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ION TRANSPORT IN PARASITIC PROTOZOA

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Summary

Many parasitic protozoa go through complex life cycles in the course of which they adapt to widely different environments; ion transport processes are expected to play a role both in pathogenicity and in adaptation. So far, studies on ion transport have been virtually limited to *Leishmania*, *Plasmodium* and *Entamoeba*. The distribution of ion pumps in the former two organisms generally appears to conform to the picture established for other protozoa, i.e. a proton-motive P-ATPase in the plasma membrane provides the driving force for H^+-coupled secondary-active transport, a proton-motive V-ATPase in the digestive vacuoles is responsible for vacuolar acidification, and an F-ATPase (ATP synthase) is found in the mitochondria. The situation in *Entamoeba*, an archaic organism that lacks mitochondria, could be different from that in the two other parasites in that a V-ATPase may be present and active both in the plasma membrane and in the membranes of the endocytic vesicles.

Introduction

In this contribution I would like to make a case for investigating ion transport in parasitic protozoa. Of course, a major rationale for studying any aspect of these organisms is biomedical; but in addition, parasitic protozoa are fascinating cells with often complex life cycles, reflecting their adaptation to widely different environments. Undoubtedly, ion transport processes play a role both in pathogenicity and in the transitions between the different cell stages. I would like to draw special attention to our own subject of investigation, *Entamoeba*. This organism is thought to have branched off very early from the evolutionary line leading to our own cells and may have retained some characteristic properties of our common ancestor, the protoeukaryote.

A recent textbook on molecular parasitology (Hyde, 1990) lists about fifteen different genera of parasitic protozoa that have been investigated on the molecular level. Within this group, ion transport studies have been limited to *Leishmania* (with some work on the related trypanosomes), *Plasmodium* and *Entamoeba*. I will give a brief overview of what is known on ion transport in the first two organisms; much additional information can be found in several recent reviews (Zilberstein, 1991; Tanabe, 1990*b*, 1991). I will then proceed to discuss in somewhat more detail our own data on *Entamoeba*.

Key words: ion transport, parasitic protozoa, *Entamoeba histolytica*.

Ion transport in *Leishmania donovani*

Leishmania donovani causes serious visceral disease. Together with the trypanosomes, *Leishmania* belongs to the Kinetoplastidae, a group of organisms characterized by the presence of an electron-dense structure (the kinetoplast) consisting of mitochondrial DNA. *Leishmania* cells spend part of their life cycle as promastigotes in the slightly alkaline (pH≈7.5) midgut of an insect vector (sand fly), the other part as amastigotes within the acidic (pH≈5.0) phagolysosomes of host macrophages (Hyde, 1990).

In view of the peculiarities of this life cycle, studies of ion transport in *Leishmania* have primarily centred around (i) the capacity of the cells to adapt to widely different extracellular pH values, and (ii) the problems of to what extent and how the promastigote–amastigote transition is triggered by a decrease in external pH. Within this framework, the following ion transport systems are particularly relevant (Fig. 1, left).

The proton-motive P-ATPase

Plasma membranes of *L. donovani* contain a proton-motive P-ATPase (Zilberstein and Dwyer, 1988; Liveanu *et al.* 1991). This H^+ pump is probably coded for by a pair of tandemly linked genes with slightly different nucleotide sequences (Meade *et al.* 1987). Interestingly, transcripts from the upstream gene are most abundant in exponentially growing promastigotes, whereas transcripts of the downstream gene dominate in

