Journal of Bioenergetics and Biomembranes, Vol. 35, No. 4, August 2003 (© 2003)

Vacuolar Proton Pumps in Malaria Parasite Cells

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The malaria parasite is a unicellular protozoan parasite of the genus *Plasmodium* that causes one of the most serious infectious diseases for human beings. Like other protozoa, the malaria parasite possesses acidic organelles, which may play an essential role(s) in energy acquisition, resistance to antimalarial agents, and vesicular trafficking. Recent evidence has indicated that two types of vacuolar proton pumps, vacuolar H^+-ATPase and vacuolar H^+-pyrophosphatase, are responsible for their acidification. In this mini-review, we discuss the recent progress on vacuolar proton pumps in the malaria parasite.

KEY WORDS: Malaria parasite; *Plasmodium falciparum*; vacuolar H^+-ATPase; vacuolar H^+-pyrophosphatase.

INTRODUCTION

Plasmodium is a parasitic unicellular protozoan that causes one of the most serious infectious diseases for human beings. The malaria parasite has a complex life cycle (Fig. 1). Upon invasion of erythrocytes in a host, the parasite lies dormant for some hours and then starts rapid growth followed by cell division, resulting in the generation of 20–30 new parasite cells. During this development, the malaria parasite develops complex membrane systems outside itself, and takes up hemoglobin and digests it in food vacuoles, speculative counterparts of mammalian lysosomes, followed by energy acquisition for growth and cell division (Halder, 1996; Lingelbach and Joiner, 1998). The digestive vacuoles (food vacuoles) are also known to be the site of accumulation of antimalarial agents (Halder, 1996; Lingelbach and Joiner, 1998). However, even at the present time, little is known about the nature of the proton pumps as well as of acidic organelles in *Plasmodium*. This is partly due to the difficulty of application of various biochemical and molecular biological approaches due to the extremely high AT ratio of the *Plasmodium* genome. In this mini-review we describe the progress on vacuolar proton pumps and acidic organelles in *Plasmodium* achieved within the last couple years.

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V-ATPase

V-ATPase is a multi-subunit protein complex comprising distinct catalytic and membrane sectors, whose subunit structure is well conserved among animals, plants, fungi, and even some bacteria. V-ATPase in *Plasmodium* has been studied in *P. falciparum*, which causes the most severe human malaria. The cDNAs encoding V-ATPase subunits *A* and *B* have been cloned and sequenced, and immunological counterparts of these subunits have been identified (Karcz *et al.*, 1993, 1994). The gene for subunit *A*, localized on chromosome 13 as a single copy, encodes a polypeptide of 611 amino acids that exhibits about 60% amino acid identity to known counterparts from various origins (Karcz *et al.*, 1993). The gene for subunit *B*, localized on chromosome 4, encodes a polypeptide of 494 amino acids exhibiting about 70% amino acids identity (Karcz *et al.*, 1994). Important regions for V-ATPase function, which includes the ATP-binding motif, are well conserved. Very recently, the cDNA for the c subunit proteolipid has been successfully cloned and sequenced (Yatsushiro *et al.*, manuscript in preparation). The proteolipid gene, located on chromosome 5, encodes an extremely hydrophobic polypeptide of 165 amino acids

