2007). Several other studies using whole-cell patch-clamp have suggested upregulation of otherwise quiescent human channels after infection (Staines *et al*., 2007), but we have not been able to confirm those findings.

A second important advance was the development of a robust and quantitative method for screening PSAC inhibitors (Wagner *et al*., 2003). This new method utilized transmittance measurements to track osmotic lysis of infected cells in organic solutes such as sorbitol, a sugar alcohol with high PSAC permeability but negligible uptake by uninfected erythrocytes (Ginsburg *et al*., 1985). This method revealed PSAC's atypical pharmacology that differs from mammalian anion channels (Lisk *et al*., 2006). When this transmittance assay was miniaturized into microplate wells, high-throughput screens for inhibitors could be carried out using large collections of chemicals. Several screens have thus been carried out (Pillai *et al*., 2010; Nguitragool *et al*., 2011; Pain *et al*., 2016), leading to potent and specific inhibitors that block the channel at low nanomolar concentrations. Single channel patch-clamp studies found that each of these inhibitors acts on the same channel, adding to the evidence for a primary role of PSAC in host cell permeability after infection (Pillai *et al*., 2010; Nguitragool *et al*., 2011).

Gene identification and a new molecular era in studying infected cell permeability

High-throughput screening also led to the identification of ISPA-28, an isolate specific PSAC antagonist that selectively blocks channels associated with a single laboratory parasite clone known as Dd2, originally derived from a patient in Indochina (Guinet *et al*., 1996). This unusual inhibitor was ineffective against unrelated parasite strains harvested from other patients around the world. ISPA-28 implicated parasite genetic elements in formation of the channel: polymorphisms in these parasite genes presumably accounted for why the compound blocks channels from one parasite clone but not those from others. By examining channels on cells infected with progeny from a genetic cross between the Dd2 clone and the HB3 parasite (originally from Honduras), a single genomic locus on the parasite chromosome 3 was implicated in channel block by ISPA-28. Subsequent DNA transfections revealed that two related *clag3* genes determine the PSAC phenotype (Nguitragool *et al*., 2011). These two genes undergo epigenetic switching (Cortes *et al*., 2007; Rovira-Graells *et al*., 2015), so that individual parasites express only one copy and reserve the other for expression at a later time (Voss *et al*., 2014).

The encoded CLAG3 protein localizes to the host erythrocyte membrane (Nguitragool *et al*., 2014), matching the site where cell-attached patch-clamp had previously localized PSAC activity. A small variant region on the protein is exposed at the host membrane and appears to be where ISPA-28 binds. Several subsequent studies have provided independent evidence for the central role of CLAG3 in formation of PSAC. For example, PSAC mutants generated by *in vitro* selection of parasite cultures with either blasticidin S or leupeptin silence the *clag*3 genes or carry a mutation in a critical transmembrane domain (Hill *et al*., 2007; Sharma *et al*., 2013; Mira-Martinez *et al*., 2013; Sharma *et al*., 2015). These channel mutants grew under this selection because they reduce PSAC-mediated uptake of these toxins while preserving channel-mediated nutrient uptake at levels that sustain parasite growth. This selective reduction of toxin permeability enables parasite survival, but the structural basis of altered permeability in both PSAC mutants remains unclear. Genetic mapping of two independent phenotypes—parasite growth inhibition by ISPA-28 and reduced channel activity upon extracellular protease treatment—have also mapped the *clag3* chromosomal locus (Pillai *et al*., 2012; Nguitragool *et al*., 2014). Mapping using growth inhibition by ISPA-28 also validates PSAC as an essential parasite target for development of antimalarial therapies; by finding that parasite killing by PSAC inhibitors is accentuated under culture conditions with reduced concentrations of key nutrients, this study also revealed that PSAC's essential function is to mediate nutrient uptake from host plasma (Pillai *et al*., 2012). Finally, the role of parasite proteins in PSAC formation has been further supported by DNA transfection to produce a conditional knockdown of the parasite-encoded translocon that facilitates export of proteins into erythrocyte cytosol (Beck *et al*., 2014).

Questions For Future Research

Identification of *clag3* genes as determinants of nutrient uptake has resolved several long standing debates about the increased permeability (Staines *et al*., 2007). By enabling molecular studies that show an essential role in nutrient uptake, they have also stimulated drug discovery against this important target. However, several key questions remain, as we itemize in the remainder of this review.

How do these proteins determine PSAC activity?

Maybe most importantly, it is still uncertain whether CLAG3 protein forms the channel directly or if it functions in some

other way to increase erythrocyte permeability. One question relates to the three *clag* genes on other parasite chromosomes (Kaneko *et al*., 2001; Iriko *et al*., 2008). Do these *clag2*, *clag8*, and *clag9* genes also encode pore forming proteins at the host membrane or do they serve unrelated functions, as has been proposed (Holt *et al*., 1999; Nacer *et al*., 2011; Goel *et al*., 2010)? If so, do each of those channels have identical properties or, for example, does each subtype mediate uptake of specific nutrients? An additional complexity is that CLAG proteins associate with two unrelated parasite proteins, termed RhopH2 and RhopH3 (Cooper *et al*., 1988; Lustigman *et al*., 1988; Ling *et al*., 2004), to form a heteromeric complex known as the RhopH complex. Like *clag* genes, these genes are conserved in all malaria parasites and appear to be essential. What are the functions of these associated proteins? One possibility is that the entire complex of three distinct proteins forms the functional channel at the host membrane; another is that one or the other protein is only a chaperone involved in transporting the other members to the host cell surface. DNA transfections in cultured parasites should address some of these unknowns, but new methodologies such as heterologous expression of these parasite proteins and determination of protein structures may also be required.

