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Initiation of *Plasmodium* sporozoite motility by albumin is associated with induction of intracellular signaling

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Abstract

Malaria infection is initiated when a mosquito injects *Plasmodium* sporozoites into a mammalian host. Sporozoites exhibit gliding motility both in vitro and in vivo. This motility is associated with the secretion of at least two proteins, circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP). Both derive from micronemes, which are organelles that empty out of the apical end of the sporozoite. Sporozoite motility can be initiated in vitro by albumin added to the medium. To investigate how albumin functions in this process, we studied second messenger signaling within the sporozoite. Using pharmacological activators and inhibitors, we have concluded that gliding motility is initiated when albumin interacts with the surface of the sporozoite and that this leads to a signal transduction cascade within the sporozoite, including the elevation of intracellular cAMP, the modulation of sporozoite motility by Ca²⁺ and the release of microneme proteins.

Keywords

Plasmodium; Sporozoite; Motility; Albumin; Intracellular signaling; cAMP; Ca²⁺

1. Introduction

Malaria infection is initiated when a mosquito injects *Plasmodium* sporozoites into the skin of a mammalian host (Sidjanski and Vanderberg, 1997; Vanderberg and Frevert, 2004; Amino et al., 2006). These sporozoites then move into dermal blood vessels, from which they reach their target destination in hepatocytes (Vanderberg and Frevert, 2004; Amino et al., 2006). A striking feature of *Plasmodium* sporozoites, as well as invasive stages of other apicomplexan protozoa, is that they exhibit gliding motility, which results in translocation of the organism without any flexing or undulation of its body, nor participation of appendages such as cilia, flagellae or pseudopods. Gliding motility has been extensively studied with malaria sporozoites due to the ease of initiating it in vitro with sporozoites removed from salivary glands and suspended in medium containing albumin (Vanderberg, 1974). Induction of sporozoite motility within the skin is a functionally appropriate physiological response to exposure to albumin upon introduction of sporozoites by mosquitoes into mammalian hosts. Albumin was the first defined

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Note: Supplementary data associated with this article.

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effector shown to induce a specific cellular process in *Plasmodium*, yet its mechanism of action remains unclear.

Among the fundamental characteristics of gliding motility, as shown in numerous studies with a wide range of apicomplexans including *Cryptosporidium*, *Eimeria*, *Plasmodium*, *Sarcocystis*, *Toxoplasma*, is that Ca^{2+} acts as an intracellular second messenger that stimulates internal organelles known as micronemes to release proteins from the parasite's anterior end and these proteins are driven posteriorly along the parasite's exterior by an actin-myosin linear motor (Stewart and Vanderberg, 1991; Entzeroth et al., 1992; Carruthers et al., 1999; Bumstead and Tomley, 2000; Gantt et al., 2000; Wetzel et al., 2005).

Intracellular signaling typically involves an external stimulus (chemical, electrical or mechanical) that triggers a signal transduction cascade which leads to a rise in intracellular free Ca^{2+} $[\text{Ca}]_i$. Elevated $[\text{Ca}]_i$ activates the secretory vesicle/plasma membrane fusion machinery directly, resulting in discharge of the vesicular contents into the external medium (Carruthers and Sibley, 1999). Microneme proteins function in invasion of host cells as well as in gliding motility (Carruthers et al., 1999). Two *Plasmodium* sporozoite microneme proteins that have been shown to be released during motility and invasion are circumsporozoite protein (CSP) (Stewart and Vanderberg, 1991; Khan et al., 1992) and thrombospondin-related anonymous protein (TRAP) (Spaccapelo et al., 1997; Gantt et al., 2000).

Studies with apicomplexans have used several different approaches to provide evidence of microneme secretion, including quantification of microneme proteins secreted into the medium, immunofluorescence studies to detect microneme proteins expressed on the parasite surface, fine structural analysis of microneme contents and their appearance on the parasite surface after microneme discharge, and gliding motility. Such studies have been done with apicomplexan parasites that include *Cryptosporidium* (Chen et al., 2004); *Eimeria* (Bumstead and Tomley, 2000); *Plasmodium* (Stewart and Vanderberg, 1991; Gantt et al., 2000; Ono et al., 2008); and *Toxoplasma* (Carruthers and Sibley, 1999; Wetzel et al., 2004).

The use of *Plasmodium* sporozoite motility as an ongoing indicator of microneme secretion has considerable experimental advantages because albumin-induced sporozoite motility may be observed in vitro for several hours (Vanderberg, 1974), in contrast to observations of cell invasion, a process that takes place relatively quickly. In the present study, we have concluded that gliding motility is initiated when albumin interacts with the surface of the *Plasmodium* sporozoite, ultimately leading to a signal transduction cascade within the sporozoite, which includes the elevation of intracellular cAMP, the modulation of sporozoite motility by Ca^{2+} and the release of microneme proteins. How the external albumin signal is transduced at the sporozoite surface to initiate intracellular signaling remains to be established.