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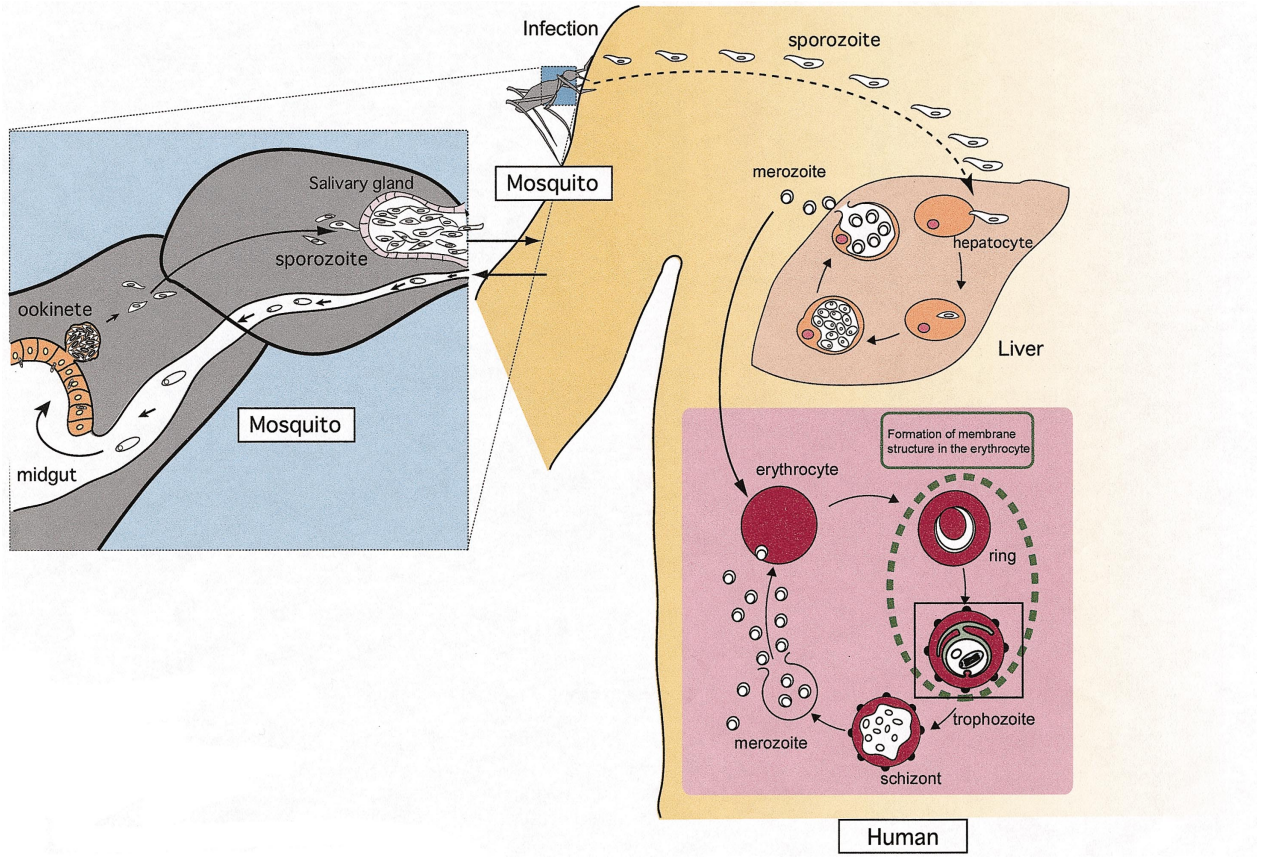

Fig. 1. Complex life cycle of the malaria parasite. The malaria parasite enters mosquito cells through the midgut and forms an ookinete. Then, the ookinete transforms into a sporozoite in the salivary gland. The mosquito injects the sporozoite stage of the malaria parasite during the ingestion of blood. The sporozoite enters a hepatocyte, and enters the merozoite stage of the parasite. Then, the merozoite is released from the hepatocyte into the blood stream and then enters an erythocyte. After erythrocyte invasion, the parasite synthesizes a membrane structure outside it self, and starts to absorb nutrients and to extrude metabolites during the ring and trophozoite stages, which last about 30 h. The parasite also takes up erythrocyte cytoplasm, particularly haemoglobin, and digests it in food vacuoles. Then, the parasite divides into more than 10 particles (schizont stage) and enters the blood stream to start the cycle again upon punctulation. The merozites again infect the mosquito during the ingestion of blood. Vacuolar proton pumps may be involved in each step of the life cycle.

exhibiting 52% identity to known proteolipids. These V-ATPase genes are transcribed throughout all stages of the life cycle.

Trial to isolate cDNAs for other subunits is not challenged at present, because of the difficulty of complementation of parasite cDNAs with yeast null mutants. However, the malaria genome project has advanced to the second round stage of the study on V-ATPase. The putative genes encoded V-ATPase subunits were nominated, which are summarized in the Table I. Besides subunits *A*, *B*, and *c*, essentially all subunits of V1 sector as well as V0 can be identified. The only exception is the c′ subunit gene, which has been shown to be an essential subunit in yeast and *C. elegans* (Oka *et al.*, 1998; Umemoto *et al.*, 1991), but not found in the *Plasmodium* genome. Although studies on the subunit expression at the protein level are required, the presence of essentially all genes for V-ATPase subunits strongly suggests that authentic V-ATPase is functional in the malaria parasite. The deduced amino acid sequences of the individual genes indicated that there is some similarity to countersubunits of plant and animal origin. Overall, the similarity is much greater to those of plant origin than those of animal origin. Interestingly, *Plasmodium* expresses functional plant type H^+-pyrophosphatase (VPPase). The coexpression of plant-type vacuolar proton pumps suggests that *Plasmodium* possesses a similar acidic organelle system to those of the plant kingdom. The properties of *Plasmodium* VPPase are discussed later. It is noteworthy that there are no changes in the sequences of the coding regions of the genes encoding the *A*, *B*, and *c* subunits between a few chloroquine-sensitive strains and chloroquine-resistant strains (Karcz *et al.*, 1993, 1994;

Table I. Subunits of V-ATPase in *P. falciparum* Predicted in the Malaria Genome Project

