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Na\(^+\) regulation in the intraerythrocytic malaria parasite

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Publications

The following publication and Letter to the Editor arose from work performed as part of this degree, and form the basis for part of Chapter 6.


Abstract

The maintenance of a low intracellular \([\text{Na}^+]([\text{Na}^+])_i\) is a crucial aspect of cellular physiology. In mammalian cells this is achieved through the extrusion of \(\text{Na}^+\) via the well-characterised \(\text{Na}^+/\text{K}^+\)-ATPase. Approximately 12 hr after invasion of the human erythrocyte by the malaria parasite there is a profound increase in the permeability of the erythrocyte membrane to a wide range of solutes, including \(\text{Na}^+\). \(\text{Na}^+\) enters the infected erythrocyte via parasite-induced ‘New Permeability Pathways’ and there is, as a result, an increase in \([\text{Na}^+]\) in the erythrocyte compartment, with \([\text{Na}^+]\), eventually reaching levels similar to those in the extracellular plasma (~130 mM). The parasite itself maintains a low \([\text{Na}^+]\). The resulting large inwardly-directed electrochemical \(\text{Na}^+\) gradient across the parasite plasma membrane energises the accumulation within the parasite of at least one essential nutrient (inorganic phosphate).

The aim of this thesis was to characterise the mechanisms involved in \(\text{Na}^+\) regulation in the mature asexual ‘trophozoite’ stage of the human malaria parasite \textit{Plasmodium falciparum}. The \(\text{Na}^+\)-sensitive, fluorescent dye Sodium-binding BenzoFuran Isophthalate (SBFI) was used to measure \([\text{Na}^+]\) in parasites functionally isolated from their host cells by saponin-permeabilisation of the host erythrocyte membrane. Under physiologically relevant conditions the resting \([\text{Na}^+]\) in isolated trophozoites was estimated to be ~11 mM. Maintenance of \([\text{Na}^+]\) was sensitive to the P-type ATPase inhibitor orthovanadate, consistent with \(\text{Na}^+\) extrusion being via a P-type \(\text{Na}^+\)-ATPase, similar to the ENA \((\text{exitus natru}; \text{exit of sodium})\)-type ATPases that operate in some other protozoa, fungi and lower plants. ENA ATPases have been predicted to antiport \(\text{H}^+\) and the data obtained here are consistent with this being true of the \textit{P. falciparum} \(\text{Na}^+\) extrusion system.

The \textit{P. falciparum} genome encodes a number of putative P-type ATPases; one of these, \textit{PfATP4}, was found to share significant sequence similarities to ENA ATPases of other protozoa. A recent study showed that mutations in \textit{PfATP4} confer resistance to a newly-described class of antimalarials, the spiroidolones. The effect of the spiroidolones on ion regulation was therefore investigated. Several spiroidolones were shown to cause a profound disruption of \([\text{Na}^+]\) regulation.

parasites with mutant PfATP4 there was both an impairment of Na\(^+\) regulation and a
decrease in the spiroindolone-sensitivity of Na\(^+\) regulation. These results are
consistent with PfATP4 being a Na\(^+\)-ATPase and the target of the spiroindolones.

The physiological role of another putative Na\(^+\) transporter, the Na\(^+\)/H\(^+\)-exchanger
PfNHE was also investigated, as previous studies on its contribution to regulation of
[Na\(^+\)]\(_i\) and intracellular pH (pH\(_i\)) have been controversial. On the basis of a
bioinformatics analysis it was predicted that the protein functions as an amiloride-
insensitive, plasma membrane Na\(^+\)-extruder, like its closely related plant
homologues. However physiological studies revealed no significant role for such an
NHE in either pH\(_i\) or [Na\(^+\)]\(_i\) regulation in the \textit{P. falciparum} trophozoite.