2. Materials and methods

2.1. Parasites

Plasmodium berghei parasites were from clones whose sporozoites constitutively express either Enhanced Green Fluorescent Protein (EGFP) (Natarajan et al., 2001) or Red Fluorescent Protein (RFP) (Frevert et al., 2005). *Plasmodium yoelii* sporozoites expressing EGFP were also used (Tarun et al., 2006). Sporozoites were obtained by dissection of salivary glands of infected *Anopheles stephensi* mosquitoes that had previously fed on gametocyte-carrying BALB/c mice (from Taconic Farms Inc., Germantown, NY, USA). Our protocol for maintenance and care of experimental animals was approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Our animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (Rockville, MD, USA).

2.2. Fluorescence microscopy

Gliding motility of EGFP expressing *P. berghei* or *P. yoelii* sporozoites in response to different treatments was monitored by time-lapse digital microscopy using a Leica MZ16FA fluorescence stereoscopic microscope with a 2.0X stereoscopic objective lens. Illumination for fluorescence studies was with an EXFO X-Cite 120 F1 illumination system and with a GFP2 filter set (restricting excitation to 480 ± 20 nm and fluorescence signal emission to wavelengths longer than 510 nm). Sporozoite preparations in various test media were placed on slides as 10 μ l drops, each was covered with a 22 mm² coverslip, and sporozoites were allowed to settle for several min, after which sporozoite motility was observed and recorded by fluorescence videomicroscopy for 30 s per field. For each preparation, at least 40 sporozoites were recorded from at least five randomly selected fields. Images were recorded with a Leica DFC350 FX digital camera using Leica FW4000 software and saved as digital files for further analysis and processing. Movies were subsequently analyzed to determine percentages of gliding sporozoites on each slide. Preparations were coded and read blindly. Examples of results within typical microscope fields are shown in Supplementary Movies S1 and S2. For processing of captured images we used a deconvolution program ('Auto DeBlur', AutoQuant Imaging, Inc., Troy, NY, USA). Movie frames were processed to prepare a single total projection for each 30 s movie, as seen in Fig. 1A and B. Others have quantified sporozoite motility by viewing CSP tracks left behind sporozoites after slides were immunostained (Coppi et al., 2006; Ono et al., 2008). However, sporozoites that do not settle and attach cannot leave tracks. Thus, this technique, first described as a qualitative procedure (Stewart and Vanderberg, 1988), may not give reliable quantitative results; it only records sporozoites able to settle and remain attached to a slide during incubation. This may skew data because non-attached sporozoites are not recorded. We preferred to assess a sample of all sporozoites in a given medium by videorecording motility within samples over a defined time. This allowed us to assess and quantify motility at leisure after the experiments, either by viewing movies or total projections prepared from those.

2.3. Statistics

Statistical analysis and data representation were performed with GraphPad Prism 5.01, 2007 (GraphPad Software Inc., La Jolla, CA, USA) and SigmaPlot 8.0, 2002 (SPSS Inc., Chicago, IL, USA), respectively. Data are given as means $\pm$ S.E.M. One-way ANOVA using Tukey's test as post-hoc, was used for comparison of groups. In all cases *P* values < 0.05 were considered statistically significant.

2.4. Materials

BSA was Fraction V, essentially fatty acid free and essentially globulin free from Sigma, St Louis, MO, USA. BAPTA-AM [1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetra-acetic acid tetra- (acetoxymethyl) ester], a membrane-permeable form of BAPTA that is a selective chelator of intracellular Ca²⁺ stores, was obtained from Sigma, St Louis, MO, USA. A stock solution of 25 mM BAPTA-AM dissolved in pure DMSO (Sigma) was mixed with medium M199 without Ca²⁺ and without phenol red (Medium 199 [H]; Biosource, Rockville, MD, USA), to provide a final concentration of 100 μ M, prior to making serial dilutions of BAPTA-AM. We also tested the effects of 0.1 μ M calcimycin (A23187), a highly specific calcium ionophore (Sigma, St Louis, MO, USA) or a combination of BAPTA-AM and A23187. As a control, some sporozoites were resuspended in 0.4% DMSO, which had been used as a diluent for BAPTA-AM. To obtain calcium-containing media, we added 1 mM CaCl₂ to the calcium-free medium. To test the effects of [Ca²⁺]_e in the media on sporozoites, we used 1 mM BAPTA (Sigma, St Louis, MO, USA) to chelate any residual calcium in calcium-free medium M199. We also tested another chelator of [Ca²⁺]_e (5 mM EGTA, pH 7.2; Sigma).