		P. falciparum				Yeast	
	Subunit	MW (kDa)	Gene number	Chromosome	Homology to yeast counterpart (%)	Gene	MW (kDa)
V_1	A	71.8	PF13 0065	13	56.2	*VMA 1*	69
	B	55.7	PFD0305c	4	62.5	*VMA 2*	57
	C	45.2	PFA0300c	1	26.8	*VMA 5*	42
	D	31.1	PF13 0227	13	47.3	*VMA 8*	32
	E	28.9	PFI1670c	9	30.7	*VMA 4*	27
	F	16.2	PF11 0412	11	41.3	*VMA 7*	14
	G	14.2	PF13 0130	13	32.0	*VMA 10*	13
	H	53.8	PF13 0034	13	25.6	*VMA 13*	54
V_0	a	122.9	PF08 0113	8	29.4	*VPH 1/STV 1*	95
	d	47.8	PF14 0615	14	34.9	*VMA 6*	36
	c	17.0	PFE0965c	5	51.5	*VMA 3*	17
	c'		—			*VMA 11*	17
	c"	22.1	MAL13P 1.271	13	48.8	*VMA 16*	23

Note. So far, cDNAs encoding subunits *A*, *B*, and *c* have been cloned. The malaria genome project predicts the presence of genes for V-ATPase subunits. The numbers of these genes, molecular weights, numbers of amino acid residures, and the homology to yeast counterparts of the predicted subunits are indicated. MW = Molecular weight.

Yatstushiro *et al.*, manuscript in preparation). These results suggest that V-ATPase is not be involved in the chloroquine resistance phenotype.

As to the localization of V-ATPase, Cowman and his colleagues have pointed out that immunoreactivity is not confined with membranes of the food vacuoles, thus making it unlikely that the only role of V-ATPase is the regulation of food vacuoles (Karcz *et al.*, 1993, 1994). The localization of the V-ATPase moiety at the subcellular level has been extensively studied using an immunoelectron microscopical procedure with antibodies against subunits *A* and *B* (Hayashi *et al.*, 2000). Electron microscopy clearly indicated that there are complex subcellular organelles in infected erythrocytes: food vacuoles can be identified as large vacuole-like organelles containing crystals of haemozoin, a nonhydrolyzable metabolite of haemoglobin. There are small clear vesicles, a single mitochondrion, and a complex outer membrane network (parasitophorus vacuolar membrane network). Immunogolds for V-ATPase subunit *A* or subunit *B* selectively labeled food vacuoles (Fig. 2(A) and (C)), small clear vesicles (Fig. 2(A) and (D)), and the plasma membrane (Fig. 2(A) and (E)). Immunogolds were also occasionally observed in the parasitophorus vacuolar membrane (Fig. 2(F)), whereas essentially no labeling was seen in the nuclear envelope, nucleus (Fig. 2(G)) and mitochondrion (Fig. 2(H)). Thus, it is clear that V-ATPase in *P. falciparum* cells is localized in at least three distinct major compartments; food vacuoles, small clear vesicles and the plasma membrane. Quantitatively, the absolute number of V-ATPase molecules in the plasma membrane and food vacuoles was roughly 16,000 and 2200 particles based on the assumption that a parasite cell contains a single ball-shaped plasma membrane and a ball-shaped food vacuole with diameters of 2.5 and 0.8 μm, respectively (Hayashi *et al.*, 2000). It is noteworthy that the cytoplasmic side, but not the luminal side, of these organelles was selectively labeled by the immunogolds, suggesting that V1 faces the cytoplasm. This orientation of the V-ATPase moiety is the same as that in various known organelles of plants and animals, and implies the direction of the proton transport across these membranes.

It is important to ask whether the V-ATPase in these organelles is functional. The use of detergent-permeabilized parasites indicated the ATP-dependent acidification of intracellular organelles, which was sensitive to bafilomycins and concanamycins, indicating functional operation of V-ATPase (Hayashi *et al.*, 2000; Saliba *et al.*, 2003; Saliba and Kirk, 1999). It is tempting to speculate that the V-ATPase identified in the plasma membrane