This study constitutes a significant advance in our understanding of fundamental
aspects of the cell physiology of the intraerythrocytic parasite, as well as shedding
light on the mode of action of what promises to be an important new class of
antimalarials, the spiroindolones.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AM</td>
<td>acetoxymethyl</td>
</tr>
<tr>
<td>AMDP</td>
<td>aminomethylenediphosphonate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>[ATP]i</td>
<td>intracellular [ATP]</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein</td>
</tr>
<tr>
<td>BCF-RPMI</td>
<td>bicarbonate-free RPMI</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>clone#2</td>
<td>NITD609-RDd2-clone#2</td>
</tr>
<tr>
<td>CPA</td>
<td>cation proton antiporter</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DV</td>
<td>digestive vacuole</td>
</tr>
<tr>
<td>ENA</td>
<td>exitus nattru</td>
</tr>
<tr>
<td>Fura-2</td>
<td>2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethansulphonic acid]</td>
</tr>
<tr>
<td>hr</td>
<td>hour/s</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50 % inhibitory concentration</td>
</tr>
<tr>
<td>MES</td>
<td>3-(N-morpholino)propane sulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
</tr>
<tr>
<td>[Na&lt;sup&gt;+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>intracellular [Na&lt;sup&gt;+&lt;/sup&gt;]</td>
</tr>
<tr>
<td>[Na&lt;sup&gt;+&lt;/sup&gt;]&lt;sub:o&lt;/sub&gt;</td>
<td>extracellular [Na&lt;sup&gt;+&lt;/sup&gt;]</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Institute</td>
</tr>
<tr>
<td>NHA</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; antiporter</td>
</tr>
<tr>
<td>NhaP</td>
<td><em>Pseudomonas aeruginosa</em> Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; antiporter</td>
</tr>
<tr>
<td>NHE</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; exchanger</td>
</tr>
<tr>
<td>NHX</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; exchanger</td>
</tr>
</tbody>
</table>
NITD  Novartis Institute for Tropical Diseases
NMDG⁺  N-methyl D-glucamine
NPP  New Permeability Pathways
PBS  phosphate-buffered saline
PfATP4  *Plasmodium falciparum* ATPase 4
PfNHE  *Plasmodium falciparum* Na⁺/H⁺ exchanger
PfNHE-kd  *Plasmodium falciparum* Na⁺/H⁺ exchanger knock-down
pHᵢ  intracellular pH
pH₀  extracellular pH
PPI  inorganic pyrophosphate
PPM  parasite plasma membrane
PV  parasitophorous vacuole
PVM  parasitophorous vacuole membrane
RPMI  Roswell Park Media Institute
s  second/s
SBFI  Sodium-binding BenzoFuran Isophthalate
S.E.M.  standard error of the mean
SOS  salty overly sensitive
WHO  World Health Organisation
Δψ  membrane potential
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Chapter 1:

Introduction
1.1. Introduction

In the most recent ‘World Malaria Report’ it was estimated that in 2010, 106 countries were endemic for malaria (World Health Organisation, 2010c). Despite concerted control efforts worldwide, malaria remains an extreme burden, and investigating the physiology of the causative parasite is important both for understanding disease pathology, and for the development of novel drugs against the parasite.

This thesis is focused on the mechanism of Na\(^+\) homeostasis in the parasite, both as a basic physiological process and also with regards to Na\(^+\) transporters as potential antimalarial drug targets and as mediators of drug resistance.

1.2. Malaria

1.2.1. History of the disease

The disease malaria takes its name from the Italian phrase “mal air” as, historically, the disease was associated with marsh/swamp air. Malaria is an ancient disease; the clinical symptoms are described in the Chinese Canon of Medicine, dating from ~2700 BC (Neghina et al., 2010) and genetic evidence of malaria was found in the mummified Egyptian pharaoh Tutankhamun who ruled in ~1300 BC (Hawass et al., 2010). The disease was distributed worldwide in the 1800s, and it undoubtedly shaped human history, as foreign armies struggled to invade malarious regions, and countries were unable to develop economically for centuries (Neghina et al., 2010).