SQ22536 (9-[tetrahydro-2'-furyl] adenine), a cell-permeable adenylyl cyclase inhibitor; H89, a protein kinase (PKA) inhibitor; Forskolin, a cell-permeable diterpenoid that possesses adenylyl cyclase activating properties; IBMX (3-isobutyl-1-methylxanthine), a non-specific inhibitor of cAMP phosphodiesterases (cAMP-PDE); and dibutyryl-cAMP (db-cAMP), a cell-permeable cAMP analog that activates PKA; Mastoparan-7 and 17; Zaprinast; Cholera toxin (CT) and Pertussis toxin (PTX) were all from Sigma. Rottlerin, an additional PKA inhibitor was obtained from Calbiochem.

3. Results

3.1. Parasite intracellular Ca^{2+} is required for sporozoite motility (BAPTA-AM inhibits sporozoite motility; its effects are reversed by the calcium ionophore A23187)

To test whether intracellular Ca^{2+} ($[\text{Ca}]_i$) is required for sporozoite motility, we examined the effects of BAPTA-AM, a cell-permeant, highly specific chelator of $[\text{Ca}]_i$, on motility of *P. berghei* sporozoites expressing either enhanced EGFP or (RFP). The results (Fig. 2) showed a concentration-dependent, highly significant effect of BAPTA-AM in inhibiting sporozoite motility between the tested concentrations of 25 to 100 μM ; this inhibitory effect was shown not to be due to the DMSO carrier of BAPTA-AM. The inhibition of motility reached a maximum of 70% at 100 μM BAPTA-AM, which we used as the standard inhibiting concentration for subsequent experiments. Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP (data not shown).

To show that this inhibition by BAPTA-AM was due to a reversible blocking of the mobilization of cytosolic Ca^{2+} rather than to killing of the parasites, we attempted to reverse its chelating effects on $[\text{Ca}]_i$ by use of the calcium ionophore, A23187. The results (Fig. 2) showed that even at the highest concentration of BAPTA-AM used, the inflow of extracellular Ca^{2+} ($[\text{Ca}]_e$) into the parasite induced by A23187 was able to almost completely reverse the inhibitory effects of BAPTA-AM on sporozoite motility. (Addition of A23187 in the presence of 1 mM CaCl_2 restored 89.8% of the motility inhibited by BAPTA-AM.) Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP.

3.2. Intracellular stores of Ca^{2+} are depleted over time; replenishment with medium containing Ca^{2+} re-induces sporozoite motility

To assess the requirement for $[\text{Ca}]_e$ in sporozoite motility, parasites were suspended in albumin-containing Medium 199 either with Ca^{2+} , or in the Ca^{2+} -free medium with addition of 1 mM BAPTA (a cell-impermeant chelator of Ca^{2+}) to chelate residual extracellular Ca^{2+} . The results (Fig. 3) showed that active sporozoite motility within the Ca^{2+} -containing media continued over the full 90 min observation period. Sporozoites in the Ca^{2+} -free media, however, exhibited a gradual decrease in motility over the 90 min course of the experiments, thus implying that reserve stores of $[\text{Ca}]_i$ could serve to sustain sporozoite motility for relatively long periods of time but were eventually depleted. Similar results were observed when 5 mM EGTA was used in lieu of BAPTA to chelate extracellular Ca^{2+} (data not shown).

To test whether the calcium-depleted sporozoites were still viable rather than having been killed by the deficiency of calcium or by exposure to calcium chelators for extended periods of time, we attempted to restore motility in these calcium-depleted sporozoites by washing them free of chelator and re-suspending them in calcium-containing control medium. After the 90 min time point, sporozoites from each preparation were washed by centrifugation at 10,000 g at 4 °C for 3 min, and resuspended in fresh medium M199 containing 1 mM CaCl_2 and 2% albumin. Sporozoites were incubated for another 30 min at 24 °C after which their motility was assessed. The results showed that active sporozoite motility was restored after re-exposure to $[\text{Ca}]_e$, thus

implying replenishment of $[Ca]_i$. Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP.

3.3.1. cAMP acts as an additional second messenger to induce sporozoite motility

Inhibiting intracellular formation of cAMP reduces sporozoite motility: To reduce levels of cAMP by inhibiting its formation via adenylyl cyclase (AC), we assessed the AC inhibitor SQ22536 for its effects on sporozoite motility. Motility of *P. berghei* sporozoites in Ca^{2+} -free medium M199 supplemented with 1 mM $CaCl_2$ and 2% albumin (positive control) was compared with medium to which had been added SQ22536. Results (Fig. 4A) showed a concentration-dependent inhibition of sporozoite motility. Inhibition of motility at 200 μ M and 500 μ M was 24.7% and 57%, respectively. Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP.