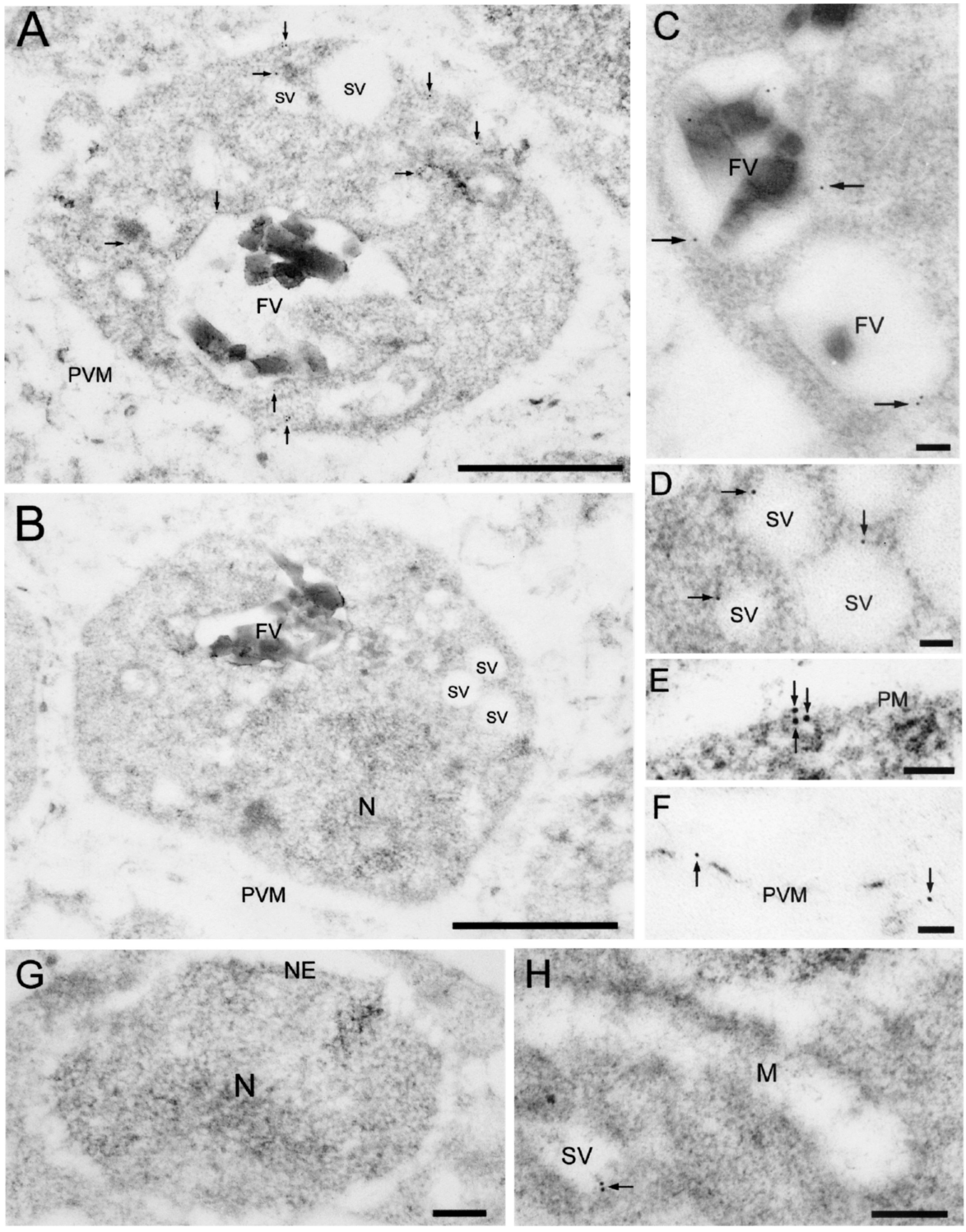

Fig. 2. Localization of V-ATPase in *P. falciparum.* Immuno-gold electron microscopy indicated that V-ATPase is mainly localized in the plasma membrane (PM), small clear vesicles (SV), food vacuoles (FV), and parasitophorus vacuolar membrane (PVM). Infected eryhtrocytes were fixed and embedded in LR White resin and then processed for the postembedding immuno-gold method. Arrows indicate gold particles. N, nucleus; M, mitochondrion; NE, nuclear envelope. The pictures are taken from a paper by Hayashi *et al.* (2000). ar $= 1$ μm.