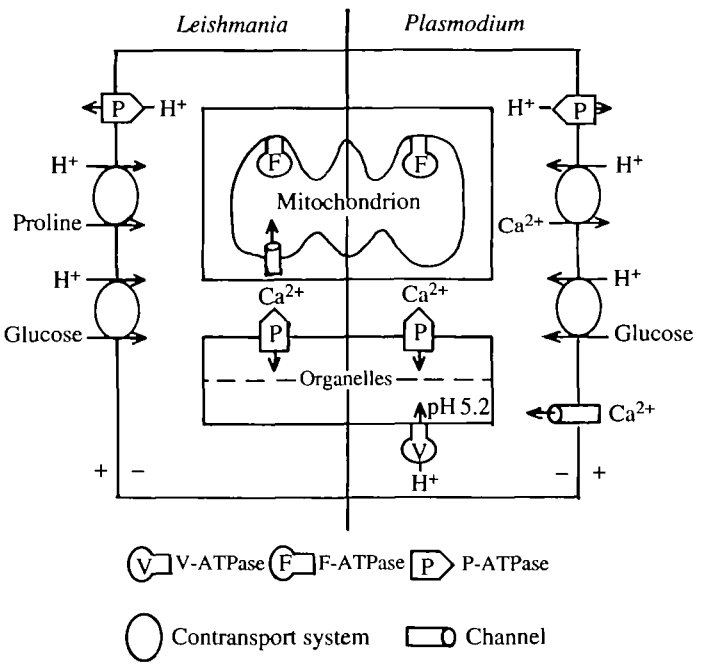

Fig. 1. Ion transport systems in promastigotes of *Leishmania* and the intraerythrocyte stage of *Plasmodium*. Transport systems that have been characterized physiologically and/or molecularly are shown. Most of the data come from *L. donovani* and *P. falciparum*, but the evidence available indicates that the diagram also applies to other species.

amastigotes; an intermediate distribution is found in promastigotes allowed to reach the stationary state. Structure and expression of the ATPase locus are conserved in different *Leishmania* strains (Meade *et al.* 1991).

The P-ATPase generates a proton-motive force (PMF) across the plasma membrane. In promastigotes, the total magnitude of the PMF has been estimated from the distribution of proline (which is thought to be cotransported with one proton; see below). The value for the PMF calculated this way is constant at about -100 mV over a wide range of external pH values (5–8). Under these same conditions, the internal pH is fixed at about 6.5; thus, at increasing extracellular pH, the contribution of the pH gradient to the PMF decreases, and this is compensated for by an increased contribution of the membrane potential (Zilberstein *et al.* 1989). This implies that additional ion transport pathways must participate in establishing the membrane potential. For amastigotes, there is some evidence that the membrane potential is partly set by a K^+ diffusion potential (Glaser *et al.* 1992).

H^+/substrate cotransport systems in the plasma membrane

In *L. donovani*, both proline and glucose are transported together with protons; consequently, the driving force for their accumulation is provided by the PMF (Zilberstein and Dwyer, 1985). Because insect guts contain large amounts of proline, one would expect this amino acid to be a more important source of energy for the promastigote than for the amastigote stage. In agreement with this expectation, there is physiological evidence that *L. donovani* possesses two proline/H^+ symport systems with different pH optima (5.5 and 7.5), with transport through the high-pH system being fastest. Like the P-ATPases (see above), these two systems are differentially expressed as a function of growth stage; the low-pH system can be induced in promastigotes by lowering culture pH (Zilberstein, 1991).

In contrast to H^+/proline symport, the expression of H^+/glucose symport activity across the plasma membrane is apparently not affected by culture pH (Zilberstein, 1991). Note in this context that, in kinetoplastids, glycolysis is compartmentalized in subcellular organelles called glycosomes (Opperdoes, 1987).

Other ion transport systems

The leishmanial mitochondrion contains the usual F-ATPase; it cross reacts with antibodies against the β subunit of the *Escherichia coli* F-ATPase (Liveanu *et al.* 1991). From a regulatory point of view, it may be significant that one or more of the subunits of kinetoplast F-ATPase are coded for by kinetoplast DNA. Transcripts of kinetoplast genes generally undergo RNA editing, which may play a role in the regression of mitochondrial functions in the amastigote stage (Sollner-Webb, 1992).

Another ion whose transport has been investigated in *Leishmania* is Ca^{2+}. As in higher eukaryotic cells, both the mitochondria and an organellar P-ATPase appear to help keep the cytoplasmic Ca^{2+} concentration low (Philosoph and Zilberstein, 1992).

Ion transport in *Plasmodium falciparum*

Of all species causing malaria in man, *Plasmodium falciparum* is the most dangerous.