It was not until 1880 that Alphonse Laveran discovered the causative parasite in a patient’s blood, and soon after, in 1898, Patrick Manson and Sir Ronald Ross determined the link between the parasite and its mosquito vector (Guillemin, 2002). Their discoveries paved the way for malaria control and in 1955 scientist Paul Russell claimed that “malaria is well on its way towards oblivion” (Hay et al., 2004). Unfortunately this optimism for malaria elimination was premature, and in 2011 the burden of malaria is still present.
1.2.2. The causative parasite and distribution

Malaria is caused by protozoan parasites of the *Plasmodium* genus, from the phylum Apicomplexa, which includes many members of relevance to human/livestock health, including *Toxoplasma, Cryptosporidium, Babesia* and *Eimeria*. All apicomplexans have, at some point in their life cycle, a polarised distribution of specialised secretory organelles at the apical end of the cell, and move via a unique mechanism of actin-myosin-based ‘gliding’ motility (Sibley, 2010). There are five species of *Plasmodium* parasite that can cause disease in humans; *P. falciparum, P. ovale, P. vivax, P. malariae* and *P. knowlesi* (Suh et al., 2004; Cox-Singh et al., 2008). The subject of this thesis, *P. falciparum*, is the most virulent of the five species infective to humans, and is capable of causing severe malaria with critical complications. Although *P. vivax* was once considered ‘benign’ it has recently been shown that it can cause severe disease typical of *P. falciparum* (Galinski and Barnwell, 2008).

Estimating the number of malaria cases each year is extremely difficult for several reasons: (i) the dominant symptom of fever does not differentiate the disease from other infections which may lead to misdiagnoses, ii) fevers are sometimes self-medicated so the case is not reported to health authorities, iii) many people living in malaria-endemic regions are re-infected multiple times in their lifetime and as they develop a naturally acquired immunity they may be asymptomatic, and iv) the reporting system of each country may not be accurate (Hay et al., 2010a). Despite these challenges it has been estimated that there are approximately one million deaths each year (World Health Organisation 2010c), and that up to 48 % of the world population lives in malaria-endemic regions (Hay et al., 2004). For *P. falciparum*, in 2007 there were estimated to be between 349 - 552 million cases (Hay et al., 2010a).

The distribution of malaria is shown in Figure 1.1, with malaria cases found mainly in sub-Saharan Africa, South America and South East Asia. The majority of severe *P. falciparum* cases occur in sub-Saharan Africa (Feachem et al., 2010). The areas of high malaria risk coincide with poverty, with up to 58 % of malaria cases occurring in the poorest 20 % of the world population (Barat et al., 2004). Malaria
results in disruption of childhood education, reduced external investment and reduced tourism (Breman et al., 2004); this, combined with turbulent governance and civil war in some countries make the coordinated control of malaria challenging. It is a vicious cycle, with malaria-induced low socioeconomic progression hindering disease eradication efforts.

Figure 1.1. Global distribution of malaria (by country) in 2010. Countries where malaria transmission occurs are shaded in dark blue, and countries where there is ‘limited risk’ of malaria transmission are shaded in light blue. The figure is from the World Health Organisation ‘Global Health Observatory’ Map Gallery (Accessed September 2011; http://www.who.int/gho/mdg/diseases/malaria/en/index.html).

1.2.3. Symptoms and immunity

Initial symptoms of malaria include malaise, headaches, myalgia and abdominal discomfort which are followed by fever and chills, perspiration, anorexia, vomiting and worsening lassitude (World Health Organisation, 2010b). Although cyclical 48 (tertian) or 72 (quartan) hr fevers are associated with the disease, the fever pattern is often less precise (Suh et al., 2004). Severe disease is associated with development of further symptoms including coma (associated with cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure or pulmonary oedema (World Health Organisation, 2010b).
People living in endemic regions may have asymptomatic infections (despite significant parasitaemia), as there is a degree of naturally acquired immunity (with both humoral and cellular components) (Suh et al., 2004). Up to the age of approximately six months, babies are protected from symptomatic disease by maternal IgG or IgA antibodies contained in breast milk (Doolan et al., 2009). However, this protection is lost over the first year of life, and children are particularly susceptible to the disease up until the age of 2 - 5 years, after which their own naturally acquired immunity develops (Doolan et al., 2009). This immunity is lost during pregnancy (particularly in a woman’s first pregnancy), possibly due to pregnancy-related immunosupression and also sequestration of infected red blood cells (erythrocytes) in the placenta (Rogerson et al., 2007). This can cause maternal malaria, putting both the mother and unborn child at high risk of severe disease outcomes. Naturally acquired immunity is lost over a period of years when a person leaves an endemic area (World Health Organisation, 2010b); thus expatriates visiting endemic regions may suffer severe disease.