Inhibiting the downstream action of cAMP activity reduces sporozoite motility: To reduce the downstream action of cAMP by inhibiting the catalytic activity of cAMP-dependent PKA, we evaluated the PKA inhibitors H89 and Rottlerin on sporozoite motility. We added either H89 (100 versus 200 μ M) or Rottlerin (5 versus 35 μ M) to positive control medium, then maintained sporozoites at 24 °C for 30 min, after which we assessed them for gliding motility. The results (Fig. 4B) show that both inhibitors significantly reduced sporozoite motility in a concentration-dependent manner, thus suggesting the role of a PKA pathway in the induction of sporozoite motility and further implicating the involvement of cAMP as a second messenger in sporozoite motility. Inhibition of motility by H89 at 100 μ M and 200 μ M, respectively, was 31.8% and 74.3%. For Rottlerin, inhibition at 5 μ M and 35 μ M, respectively, was 40.9% and 49.5%. Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP.

3.4. Elevation of sporozoite intracellular cAMP circumvents the need for albumin to induce sporozoite motility

To test whether enhancing the synthesis of cAMP was able to induce sporozoite motility in the absence of albumin, we examined the effects of forskolin, a specific activator of AC (Insel and Ostrom, 2003). To test whether inhibiting the degradation of cAMP was similarly able to induce sporozoite motility in the absence of albumin, we examined the effects of IBMX, a non-specific inhibitor of cyclic nucleotide PDE (the enzyme that hydrolyzes cAMP to AMP). Finally, to test whether a cell-permeant cAMP agonist was also able to induce sporozoite motility in the absence of albumin, we examined the effects of db-cAMP. Studies were done as previously described, with these agents being added to albumin-free media. The results (Fig. 5) showed that each of these pharmacological agents was able to significantly initiate sporozoite motility in the absence of albumin. However, Zaprinast, an inhibitor of cyclic GMP-specific PDE failed to initiate sporozoite motility in the absence of albumin, even at levels up to 400 μ M (data not shown).

The ability to induce motility by 100 μ M forskolin, 100 μ M IBMX or 20 μ M db-cAMP was 82.3%, 107% and 146.5%, respectively (compared with the negative control without albumin). Similar results were obtained with *P. yoelii* sporozoites that expressed EGFP. When these agents were added to medium that already contained albumin, none had any significant effects on sporozoite motility (data not shown), implying that increased intracellular cAMP concentrations beyond endogenous levels were unable to enhance sporozoite motility.

3.5. Pharmacological agents known to influence heterotrimeric G-Proteins also influence sporozoite motility

To test the possible role of G-proteins in transduction of a signal from albumin across the sporozoite pellicle, we assessed the action of CT on sporozoite motility. CT normally acts

directly on the stimulatory trimeric G protein G_{α_s} , “locking” it in an active state, resulting in a sustained increase in AC activity. Motility of *P. berghei* sporozoites in Ca^{2+} -containing medium with 2% albumin (positive control) or no albumin (negative control) was compared with negative control medium with added CT. Results (Fig. 6A) showed a concentration-dependent effect of CT in initiating sporozoite motility in albumin-free medium, with 50 μ g/ml of CT significantly enhancing motility (72.9% increase in motility compared with the albumin-free negative control).

We next tested the ability of Mastoparan (Mas), a cell-permeable 14-residue peptide toxin from wasp venom, to inhibit sporozoite motility (Higashijima et al., 1988). Mas has been shown to specifically activate the $G_{\alpha_i}/G_{\alpha_o}$ inhibitory subgroup of trimeric G proteins (Higashijima et al., 1990); Mas-7 is an especially active mutant form. Studies were done as previously described, with Mas-7 added to albumin-containing media. The results (Fig. 6B) showed a significant concentration-dependent reduction of sporozoite motility by Mas-7 at 20 and 40 μ g/ml (29.3% and 40.0% decreases in motility, respectively, compared with the albumin-containing positive control). Pre-treatment of sporozoites with PTX abolished the ability of Mas-7 to inhibit sporozoite motility, whereas PTX alone had no detectable effects on motility (data not shown). PTX selectively catalyzes the ADP-ribosylation of $G_{\alpha_i}/G_{\alpha_o}$, thus preventing activation of these G proteins (Katada and Ui, 1982; Bokoch and Gilman, 1984).

Because Mas is known also to interact nonspecifically with membranes, we used as a control the Mas mutant, Mas-17, which also is membrane-active but lacks the specific heterotrimeric G-Protein-activating properties of Mas (Huber et al., 1997). Mas-17, even at 40 μ g/ml, did not inhibit sporozoite motility (Fig. 6B).