Like *Leishmania*, it spends part of its life cycle in an insect vector (the *Anopheles* mosquito), the other part within the human host. After a developmental stage in the liver, it invades the host erythrocytes where it grows and matures, enveloped continuously by a parasitophorous membrane. At this stage it obtains most of its amino acids by endocytosing and degrading red cell haemoglobin. Classical antimalarial chemotherapy, which includes amino alcohols such as quinacrine and chloroquine, is directed against the intraerythrocyte stage. A huge problem is posed by the fact that the parasites are becoming increasingly resistant to these compounds (Hyde, 1990). A major rationale for studying ion transport processes in *P. falciparum* is that they may play a role both in the action of these antimalarial drugs and in parasite resistance to them. Specifically, all of these compounds are weak permeant bases and, as such, are accumulated within the acidic food vacuole of the parasite (Yayon *et al.* 1984); even so, at therapeutic concentrations they do not raise the pH within the vacuole to any significant extent (Yayon *et al.* 1985). Because these compounds accumulate according to a pH gradient, the driving force for their accumulation is indirectly provided by the proton-motive ATPase in the vacuolar membrane. The drugs damage the parasite irreversibly, possibly by inhibiting a haem polymerase; the latter enzyme precipitates (and thus inactivates) the toxic free haem that is released as a degradation product of haemoglobin (Slater and Cerami, 1992). Parasites that are resistant to these drugs keep the intracellular drug concentration low; they do so by enhancing the rate of drug efflux (Krogstad *et al.* 1987, 1992). In resistant cells, vacuolar pH appears to be higher than in sensitive cells (Geary *et al.* 1986). This observation has led to the proposal (Ginsburg and Krugliak, 1992) that resistance is due to a decrease in activity of the V-ATPase. In agreement with this hypothesis, inhibition of the V-ATPase by bafilomycin A_1 decreases the susceptibility of the parasites to chloroquine (Bray *et al.* 1992). Alternatively, there is evidence for the involvement of a multidrug-resistance P-glycoprotein homologue (Cowman, 1991; see, however, Krogstad *et al.* 1992). It has recently been reported (Valverde *et al.* 1992) that expression of the human P-glycoprotein generates volume-regulated, ATP-dependent, chloride-selective channels. Taken together, these data open up the possibility that malarial drug resistance could at least in part be due to suppression of a P-glycoprotein-associated chloride flux: similar to the situation in intracellular organelles containing a defective cystic fibrosis transmembrane regulator (Al-Awqati *et al.* 1992), this would inhibit the conversion of the V-ATPase-generated membrane potential into a pH gradient.

In somewhat more detail, the following elements are thought to contribute to ion transport in intraerythrocyte *Plasmodium* (Fig. 1, right). As in *Leishmania*, an H^+-ATPase in the plasma membrane (which according to its sensitivity towards inhibitors is classified as P-type) is responsible for building up a PMF consisting of a membrane potential of about -90 mV and a pH difference of less than 1 unit (Mikkelsen *et al.* 1986); the PMF drives the uptake of glucose through H^+/glucose symport (Tanabe, 1990*a*) and may also drive the extrusion of Ca^{2+} (which is taken up electrogenically; Tanabe, 1990*b*) through nH^+/Ca^{2+} ($n \geqslant 3$) antiport (Kramer and Ginsburg, 1991). In addition to the latter exchange system, a gene has been cloned that probably codes for an organellar P-type Ca^{2+}-ATPase (Murakami *et al.* 1990). As would be expected, Ca^{2+} transport and compartmentation in the intraerythrocytic parasite are influenced by the permeability

properties of the infected red cell (which are different from those of the uninfected red cell) (Kramer and Ginsburg, 1991). The digestive vacuoles are thought to contain the usual V-ATPase; this hypothesis is supported by the inhibitor profile of this ATPase (Choi and Mego, 1988; Bray *et al.* 1992) and by immunological cross reactivity with antibodies to the *Neurospora* V-ATPase (H. Ginsburg, personal communication). Finally, the mitochondrion of *Plasmodium* presumably contains an F-ATPase. However, it is not yet clear whether in the intraerythrocytic, microaerophilic stage this ATPase works in the usual synthetic mode (Kanaani and Ginsburg, 1989) or in a hydrolytic mode (building up a PMF by hydrolyzing ATP imported from the cytoplasm; Fry, 1991).