1.2.4. Disease control and eradication

In the absence of a vaccine, malaria control is reliant on chemotherapy and vector control measures. Many international initiatives support prevention and treatment programs; these include the World Health Organisation Roll Back Malaria program, the Multilateral Initiative in Malaria, the Medicines for Malaria Venture, the Malaria Vaccine Initiative, and the Global Fund to Fight AIDS, TB and Malaria (Tuteja, 2007). Recently, the Bill and Melinda Gates Foundation also announced their support of malaria eradication (Roberts and Enserink, 2007). Although it is thought that morbidity and mortality can be reduced with our current arsenal of defenses against malaria, it is predicted that new tools and drugs will be needed for disease eradication (Mendis et al., 2009).

Vector control is achieved by indoor residual spraying (to kill the mosquitoes or deter their entry into the dwelling), and the use of insecticide treated bed-nets (to kill the mosquito and/or prevent biting). An infected mosquito can transmit the disease for up to two months (Tuteja, 2007) and factors such as prevalence of the mosquito in a certain area, longevity, biting frequency and human behaviour such as working...
or living in poorly constructed houses near mosquito breeding sites are all key aspects to consider when attempting to modulate/disrupt vector-human interaction (Mendis et al., 2009). There has been one field study in Benin that suggests that the benefits of using both indoor residual spraying and insecticide-treated nets are additive (Ngufor et al., 2011). However when indoor residual spraying and insecticide treated nets are combined, the use of multiple classes of insecticides (the major classes being pyrethroids, organochlorides, carbamates and organophosphates) is recommended to prevent mosquito-resistance to insecticides (Okumu and Moore, 2011). The introduction of larvivorous (larvae-eating) fish into lakes and wells (including the guppy, Poecilia reticulate, and the mosquitofish, Gambusia affinis) is gaining appeal as a mosquito control measure, though its effects on water system biodiversity need further investigation (Ghosh and Dash, 2007).

Chemotherapy is essential to malaria control programs; however, parasite resistance to currently used antimalarials is widespread and there is therefore an ongoing need to develop new antimalarial drugs. Historically, the first antimalarial remedy was cinchona tree bark, which was used by Incan tribes for the treatment of fever (Baird, 2005). In the 1500s, Jesuit priests spread information on the use of cinchona bark from South America to mainland Europe, but it was not until 1820 that the active ingredient quinine was isolated from the bark, in which it is found to constitute 13% of bark dry weight (Burrows et al., 2011). The structures of quinine and other antimalarials mentioned in Section 1.2.4 are shown in Fig. 1.2. Despite side effects including tinnitus, hearing loss and dizziness, quinine is still the first drug recommended by the World Health Organisation for the treatment of pregnant women, and the second-line drug recommended for standard malaria treatment (World Health Organisation, 2010a).

Supply problems for quinine during World War II (as well as the unwanted side effects) led to the development of chloroquine as an antimalarial. Quinine and chloroquine both contain the quinoline chemical moiety, a benzene fused via two carbons to a pyridine. Chloroquine was soon deemed a ‘wonder drug’ as it was cheap, effective and safe, and it, along with the infamous insecticide DDT, was the stalwart of the 1950s/1960s Global Malaria Eradication Program.
Unfortunately parasite resistance to chloroquine emerged in multiple independent loci (Thailand and Columbia in the 1950s, and New Guinea and eastern Sub-Saharan Africa in the 1970s) and chloroquine resistance is now widespread (Wellems and Plowe, 2001; Hyde, 2007), rendering it largely useless as an antimalarial.