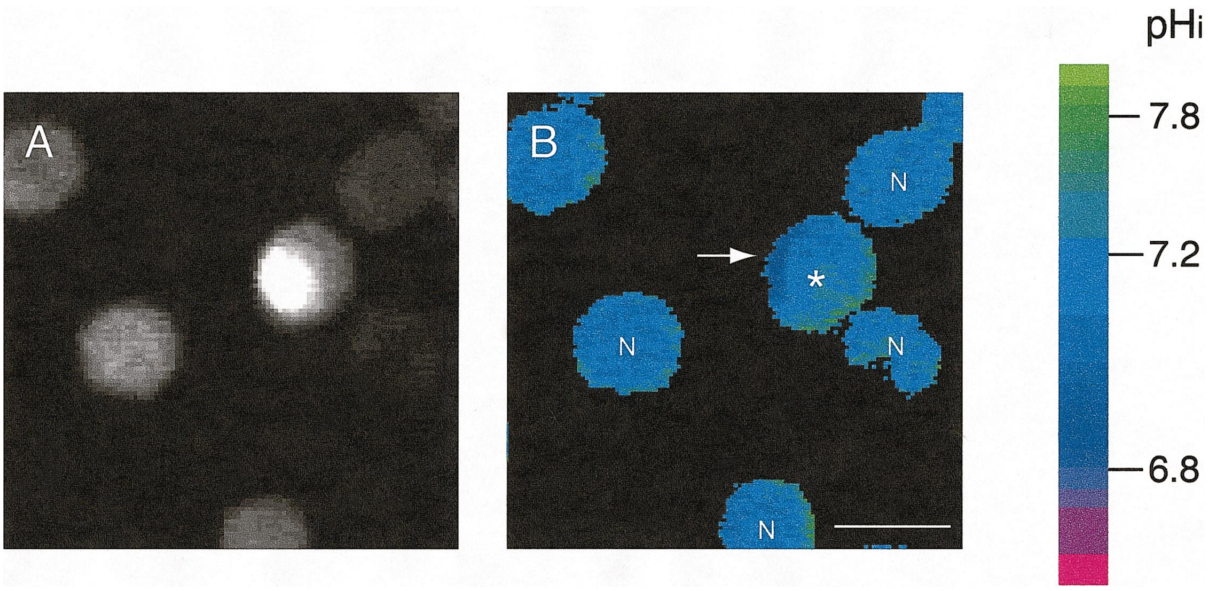

Fig. 3. Evidence for proton extrusion from infected *P. falciparum* cells. Infected erythrocytes exhibited bafilomycin-sensitive proton extrusion that acidifies a restricted area of the erythrocyte cytoplasm, an electrochemical gradient of protons being formed across the parasite cell membrane. The pH gradient may be involved in the uptake of nutrients.

extrudes protons from a parasite cell into the cytoplasm of an infected erythrocyte, causing acidification of a restricted region outside the plasma membrane. To monitor such presumptive proton extrusion from a parasite cell, the cytoplasmic pH of 2′,7′-bis-(2-carboxyethyl)-5,6-carboxylfuorescein (BCECF)-loaded parasitized erythrocytes was fluorometrically measured (Hayashi *et al.*, 2000). The cytoplasmic pH of parasitized erythrocytes and infecting parasite cells as well as uninfected erythrocytes was found to be essentially the same (7.30 ± 0.02, 30 determinations for four independent preparations) (Hayashi *et al.*, 2000), which is consistent with that previously reported (Saliba and Kirk, 1999; Wünsch *et al.*, 1998). It was found that a crescent shaped acidic region of pH 6.9 ± 0.03 is always present near the parasite cells at the trophozoite stage in erythrocytes (Fig. 3). Since the acidic pH is neutralized by bafilomycins and concanamycins, but not insensitive to vanadate and oligomycin, supports that V-ATPase at the plasma membrane pumps protons extracellularly and acidifies restricted areas near the membrane (Hayashi *et al.*, 2000). The physiological significance of the acidic region outside the parasite is discussed later.

It is difficult to directly measure V-ATPase-mediated proton transport in small clear vesicles, since a procedure for isolation of small particles has not been established yet. To estimate the proton transport, we applied the amine-induced vacuolation procedure, which was established more than 20 years ago (Poole and Ohkuma, 1981). This procedure is based on the ΔpH-dependent accumulation of amines in acidic compartments followed by the entry of water, causing vacuolation. The vacuolated organelles can be regarded as acidic compartments in which protons are actively supplied. Some examples are presented (Fig. 4). Upon either ammonium chloride- or methylamine-evoked vacuolation, many vacuolated structures can be observed. Some of the latter originate from food vacuoles, because they contain heamozoin, and some seem to originate from small clear vesicles. Such vacuolation was not observed when infected erythrocytes were treated with bafilomycin or concanamycins, suggesting that electrogenic proton entry occurs in small vesicles as well as food vacuoles. Overall, it can be concluded that V-ATPase in the plasma membrane and small clear vesicles as well as food vacuoles is functional under physiological conditions. The role of the small clear vesicles is unclear at present. It is possible that the acidic pH of the small vesicles might be involved in the trafficking pathways, as in the cases of other eukaryotic cells.

VACUOLAR H^+-TRANSLOCATING PYROPHOSPHATASE (V-PPase)

Plant vacuoles possess proton pumping pyrophosphate (V-PPase) as another vacuolar proton pump. This enzyme translocates protons across a membrane by using potential energy liberated on hydrolysis of the phosphoanhydride bond of inorganic phosphate (reviewed in Rea and