Ion transport and functional compartmentation in *Entamoeba histolytica*

E. histolytica is the causative agent of amoebic dysentry in man. Pathogenic strains penetrate the gut wall and may cause abscesses in internal organs such as the liver and brain (Hyde, 1990). Structurally, *Entamoeba* is a very simple eukaryote lacking mitochondria, peroxisomes, a rough endoplasmic reticulum (ER) and a well-defined Golgi apparatus (Martínez-Palomo, 1982). Based partially on these properties, *Entamoeba* has been classified by Cavalier-Smith (1987) as an Archezoon; this classification implies that it may have branched off very early in evolution. In agreement with this view, we have recently found that, with regard to the amino acid sequence of its ubiquitin, *E. histolytica* is an unambiguous outgroup to all other taxa (including trypanosomes) whose ubiquitin sequences have been established (Wöstmann *et al.* 1992). In view of this information, it seems reasonable to expect that the study of ion transport systems in *Entamoeba* may provide clues as to the evolutionary development of transport systems in higher eukaryotic cells. This expectation applies to the molecular structure of the transporters; however, it also applies to their physiological function. To put the latter aspect into its conceptual context, I start by briefly summarizing our model for the functional compartmentation of *E. histolytica*; a more detailed discussion of this model (including experimental evidence) is given by Bakker-Grunwald (1991).

Functional compartmentation in Entamoeba histolytica

Functional compartmentation in eukaryotes has probably evolved roughly as depicted in Fig. 2 (Bakker-Grunwald, 1991). With the acquisition of cytoskeletal elements, the cell developed the capacity for endocytosis and exocytosis (stage 2). This capacity was a prerequisite for the formation of a discrete nucleus surrounded by a nuclear membrane in communication with a proto endoplasmic reticulum (stages 3 and 4). An unresolved sequence of multiple events (including endocytobiosis) then led to the development of the higher eukaryotes (stage 5). We think that, at least in some respects, *Entamoeba* got stuck at stage 4. Specifically, the cells appear to possess an undifferentiated membrane compartment consisting of the plasma membrane together with an intracellular vesicle pool; the latter presumably has a membrane composition identical to that of the plasma membrane, and the two membrane fractions constitutively communicate with each other by endo- and exocytosis. In contrast to the situation in higher (Fig. 2, stage 5) eukaryotic cells, this communication is not restricted by coat structures such as clathrin. Possibly

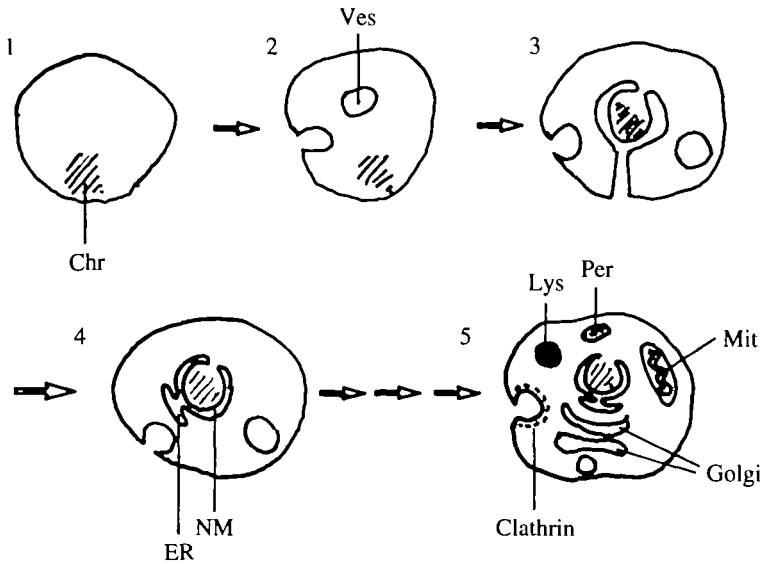

Fig. 2. Evolutionary development of functional compartmentation in the eukaryotic cell. Chr, chromosomes(s); Ves, endocytic vesicle; NM, nuclear membrane; Per, peroxisome; Mit, mitochondrion; ER, endoplasmic reticulum; Lys, lysosome (from Bakker-Grunwald, 1991).