In response to chloroquine resistance, several other antimalarials were developed. Another quinoline based drug, amodiaquine was developed in the 1960s and although some chloroquine/amodiaquine cross resistance does occur it can still be used in many areas of chloroquine resistance in Africa (Sa et al., 2009).

Sulfadoxine-pyrimethamine was introduced in 1971, and was widely used to treat chloroquine-resistant malaria. However, parasite resistance to sulfadoxine-pyrimethamine developed very quickly in the field after only 1-2 years (Sibley et al., 2001). Mefloquine (another quinoline), was introduced in the 1980s, but it too succumbed to resistance in some areas after only a few years (Baird, 2005). Additionally, mefloquine is associated with insomnia, psychiatric disorders and gastrointestinal discomfort (Baird, 2005). Highlighting the usefulness of the
quinoline moiety, another quinoline derivative, lumefantrine, was developed, and it is now the most commonly used drug in combination therapy, with 56 countries using it as their first- or second-line treatment option (World Health Organisation, 2010a).

The most recent substantial development in our antimalarial arsenal was the isolation of artemisinin from the sweet wormwood shrub, *Artemisia annua*, where it is found in the leaves at around 0.1 - 1.8 % w/w mass (Burrows *et al.*, 2011). Although it was discovered and used in China in the 1980s, it was not until the 1990s that it was utilised throughout the tropical world (Dondorp *et al.*, 2011). Various derivatives of artemisinin with superior pharmacokinetic properties have been synthesised. These include dihydroartemisinin, artemether and artesunate (Burrows *et al.*, 2011). To prevent widespread artemisinin resistance, the World Health Organisation recommends that artemisinin derivatives be used in combination therapy with another antimalarial, with the combinations artemether–lumefantrine, artesunate–amodiaquine, artesunate–mefloquine, artesunate–sulfadoxine–pyrimethamine and dihydroartemisinin–piperaquine (another quinoline) currently in use (World Health Organisation, 2010a).

Artemisinins kill parasites very rapidly, but have a short half-life in the body; using a combination therapy therefore ensures that those parasites not killed by the initial artemisinin are killed by a longer lasting second drug. Additionally, the chances of a parasite developing resistance to two drugs with unrelated modes of action are lower than when the drugs are used in monotherapy (White *et al.*, 1999). However, it is of utmost concern that resistance to artemisinin and artemisinin combination therapies have been observed on the Thailand-Cambodia border (Jambou *et al.*, 2005; Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Dondorp *et al.*, 2011). Despite these few reports of treatment failure, artemisinin combination therapies are currently effective worldwide. However if resistance were to increase there are no alternative effective antimalarials ready for deployment.
1.2.5. The life cycle of \textit{P. falciparum}

The complex life cycle of \textit{Plasmodium} involves both asexual reproduction in the human host and sexual reproduction in the mosquito midgut. The stages of the life cycle are summarised in Figure 1.3.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{life_cycle.png}
\caption{Life cycle of \textit{Plasmodium} spp. (A) Male and female gametes fuse in the mosquito midgut to form a zygote. The zygote differentiates into a ookinete which encysts as an oocyst on the external epithelial wall and produces sporozoites. The sporozoites are injected into the human host during the mosquito bloodmeal. (B) The sporozoites travel from the dermis, to the bloodstream, and then to the liver. In the case of \textit{P. vivax} and \textit{P. ovale} the parasites can form a dormant hypnozoite stage. Alternatively, significant differentiation and multiplication occurs, with the results that thousands of merozoites are released into the bloodstream. (C) Merozoites invade the erythrocytes of the host and undergo a cycle of intraerythrocytic development. (D) A small proportion of parasites differentiate to form the male and female gametocytes, which are round in most species, but falciform in \textit{P. falciparum}. The male and female gametocytes are ingested during the bloodmeal of the mosquito. This figure is adapted from Suh et al. (2004).}
\end{figure}
Plasmodium parasites are carried by female mosquitoes of the Anopheles genus, of which there are approximately 50 - 60 species that are capable of successful disease transmission to humans (Cohuet et al., 2010; Hay et al., 2010b). When gametocytes are ingested during the bloodmeal, they mature in the mosquito midgut into gametes. This takes place within 10 min of ingestion and is initiated in response to a drop in temperature (~5 °C), an increase in pH from ~7.2 to 8 and the presence of xanthurenic acid in mosquito midgut (Billker et al., 1997; Billker et al., 1998). In the course of gamete maturation (gametogenesis), the microgamete (male) replicates its genome three times to form eight flagellated microgametes, which are motile. The macrogamete (female) is fertilised by a male, flagellated microgamete to form a zygote. This zygote differentiates into a motile ookinete form, which can travel through the midgut epithelium to encyst as an oocyst between the epithelium and basal lamina (Pradel, 2007).