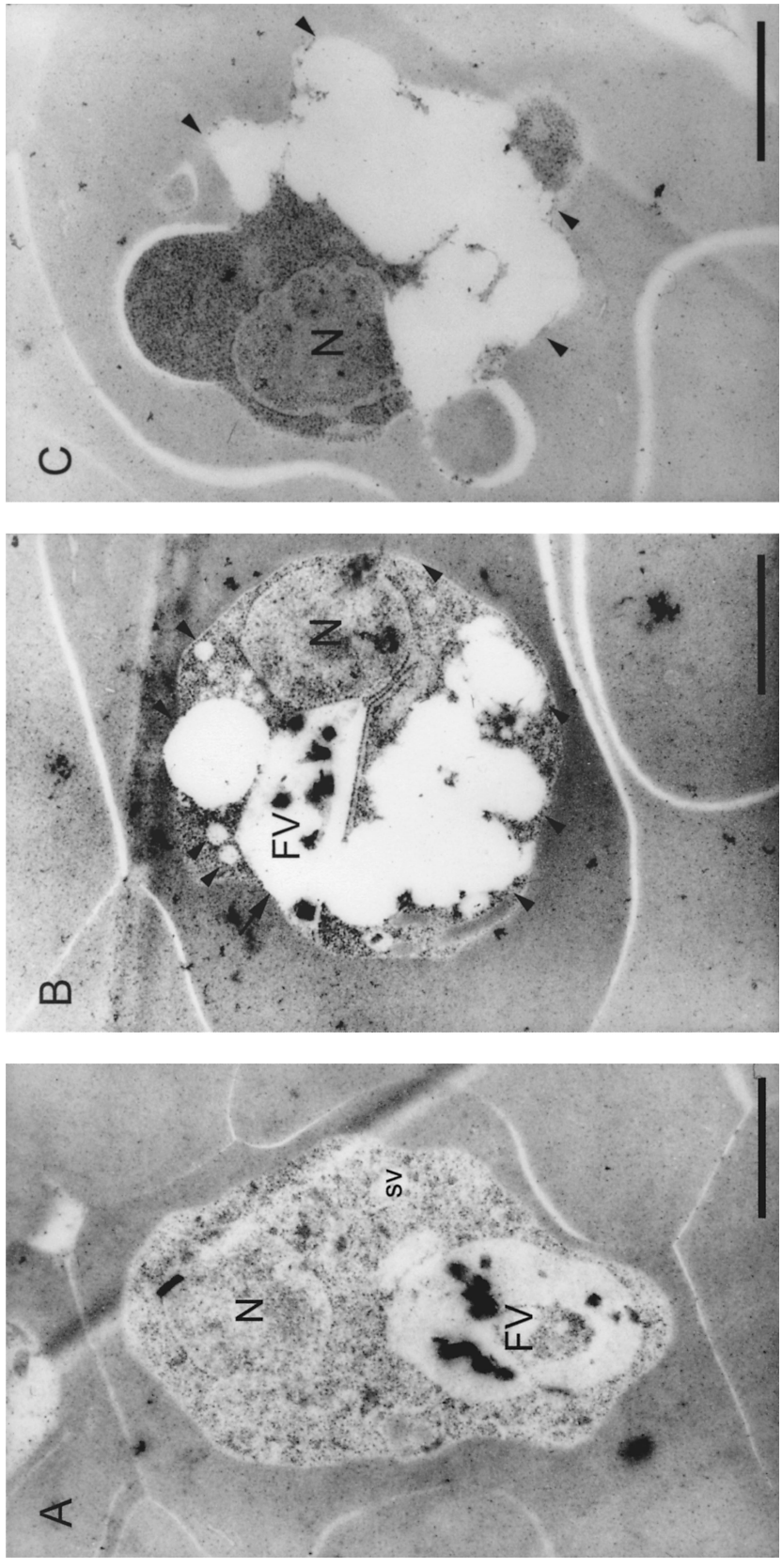

Fig. 4. Amine-induced vacuolation suggests that the acidic pH in small clear vesicles as well as food vacuoles is maintained through an active mechanism. Infected erythrocytes were not treated (A) or treated with ammonium chloride at 50 mM (B) or methylamine at 10 mM (C) for 30 min. Samples were fixed, and thin-sectioned specimens were made according to Hayashi *et al.* (2000), and observed under an electron microscopy. Vacuoles originating from a food vacuole (FV, an arrow) and small vesicles (SV, arrowheads) are indicated. Vacuolation was not observed if parasite cells were pretreated with concanamycin B at 1 μM. Bar = 1 μm. N, nucleus.

Poole, 1993). Pyrophosphate is produced through several major metabolic pathways including synthesis of DNA and RNA, and should be an abundant and cheap energy source even in the malaria parasite. The occurrence of V-PPase seems to be specific for the plant and bacterial kingdoms, there being no evidence so far of the expression of V-PPase in the animal kingdom. V-PPase constitutes a single and highly conserved polypeptide with an apparent molecular mass of 56–78 kDa and localized in vacuolar membranes with the same orientation as V-ATPase. Thus, the addition of pyrophosphate and ATP can drive V-PPase and V-ATPase, causing the energization of vacuoles. The plant V-PPases are well conserved membrane proteins with a 15- or 16-transmembrane topology (Rea and Poole, 1993). The malarial parasite also possesses V-PPase (reviewed in McIntosh and Vaidya, 2002).