4. Discussion

After injection into the skin by mosquitoes, malaria sporozoites must reach the blood to continue their development in the liver. Most, if not all, sporozoites are released into avascular skin and s.c. tissue, then use gliding motility to reach and invade dermal blood vessels. Sporozoites are arrested during passage through liver sinusoids, then move through Kupffer cells to invade hepatocytes (Frevert et al., 2006). A notable characteristic of sporozoites is an ability to move with apparently little effort in and out of cells (Vanderberg et al., 1990; Mota et al., 2001). After invading a hepatocyte, sporozoites continue to move through several, ultimately remaining in one and differentiating into the next stage of parasite development, the hepatocytic exoerythrocytic form. Few cells have such a wide-ranging and adventurous existence. Underlying all is the ability of sporozoites to engage in gliding motility.

Some sporozoites exhibit gliding motility during passage through the mosquito from oocyst to salivary gland (Vanderberg, 1975); sporozoite motility within the glands and their ducts has been elegantly documented (Frischknecht et al., 2004). Gliding may be observed close to squashed gland preparations, suggesting mosquito-induced motility factors; motility is lost when sporozoites are diluted in medium without serum or albumin. Sporozoite motility within such diluted suspensions is triggered by albumin (Vanderberg, 1974). Two sporozoite proteins associated with gliding motility, CSP and TRAP, are secreted from the parasite's apical end (Stewart and Vanderberg, 1991; Spaccapelo et al., 1997; Sultan et al., 1997), then leave trails behind the gliding sporozoites (Stewart and Vanderberg, 1991; Kappe et al., 1999).

Studies with the apicomplexan protozoan *Toxoplasma* showed that microneme discharge follows intracellular stimulation by Ca^{2+} , which is an essential component of a signal transduction pathway that controls *Toxoplasma* microneme discharge and apical attachment (Carruthers and Sibley, 1999). Because albumin triggers motility in *Plasmodium* sporozoites,

we decided to assess the relationship between sporozoite exposure to albumin and intracellular signaling.

We tried, first, to confirm a role for Ca^{2+} signaling in *Plasmodium* sporozoite motility. We found that BAPTA-AM, a cell-permeant, $[\text{Ca}]_i$ chelator, showed a concentration-dependent inhibition of sporozoite motility. A reversal was seen when the calcium ionophore A23187 was added, indicating that BAPTA-AM acted by blocking mobilization of cytosolic Ca^{2+} rather than killing parasites. Using a different assessment of microneme secretion (translocation of TRAP to exterior), Gantt et al. (2000) had similar results; incubation of sporozoites with A23187 increased the numbers of sporozoites with TRAP surface caps. This was partially reversed by pre-treatment with BAPTA-AM.

Although $[\text{Ca}]_i$ plays a significant role in microneme secretion, motility and invasiveness by apicomplexans, there has been controversy over the role of $[\text{Ca}]_e$. Pezzella et al. (1997) reported that EGTA chelation of extracellular Ca^{2+} decreased *Toxoplasma* invasion. However, Lovett and Sibley (2003) reported normal invasiveness when the pH of the EGTA-containing medium was corrected, suggesting that intracellular Ca^{2+} stores were sufficient to maintain cellular functions. We now show that *Plasmodium* sporozoites continue motility in Ca^{2+} -free medium but with a decrease over time, thus indicating that $[\text{Ca}]_i$ can maintain motility in the absence of Ca^{2+} but that intracellular stores are eventually depleted. Replenishing these stores by adding Ca^{2+} to the medium restores sporozoite motility.

In apicomplexans, as in other cell types, cAMP is a central element of intracellular signaling. We used a pharmacological approach, namely inhibitors and enhancing agents to test cAMP for its effects on sporozoite motility. First, we used SQ22536 to inhibit AC (an enzyme leading to cAMP synthesis); we showed a concentration-dependent effect of SQ22536 in inhibiting motility. Pharmacological approaches must be used with caution, as some agents may have additional functions beyond affecting signaling. For instance, SQ22536 is structurally related to adenosine, and may also act as an adenosine antagonist (Schulte and Fredholm, 2002). Yet, a pharmacological approach is sometimes the only feasible way of dealing with parasites that can neither be cultured nor purified; it is best done by evaluating more than one agent, each of which inhibits a different target. Davies et al. (2000) recommended approaches to validate studies using PKA inhibitors in cell-based assays. Among these is that “the same effect is observed with at least two structurally unrelated inhibitors of the protein kinase” (Davies et al., 2000). We attempted to follow these strategies as much as possible. Table 1 summarizes agents we used and their effective concentrations; these are compared with other systems.