because of this lack, it is basically (i.e. in the absence of a specific stimulus such as that provided by the interaction with target cells) stochastic; by this I mean that a newly internalized vesicle, after mixing with the intracellular pool, has the same probability as any of the other vesicles of being exocytosed next. This membrane compartment and the intracellular lumen enclosed by it perform a range of functions that in higher eukaryotic cells are taken over by such diverse organelles as lysosomes and cytotoxic vesicles. In addition, there appears to be a separate biosynthetic compartment, corresponding to the endoplasmic reticulum/Golgi organelle of higher eukaryotic cells.

Ion transport in Entamoeba histolytica.

E. histolytica takes up by pinocytosis about 30% of its own volume per hour. At the high ($>100\,mmol\,l^{-1}$) extracellular NaCl concentration of the growth medium (or of serum, in the case of invading parasites), this results in an appreciable Na^+ (and associated Cl^-) load that far exceeds trans-plasma membrane influx (Bakker-Grunwald *et al.* 1986). We have previously noticed that cells lose NaCl (and osmotically associated water) upon inhibition of vesicular traffic, and we interpreted this to mean that the amoebae get rid of at least part of the pinocytic Na^+ load by a vectorial transmembrane movement of Na^+ from the vesicles into the cytoplasm and out of the cell (Bakker-Grunwald *et al.* 1985, 1986). This would require a Na^+ 'pump' in the plasma membrane in series with a Na^+ 'leak' in the vesicle membranes; I will speculate on the nature of the Na^+ 'pump' below. Recently, Dr Löhden-Bendinger in my laboratory has confirmed, in collaboration with Dr K. Zierold (MPI Dortmund), that the Na^+ concentration within the vesicles decreases as they age (Löhden-Bendinger, 1991). To this end, she has performed

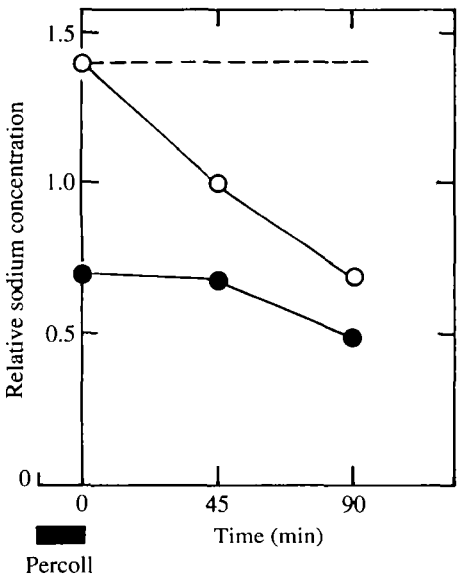

Fig. 3. X-ray microprobe analysis of the Na^+/K^+ content of pinocytic vesicles as a function of vesicle age. Amoebae were allowed to pinocytose growth medium containing Percoll (25% v/v) for 15 min at 36°C, washed, and reincubated at 36°C in growth medium containing cytochalasin B (20 μmol l^{-1}) to block exocytosis. At the indicated times, samples were centrifuged and the cells were frozen in liquid propane. Percoll-containing vesicles in ultrathin sections were identified by the Percoll silicon signal. The peak height for Na^+ normalized to that for K^+ is plotted; K^+ content does not change significantly after addition of cytochalasin B (Bakker-Grunwald *et al.* 1985). ○, vesicles; ●, cytoplasm; ---, medium. Data points are means from 10–20 independent spectra; standard deviations are indicated by bars.

a pulse-chase X-ray microprobe analysis. Amoebae were loaded for 15 min ('pulse') with a suspension of Percoll, washed, and subsequently incubated for different times in the absence of Percoll ('chase'). Because Percoll gives a strong silicium signal in the X-ray spectrum, the Percoll-containing vesicles (the age of which is predetermined by the chase time) can be unambiguously identified in freeze-dried sections of the amoebae. In this way we established that the Na^+ concentration within the vesicles decreased towards the concentration in the cytoplasm with a half-time of about 45 min (Fig. 3). As, in addition, part of the Na^+ is regurgitated by exocytosis (see above), Na^+ extrusion branches off into a transmembrane and an exocytic component (Fig. 4). We estimate that in growth medium about two-thirds of the Na^+ taken up by pinocytosis is removed across vesicle and plasma membranes; the remaining one-third is regurgitated.