It was found that pyrophosphate promotes the acidification of cell homogenates of *P. falciparum* at the trophozoite stage, as revealed by the uptake of acridine orange (Luo *et al.*, 1999). The acidification is electrogenic, requires K^+ and Mg^{2+}, and is inhibited by aminomethylenediphosphate (AMDP). Furthermore, an immunological counterpart of plant V-PPase has been identified in the subcellular compartments of the parasite cells. These results constitute the first evidence of the presence of V-PPase in *Plasmodium*. *Plasmodium* V-PPase requires Mg^{2+} for its activity, and recognizes a complex of Mg pyrophosptate, most probably Mg^{2+} pyrophosphate, as a substrate. V-PPase activity also requires a modest concentration of K^+ (around 50 mM) (Luo *et al.*, 1999; Marchesini *et al.*, 2000; McIntosh *et al.*, 2001). These properties are similar to those of plant V-PPases. Other protozoa including *Trypanosoma cruzi* (Scott *et al.*, 1998), *T. brucei* (Rodrigues *et al.*, 1999a), and *Leishmania donovani* (Rodrigues *et al.*, 1999b) possess similar V-PPases.

Molecular biological and biochemical studies have indicated the presence of an isoform that pumps protons across the membranes at the expense of hydrolysis of pyrophosphate even in the absence of K^+. Thus, plant V-PPases can be classified into two subclasses, VP1, K^+-dependent PPases (Sarafian *et al.*, 1992), and VP2, K^+-independent PPases (Drozdowicz *et al.*, 2000). In *P. falciparum*, two genes encoding corresponding VP1 and VP2 have been identified (McIntosh *et al.*, 2001). *Pf* VP1 was found to be similar in size to other known VP1s, exhibiting 60% similarity and 51% identity to the prototype VP1. The two genes were actually transcribed in the parasite cells. Thus, like in plant tissues, two types of V-PPase may function in the malaria parasite.

The subcellular localization of V-PPase may provide a clue as to the physiological significance of the enzyme. Indirect immunofluorescence microscopy with polyclonal antibodies indicated that *Pf* VP1 is present within the vacuolar membrane structure, possibly food vacuoles (Luo *et al.*, 1999; McIntosh *et al.*, 2001). It was found that *Pf* VP1-GFP fusion protein can be targetted to the plasma membrane (McIntosh *et al.*, 2001). The data obtained so far indicated the more condensed localization of V-PPase in food vacuoles, and lesser amounts of localization can be seen in the plasma membrane. Thus, it is possible that V-PPase and V-ATPase are colocalized and acidify the same compartment. The occurrence of the functional combination of V-ATPase and V-PPase has been demonstrated in acidocalcisomes (Marchesini *et al.*, 2000), and food vacuoles (Saliba *et al.*, 2003), as described later. However, evidence of the functional occurrence of V-PPase in the plasma membrane has not been obtained yet.

H^+-LINKED SECONDARY TRANSPORTERS

The malaria parasite at the intraerythrocytic stage has at least three distinct acidic compartments: food vacuoles, small clear vesicles, and a restricted extracellular space. Like other membrane systems of eukaryotes and prokaryotes, these proton pumps form an electrochemical gradient of protons across the membranes so as to energize the organelles. Thus, it is possible that H^+-linked secondary transporters operate in these organelles.

Parasites can take up various neutrients including sugars and various vitamins and drugs through the plasma membrane (Lauer *et al.*, 1997). However, information on H^+ coupled secondary transporters is scarce. Chloroquine, an antimalarial agent, is known to be taken up by the parasite via a specific transport system (Sanchez *et al.*, 1997). Since chloroquine uptake is sensitive to amiloride derivatives, inhibitors of Na^+/H^+ antiporters, Na^+/H^+ antiporter is speculated to be somehow involved in the uptake process (Sanchez *et al.*, 1997; Wünsch *et al.*, 1998). However, the presence of Na^+/H^+ antiporter at the parasite plasma membrane unclear, and the energy coupling of chloroquine uptake with such antiporters should be reconsidered.

The first evidence of a H^+ coupled transporter was obtained recently as to pantothenate (vitamin B5), a precursor of coenzyme A (CoA) and one of the absolute extracellular requirments of the parasite (Saliba and Kirk, 2001). Pantothenate enters malaria-infected erythrocytes via specific transport systems. These transport systems show the distinct features to known Na^+-dependent transporters with low affinity to a substrate (around 23 mM). The uptake is sensitive to proton conductor such as CCCP, and a stoichiometry of $1H^+$ and 1 pantothenate has been calculated (Saliba and Kirk, 2001). Once taken up, panthothenate is rapidly phosphorylated by a high-affinity

panthothenate kinase and converted to CoA (Saliba *et al.*, 1998). Another H^+ coupled transporter for lactate and pyruvate has also been reported (Elliott *et al.*, 2001). Glucose is also rapidly taken up by the parasite plasma membrane through a facilitated hexose transporter and is metabolized through glycolysis (Woodrow *et al.*, 1999), with an approximately 100-fold increase in glucose utilization ability as compared with uninfected erythrocytes. Since the malaria parasite lacks functional TCA cycles, this higher glycolysis activity increases the formation and export of lactic acid by infected cells. To avoid an increase in osmotic stress and interference with oxidation of NADH to NAD^+, the parasite must secrete lactate. Consistently, malaria parasite infection gives rise to extensive extracellular acidosis (White, 1998). Thus, it is believed that the malaria parasite may somehow extrude protons outside cells, although little is known about the mechanism underlying this proton extrusion. Isolated parasite cells can take up lactate depending on an acidic pH (Elliott *et al.*, 2001). The stoichiometry of H^+ and lactate influx is 1. The lactate transport activity is sensitive to phloretin, and the overall properties are similar to those of the H^+/monocarboxylate symporter in mammalian cells. They also showed that lactate can be secreted through the H^+/lactate or pyruvate symport system. Under physiological conditions, this symport system may function in extruding lactate and H^+.