The roles of AC and cAMP in sporozoite invasion were explored by Ono et al., 2008, who assessed TRAP appearance at the sporozoite's apical end; they reported that increased cAMP levels stimulated exocytosis and activated sporozoite invasion. They also found that activation of exocytosis by cAMP-mediated pathways reduced sporozoite migration through host cells, confirming a previous report (Mota et al., 2002). Our own study focused on motility rather than invasion, so direct comparisons may not be applicable. Nevertheless, exocytosis of TRAP is necessary for both motility (Sultan et al., 1997) and invasion. Thus, apical regulation of exocytosis may vary depending upon whether the sporozoite is gliding extracellularly versus migrating intracellularly; in the latter case exocytosis seems to become associated with reduced sporozoite migration and enhanced infectivity. Sporozoites continue their motility in AC- α knockouts (Ono et al., 2008), thus implying a role for AC- β in synthesis of cAMP within these parasites. Further evidence of the role of cAMP was shown by our finding that PKA inhibitors H89 and Rottlerin reduced sporozoite motility. cAMP generally acts by stimulating PKA, a transducer of the cAMP second messenger system. *Plasmodium* possesses an expanded family of Ca^{2+} -dependent PKAs (CDPKs) (Billker et al., 2004). H89 was previously shown to block

Plasmodium falciparum erythrocytic development (Syin et al., 2001) and abolished cAMP-induced phosphorylation in *P. falciparum* extracts.

Having shown that Ca^{2+} and cAMP act as second messengers in signaling pathways activating sporozoite motility, we next examined whether elevating cAMP concentrations with pharmacological agents could replace albumin to initiate sporozoite motility. Synthesis of cAMP is controlled by AC; thus, we used an AC activator, forskolin, to increase cAMP levels. Degradation of cAMP is controlled by PDE, which hydrolyzes cAMP to AMP; thus, we used the non-specific PDE inhibitor, IBMX, to raise cAMP levels by inhibiting its degradation. Finally, we used a cell-permeant cAMP agonist, dibutyryl cAMP, to emulate the effects of cAMP. All of these initiated sporozoite motility without a need for albumin in the medium. Indeed, these are the first agents reported to bypass the albumin normally required to activate sporozoites. Our results imply that extracellular albumin initiates an intracellular cascade of second messengers, including cAMP and Ca^{2+} , resulting in initiation of sporozoite motility.

Because cGMP-specific PDE is found in *P. falciparum* (Yuasa et al., 2005) and *Plasmodium* gametogenesis is mediated by a cGMP-dependent PKA (McRobert et al., 2008), we tested whether cGMP rather than cAMP mediates sporozoite motility. Accordingly, we tested Zaprinast, an inhibitor of cGMP-specific PDE (Ziolo et al., 2003); it failed to initiate motility in the absence of albumin, further supporting a specific role of cAMP rather than cGMP in initiating sporozoite motility.

How the effects of albumin are transduced at the parasite surface to initiate intracellular signaling is yet to be determined. The cAMP signaling system is classically activated when a ligand binds to the cell surface component of a transmembrane receptor coupled to a G protein at the inner face of the plasma membrane (Neves et al., 2002). In many systems, CT acts directly on the alpha subunit of stimulatory G proteins ($\text{G}\alpha_s$), a physiological substrate for CT, while CT has little effect on inhibitory classes of G proteins (Serventi et al., 1992). $\text{G}\alpha_s$ hydrolyzes GTP, thus activating AC and formation of cAMP (Serventi et al., 1992). We found that CT in albumin-free medium induced sporozoite motility, thus circumventing the need for albumin. This might normally imply a role for heterotrimeric G proteins in transducing an albumin signal across sporozoite membranes to initiate formation of cAMP. However, a *P. falciparum* database (<http://www.ncbi.nlm.nih.gov/pubmed/12368864?dopt=Abstract>) failed to show the presence of heterotrimeric G proteins (Harrison et al., 2003), although other G proteins were reported (Thélu et al., 1994; Gardner et al., 2002). One group, however, reported heterotrimeric G proteins within *Plasmodium* (Dyer and Day, 2000).

Another pharmacological agent affecting heterotrimeric G proteins is Mas, which mimics ligand-bound G-protein receptors (Sukumar and Higashijima, 1992), specifically activating inhibitory $\text{G}\alpha_i/\text{G}\alpha_o$ trimeric G proteins (Higashijima et al., 1990) and disrupting G protein-coupled signaling. We found that Mas-7 inhibited sporozoite motility, which might normally imply that activation of $\text{G}\alpha_i/\text{G}\alpha_o$ protein prevents albumin from initiating G protein-mediated stimulation of sporozoite motility. The inhibitory effects of Mas-7 on motility were blocked by PTX, which functions by catalyzing ADP-ribosylation of $\text{G}\alpha_i$. PTX makes the inhibitory form of $\text{G}\alpha$ incapable of exchanging GDP for GTP, thus blocking the inhibitory pathway. Because true heterotrimeric G proteins are yet to be demonstrated in *Plasmodium*, however, one must use caution in interpreting our results with Mas and CT. Mas can stimulate Ca^{2+} release without acting through heterotrimeric proteins in plants (Miles et al., 2004; Sun et al., 2007) and animals (Hirata et al., 2000, 2003). Thus, Mas and CT may be affecting other targets involved in a pathway leading to sporozoite motility. Further studies are needed.