In addition to carrying a major part of the sodium (and chloride) circulation, the membranes of the pinocytic vesicles also transport protons. The vesicles acidify to pH 5.5 immediately after their invagination (Ravdin *et al.* 1986; Löhden-Bendinger and Bakker-Grunwald, 1990); this pH is maintained as the vesicles age. Acidification is inhibited by low concentrations of bafilomycin A_1, indicating that it is caused by a V-ATPase (Löhden-Bendinger and Bakker-Grunwald, 1990). Meanwhile, we have observed what appears to be 'crosstalk' between the V-ATPase and the Na^+ circulation. Specifically, as

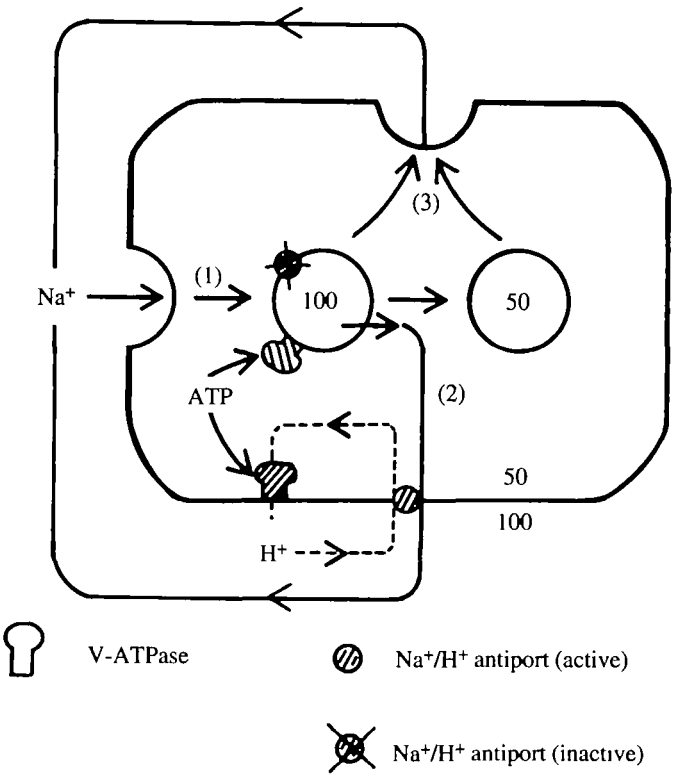

Fig. 4. Na^+ circulation in *Entamoeba histolytica*. Na^+ is taken up by pinocytosis (1) and leaves the cells both by a transmembrane pathway [(2); about 70%] and by regurgitation [(3); about 30%]. The transmembrane component may be indirectly (through Na^+/H^+ antiport) driven by a V-ATPase in the plasma membrane; to explain the net efflux of Na^+ we assume that the Na^+/H^+ antiporter in the vesicle membrane is inactive. The unbracketed numbers indicate the approximate concentrations ($mmol\,l^{-1}$) of Na^+ at the respective locations.

shown in Fig. 5, addition of bafilomycin induced a net increase in sodium content compared with the control. Because bafilomycin does not stimulate pinocytic or trans-plasma membrane uptake (Löhden-Bendinger and Bakker-Grunwald, 1990; legend to Fig. 5), the bafilomycin-induced increase in Na^+ content must have been due to inhibition of Na^+ efflux rather than to stimulation of Na^+ influx. This inhibition of efflux can best be explained if we assume that Na^+ extrusion across the plasma membrane is indirectly driven by the V-ATPase, presumably by H^+/Na^+ antiport (Fig. 4). This would require that the V-ATPase is (i) present and (ii) functional in the plasma membrane. The first claim offers no problems: this is actually what one would expect in view of the unrestricted communication between vesicles and plasma membrane (see above). The second claim, that the V-ATPase is functional both in the plasma membrane and in the vesicles, is much more fundamental and should be confirmed by independent measurements; so should the existence of the putative Na^+/H^+ transporter. Even so, the experimental evidence suggests that a V-ATPase supplies the driving force for at least one form of secondary-active transport. In this sense, our data are fully in line with the proposal (Maloney and