Information as to H^+ coupled secondary transport in food vacuoles is scarce. Although antimalarial agents such as chloroquine are well known to accumulate in food vacuoles, no specific transporters have been found. Cycloprodigiosin, a fluorescent Cl^-/H^+ exchanger having potent antimalarial activity, also accumulates into food vacuoles (Kim *et al.*, 1999). Since these antimalarials are amphipathic amines, carrier-independent partitioning according to a pH gradient can be considered for the mechanism.

Recently, an acidic calcium pool(s) abbreviated as acidocalcisome has been detected in some pathogenic protozoa including *Trypanosomes* (reviewed in De Souza, 2002; Docampo and Moreno, 2001). Typical acidocalcisomes in trypanosomatids show acidic internal pH with high electron density under an electron microscope, and has a matrix consisting of calcium, pyrophosphate, and so on (Rodrigues *et al.*, 2002). Similar acidic calcium pools have been detected in intraerythrocytic malaria parasite (Garcia *et al.*, 1998; Marchesini *et al.*, 2000). Calcium ions play important roles in signal transduction, development and infectious processes in the malaria parasite. Thus, their free concentration in the cytoplasm is carefully controlled. Intracellular free Ca^{2+} in the parasite was measured for either Fluo-3 or Fura2-loaded parasite cells. Under physiological conditions, free Ca^{2+} is maintained at about 100 nM. The Ca^{2+} concentration increased upon either addition of thapsigargin or the treatment of nigericin in the presence of K^+. Bafilomycins, AMDP and sodium fluoride gave the similar effect. From these results, it is suggested that Ca^{2+}-ATPase, V-ATPase, and V-PPase are responsible for accumulation of Ca^{2+}, as in the case with acidocalcisomes of *Trypanosomes* (De Souza, 2002; Docampo and Moreno, 2001). However, it is also possible that, like other eukaryotic cells, there are more than one acidic Ca^{2+} pools in *Plasmodium*, one is food vacuole or small clear vacuoles and another is endoplasmic reticulum (ER). Like other plant vacuolar systems, vacuolar Ca^{2+}/H^+-antiporter coupled with V-ATPase and V-PPase might be responsible for pH-dependent Ca^{2+} accumulation in food vacuoles and small clear vesicles, whilst Ca^{2+}-ATPase and Ca^{2+} channels might participate in Ca^{2+} homeostasis of ER. In any events, morphological identification of acidocalcisomes and biochemical identification of proteins responsible for Ca^{2+} transport in *Plasmodium* will clarify these issues.

CONCLUSION REMARKS

Overall, studies on vacuolar proton pumps and related secondary transporters in the malaria parasite are still at a preliminary stage. Recent development of methodologies for the expression of parasite proteins and gene-knock out parasites will be helpful for elucidating the molecular events in acidic organelles of the parasite. In this mini-review, we did not mention the progress regarding the vesicular trafficking of parasite membrane proteins. Evidence is accumulating that the parasite contains a unique membrane trafficking machinery: parasite cells secrete vesicular trafficking proteins such as *Plasmodium* homologues of Sar1p small GTP protein (Albano *et al.*, 1999) and *N*-ethylmaleimide-sensitive factor (*Pf* NSF) extracellularly (Hayashi *et al.*, 2001). These proteins seem to be responsible for development of the parasitophorus vacuolar membrane network and the targetting of some membrane proteins to the erythrocyte plasma membrane. We wonder if V-ATPase subunits are somehow involved in these trafficking processes. The participation of proteolipid in membrane fusion events was demonstrated recently (Peters *et al.*, 2001). V-ATPase in host cells seems to be important for understanding the mechanism of infection. During malaria transmission in the mosquito, it was found that *P. gallinaceum* preferentially invades V-ATPase-expressing cells in the *Aedes aegypti* midgut (Shahabuddin and Pimenta, 1998). The infection mechanism is currently unknown, but the participation of the

V0 sectors most probably proteolipid, might function as a parasite receptor. Vacuolar proton pumps might be useful indicators for revealing the molecular events in the parasitic complex life cycle of the malaria parasite.

ACKNOWLEDGMENTS

The work presented here was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, 08281105, from the Japanese Ministry of Education, Science, Culture, and Sports. S.Y. and M.H. were supported by Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists.

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