Serum albumin was reported to initiate intracellular second messenger cascades in other systems, e.g., elevation of $[\text{Ca}]_i$ in microglia (Hooper et al., 2005). Indeed, albumin has been

found to bind to a 60-kDa glycoprotein (albondin), which initiates endocytosis via signaling through $G\alpha_i$ (Minshall et al., 2000, 2002). A wide range of albumin molecules from different species, extending even to ovalbumin, were shown capable of inducing sporozoite motility (Vanderberg, 1974). Thus, a conserved epitope of the albumin molecule is likely responsible for triggering a cascade leading to sporozoite motility. How this acts through the thick pellicle of the *Plasmodium* sporozoite is yet to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Typical microscope fields of sporozoites in control media. Figures were prepared from 30 s time-lapse videos, each processed to create a single “total projection” showing sporozoite paths for the 30 s period. A) Positive control medium containing albumin; shows seven motile sporozoites and one non-motile sporozoite at lower left (= 87.5% motility). This was prepared from Supplementary Video S1. B) Negative control medium without albumin; shows one motile sporozoite at lower center and three non-motile sporozoites (= 25% motility). This was prepared from Supplementary Video S2. Bar = 50 μ m.

Fig. 2.

The intracellular Ca^{2+} chelator, BAPTA-AM [1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid tetra-(acetoxymethyl) ester] reduces motility of *Plasmodium berghei* sporozoites; its effects are reversed by the calcium ionophore A23187. *Plasmodium berghei* sporozoites were resuspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control), or with the addition of 0.4% DMSO (DMSO control), or in various concentrations of BAPTA-AM (B-AM) plus 0.4% DMSO used as a diluent for B-AM. ***($P < 0.0001$). Addition of A23187 reversed the inhibitory effects of BAPTA-AM; there was no significant difference between this mean and the positive control mean ($P > 0.05$). A23187 added alone to control medium had no significant effects on sporozoite motility. For

this and each of the subsequent Figures, each bar is the mean of three independent experiments $\pm$ S.E.M., with at least 40 sporozoites observed for each preparation in each experiment.

Fig. 3.

Extracellular Ca^{2+} $[\text{Ca}]_e$ is not essential for *Plasmodium berghei* sporozoite motility in the presence of albumin. *Plasmodium berghei* sporozoites were in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control), or in this Ca^{2+} -free medium supplemented with albumin but without CaCl_2 and with addition of 1 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) to chelate any residual extracellular calcium (bars labelled '+BAPTA'). Sporozoites were maintained at 24° C and aliquots were assessed for sporozoite motility at 30, 60 and 90 min. Positive control preparations showed no decrease in sporozoite motility over the 90 min course of the studies, whereas there were gradual decreases in motility over time in the Ca^{2+} -free medium. * ($P < 0.05$), *** ($P < 0.0001$). Motility of the calcium-depleted sporozoites was restored by washing those free of chelator at 90 min, then re-suspending them in Ca^{2+} -containing control medium. After 30 min further incubation, motility was re-assessed. Black bar at far right (120 min) indicates restoration of motility after re-exposure to $[\text{Ca}]_e$. There was no significant difference between this mean and the equivalent positive control mean ($P > 0.05$).

Fig. 4.

cAMP acts as an additional second messenger to induce *Plasmodium berghei* sporozoite motility. A) Inhibiting intracellular formation of cAMP reduces sporozoite motility. *Plasmodium berghei* sporozoites were suspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control), or in this medium supplemented with various concentrations of the adenylyl cyclase inhibitor SQ22536 (* $P < 0.05$), ***($P < 0.0001$)), B) Inhibiting cAMP activity reduces sporozoite motility. *Plasmodium berghei* sporozoites were suspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control), or with the addition of 0.4% DMSO (DMSO control) or in medium supplemented with various concentrations of the cAMP-dependent protein kinase

inhibitors H89 or Rottlerin plus 0.4% DMSO used as a diluent for H89 and Rottlerin. **($P < 0.01$), ***($P < 0.0001$).

Fig. 5.

Elevation of *Plasmodium berghei* sporozoite intracellular cAMP circumvents the need for albumin to induce sporozoite motility. *Plasmodium berghei* sporozoites were suspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control) or without albumin (negative control), or with the addition of 0.4% DMSO (DMSO control), or in negative control medium supplemented with either 20 µM dibutyryl-cAMP (db-cAMP) or 100 µM forskolin or 100 µM (3-isobutyl-1-methylxanthine) (IBMX) plus 0.4% DMSO used as a diluent for forskolin and IBMX. ***($P < 0.0001$).