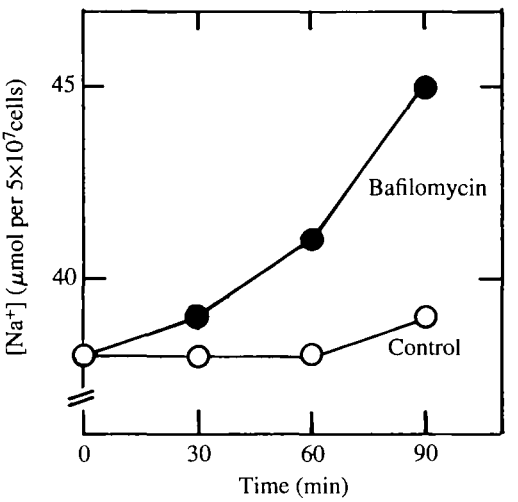

Fig. 5. Effect of bafilomycin A_1 on Na^+ content of *E. histolytica*. Amoebae were incubated at 36°C in growth medium containing bafilomycin A_1 (25 μmol l^{-1}; ●) or an equivalent volume of dimethyl sulphoxide (control; ○). At the indicated times, cells were spun down and washed, and Na^+- and K^+ contents were determined by flame photometry. K^+ content was constant and equal in the cells incubated with and without bafilomycin (not shown), indicating that the drug did not increase the permeability of the plasma membrane nonspecifically. Values are averages of duplicates, which deviated by less than 10%; the experiment is representative of two replicates.

Wilson, 1985) that both V-type H^+-ATPases and P-type H^+-ATPases originally resided on the plasma membrane of a protoeukaryote, the former providing the driving force for other transport systems. After the incorporation of chloroplasts and/or mitochondria the V-ATPases were generally relegated to newly developing internal organelles such as lysosomes and Golgi bodies, whereas the P-ATPases took over on the plasma membrane. Meanwhile, it has been established that there are exceptions to this rule: V-ATPases are found in the plasma membranes of insect epithelial cells (Wieczorek, 1992; Klein, 1992), renal cells (Gluck, 1992) and osteoclasts (Chatterjee *et al.* 1992). It seems reasonable to assume that these specialized V-ATPases have only recently returned to the cell surface, whereas the V-ATPase of *E. histolytica* may represent the original state in which the enzyme has been internalized but has not yet been banned to the internal compartments.

Conclusions

Three different parasites, three different strategies to cope with the ionic environment? Clearly not. Although *Leishmania* and *Plasmodium* are very different organisms, both of them obviously adhere to the same principles to which yeast cells or *Dictyostelium* adhere: a P-type H^+-ATPase in the plasma membrane drives H^+-coupled secondary-active transport, an organellar P-type Ca^{2+}-ATPase keeps the cytoplasmic Ca^{2+} concentration low, a V-type H^+-ATPase in the digestive vacuoles is responsible for vacuolar acidification and an F-type H^+-ATPase in the mitochondria couples PMF to

phosphate potential (in whatever direction). However, within this general framework, the specific systems involved raise equally specific questions. What makes one of the two *Leishmania* P-type H^+-pumps better adapted to an acidic environment, and what is the nature of the defect in ion transport that may contribute to drug resistance in *Plasmodium*? The answers to questions such as these obviously have potential implications for chemotherapy, but they will also broaden our general understanding of adaptation and of those forms of regulation that are based on the interplay between pumps and leaks.

And *Entamoeba*? As I have pointed out above, these cells may be different in employing a V-ATPase for functions that in higher eukaryotes are taken over by a P-ATPase. This hypothesis would fit in with the archaic functional compartmentation of this organism and suggests that the study of its ion transport systems will be particularly rewarding from an evolutionary point of view.

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CHAPTER 8. Insect plasma membranes