It is also possible that these proteins do not form a pore themselves, but rather function to activate host channels at the erythrocyte membrane. In this scenario, we would generally expect that co-immunoprecipitation experiments using CLAG3, RhopH2, or RhopH3 should identify putative interacting proteins that form functional channels. However, such proteins have not been clearly identified (Kaneko *et al*., 2001; Vincensini *et al*., 2008; our unpublished studies). While these associations may be either too brief or unstable to allow detection, these findings do favor direct pore formation by the RhopH complex. Direct formation of the pore is also suggested by the rapid PSAC block upon addition of ISPA-28, a chemical inhibitor that interacts specifically with CLAG3 in molecular studies. This suggests direct formation because inhibitors acting through enzymatic modulators of ion channels typically require up to an hour for signaling cascades to fully affect downstream activities, rather than the essentially instantaneous effects observed in patch-clamp studies. Finally, helical wheel analysis suggests that CLAG3 has an amphipathic transmembrane domain that can line a water-filled pore (Sharma *et al*., 2015).

What are the specific roles of each member of the RhopH complex?

CLAG3, RhopH2, and RhopH3 are assembled into a complex shortly after their biosynthesis in merozoites near the end of the parasite developmental cycle within erythrocytes (Ling *et al*., 2004). At the end of the cycle, these proteins are secreted into the next host cell at re-invasion. They then traffic via poorly understood mechanisms to the host membrane many hours after erythrocyte invasion to induce functional channel activity (Nguitragool *et al*., 2011). Thus, we also need to ask whether one or more of the proteins only serves to chaperone the other proteins through this complicated route to the host membrane. If all members of the complex contribute directly to pore formation, does one or more serve a modulatory role? One such role would be to sense host nutritional status and alter channel-mediated nutrient uptake, as this may improve parasite growth in malnourished hosts.

The trafficking of these proteins to the host membrane also deserves more rigorous study. One study found that CLAG3 is not exported by the Plasmodium translocon of exported proteins (PTEX) on the vacuolar membrane surrounding the intracellular parasite (Beck *et al*., 2014), though our unpublished studies have cast some doubt on this finding. How these proteins traffic past this membrane and the precise elements required for insertion into the host membrane both remain unclear. Imaging and mass spectrometry studies suggest that they require transit through Maurer's clefts (Sam-Yellowe *et al*., 2001; Vincensini *et al*., 2005), membrane-bound organelles in the host cytosol of infected erythrocytes.

Permeation through the channel and potential for drug development

As the functions of these member proteins become clearer, further study should be directed to understanding how this parasite ion channel allows selective uptake of solutes. For example, although PSAC is permeable to nutrients and solutes of varying charge and size (amino acids, sugars, purines, some vitamins, organic cations, halide anions, etc.), the channel stringently excludes the small sodium ion (Cohn *et al*., 2003; Pillai *et al*., 2013). Exclusion of a single small monovalent ion is intriguing because it is without precedent amongst other broad selectivity channels. It is also interesting because sodium exclusion is critical for parasite survival in the bloodstream; without this exclusion, net sodium uptake would produce osmotic lysis of infected cells and cause parasite death. Thus, this parasite channel appears to have evolved its most unusual selectivity for solutes as these features permit both essential nutrient uptake while preserving erythrocyte integrity within human plasma. A clear understanding of the mechanisms involved in this ability to exclude a single ion will provide fundamental insights not only into parasite biology, but into the workings of ion channels in other organisms.

Although studies have implicated isoleucine as maybe the most critical nutrient acquired via PSAC in parasite culture conditions (Divo *et al*., 1985; Liu *et al*., 2006; Pillai *et al*., 2012), other nutrients are almost certainly needed *in vivo*. It will be important to better define these essential nutritive solutes because this may guide development of antimalarial drugs that work by blocking PSAC. Whether soluble metabolic waste products produced by the intracellular parasite are expelled through this channel is also unclear and deserves study.

High-throughput screens have identified potent and specific PSAC inhibitors from diverse scaffolds (Pillai *et al*., 2010; Pillai *et al*., 2012). Inhibitors from two separate classes produce synergistic parasite killing in cultures (Pain *et al*., 2016). With these developments, there is growing interest in developing these compounds into new antimalarial therapies. Successful development will requiring finding one or more chemical inhibitors that kill parasites quickly, have appropriate absorption, distribution, metabolism, and safety in humans. These multiple requirements are difficult to meet and often require concerted effort throughout a multi-year project.

CONCLUSIONS

Malaria parasites have adapted to growth and development in erythrocytes. To enable intracellular survival, the parasite remodels its host cell and increases its permeability via an unusual broad specificity ion channel termed PSAC. Although genetic mapping and molecular studies have identified parasite proteins involved in PSAC formation, key questions remain about the precise roles and mechanisms of protein trafficking required for this activity. It is an exciting time for basic research in this field because new transfection technologies such as CRISPR should enable next-level molecular insights. These studies should reveal important insights into host-parasite interactions and permeation through ion channels. It may also lead to new, much needed therapies for malaria.

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