Fig 6.

Pharmacological agents known to act upon heterotrimeric G proteins are able to modulate *Plasmodium berghei* sporozoite motility. A) Addition of cholera toxin (CT) circumvents the need for albumin to induce sporozoite motility. *Plasmodium berghei* sporozoites were suspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control) or without albumin (negative control), or in negative control medium supplemented with CT. **($P < 0.01$). B) Inhibition of sporozoite motility by Mastoparan (Mas-7). *Plasmodium berghei* sporozoites were suspended in Ca^{2+} -free medium M199 supplemented with 1 mM CaCl_2 and 2% albumin (positive control with albumin), or in this medium supplemented with Mas-7. **($P < 0.01$), ***($P < 0.0001$). Pre-treatment of

sporozoites with pertussis toxin (PTX) reversed the inhibitory effects of 40 µg/ml Mas-7. Mas-17, a Mastoparan mutant that lacks the specific G protein-activating properties of Mas-7, did not inhibit sporozoite motility, even at 40 µg/ml.

Table 1
Pharmacological agents used: specificities and comparison with effects reported in other systems.

Tested compound	Specificity	Mammalian IC ₅₀ /EC ₅₀	Inhibition/activation range in parasites and other organisms	Current Data
SQ22536	Adenylyl cyclase inhibitor	200 μM ^a	20 mM (<i>Schistosoma mansoni</i>) ^j 5 mM (<i>Brugia pahangi</i>) ^k 1 mM (<i>Dicrocoelium</i>) ^l	200 μM = 24.7% inhibition 500 μM = 57% inhibition
IBMX	Non-specific inhibitor of cAMP and cGMP phosphodiesterases (PDE)	2–50 μM ^b	> 200 μM (<i>Plasmodium falciparum</i>) ^m 126.2 μM (<i>Trypanosoma cruzi</i>) ⁿ > 100 μM (<i>P. falciparum</i>) ^o	100 μM = 107% enhancement in albumin-free medium
Zaprinast	Specific cGMP-PDE inhibitor	0.5 μM ^c	728 μM (<i>T. cruzi</i>) ⁿ	No inhibition up to 400 μM
Forskolin	Adenylyl cyclase activator	30 μM ^d	10–100 μM (<i>S. mansoni</i>) ^p 100 μM (<i>Entamoeba histolytica</i>) ^q 100 μM (<i>P. falciparum</i>) ^r	100 μM = 82.3% enhancement in albumin-free medium
db-cAMP	Membrane-permeable cAMP analog	116 μM ^d	5 mM (<i>E. histolytica</i>) ^q 100 μM (<i>T. cruzi</i>) ^s	20 μM = 146.5% enhancement in albumin-free medium
H89	Selective, potent inhibitor of cAMP-dependent protein kinase	80 nM–2.8 μM ^e	100 μM (<i>P. falciparum</i>) ^t	100 μM = 31.8% inhibition 200 μM = 74.3% inhibition
Rotlerin	Protein kinases inhibitor	5.4 μM ^e	5–35 μM (<i>T. cruzi</i>) ^u	5 μM = 40.9% inhibition 35 μM = 49.5% inhibition
Cholera Toxin	Gα _s activator	0.2 μg/ml ^f 32 ng/ml ^g	50 μg/ml (<i>P. falciparum</i>) ^v	50 μg/ml = 72.9% enhancement in albumin-free medium
<i>Mas-7</i>	Gα _i /Gα _o activator	10 μM ^h 1–2 μM ⁱ	20–40 μg/ml (<i>T. cruzi</i>) ^w 10 μM (14.2 μg/ml) (<i>Mycoplasma hyopneumoniae</i>) ^x	20 μg/ml = 29.3% inhibition 40 μg/ml = 40% inhibition

^aReid et al., 1990;
^bBeavo et al., 1970;
^cLoughney et al., 1998;
^dBrodsky et al., 1998;
^eDavies et al., 2000;
^fAiyar et al., 1999;
^gSmit et al., 1996;

- ^hKonrad et al., 1995;
- ⁱKlinker et al., 1994;
- ^jMatsuyama et al., 2004;
- ^kGunawardena et al., 2003;
- ^lAlvarez-Curto et al., 2007;
- ^mMcRobert et al., 2008;
- ⁿAlonso et al., 2006;
- ^oYuasa et al., 2005;
- ^pHumphries et al., 2004;
- ^qFranco et al., 2002;
- ^rRead et al., 1991;
- ^sHeath et al., 1990;
- ^tSudo et al., 2008;
- ^uBelaunzarán et al., 2009;
- ^vDyer and Day, 2000;
- ^wRodriguez et al., 1999;
- ^xPark et al., 2002.