The mature oocyst releases ~3 000 - 10 000 sporozoites into the haemolymph of the mosquito (Rosenberg, 2008), and the sporozoites travel to the salivary glands to be injected (along with salivary contents, including anti-coagulants) into the vertebrate host during a blood meal. The process from gametocyte ingestion to infective sporozoite production takes about two weeks, but during this time the mosquito is not merely a carrier for the parasite; it has immune responses of its own (Yassine and Osta, 2010). The mosquito can recognise ‘pathogen associated molecular patterns’ and activates innate immune responses against the parasite, including binding of complement-like thioester-containing proteins and leucine-rich repeat proteins (Yassine and Osta, 2010). Additionally, it has recently been reported that the mosquito midgut microbial flora, particularly Enterobacter strains, can prevent parasite development (Cirimotich et al., 2011).

Some 6 - 22 sporozoites are injected into the dermis during a blood meal (Rosenberg, 2008), and they migrate using actin/myosin-powered ‘gliding motility’ until they contact an endothelial cell, which they traverse to enter the blood stream (Moreira et al., 2008). Once they reach the liver, the sporozoites cross the sinusoidal barrier, into the liver parenchyma where they find a hepatocyte to invade, based upon the sulfation status of heparin sulphate proteoglycans on the hepatocyte surface (Sinnis et al., 2007). In P. vivax (and, it is presumed, P. ovale), hypnozoites can be formed.
These are dormant stages with a low antigenic profile (Galinski and Barnwell, 2008). The hypnozoite can reactivate many years later; however, the signals leading to its 'awakening' are unknown. For sporozoites that continue through the lifecycle, significant differentiation and multiplication occurs in the hepatocytes over ~5 days before the hepatocyte ruptures to release ~10 000 - 30 000 merozoites into the blood stream (Rosenberg, 2008).

Merozoites invade the erythrocytes of the host. Understanding the 'intraerythrocytic' stage is critical as it is responsible for many of the clinical symptoms of the disease. Figure 1.4 shows a cartoon of the intraerythocytic cycle which, in P. falciparum, takes 48 hr.

Figure 1.4. The intraerythrocytic stage of the life-cycle of the malaria parasite. Merozoites invade the erythrocytes of the host, where they develop into 'ring' stage parasites. The metabolically active trophozoite stage is when much of the significant protein, RNA and DNA synthesis occurs. Asexual reproduction, by the process of schizogony, leads to the formation of a schizont, which bursts to release new merozoites into the blood stream. These merozoites are capable of beginning the cycle again. The figure is from Kirk (2001).
The merozoite stage of the parasite life cycle has been the subject of intensive study. Merozoite surface antigens have been proposed as potential antimalarial drug targets (O'Donnell and Blackman, 2005) and as candidate vaccine targets (Richards and Beeson, 2009). Merozoites are highly polarised, with a grouping of invasion organelles at the apical end, including the rhoptries and micronemes. Erythrocyte invasion is a multi-step event involving initial non-specific contact, apical reorientation, tight-junction formation, entry into the erythrocyte and erythrocyte resealing (Gilson and Crabb, 2009). Receptor mediated binding of the merozoite to the erythrocyte, and tight junction formation rely on the coordinated release of the microneme and rhoptry contents, which contain key parasite ligands, kinases and virulence factors. It has been shown that exposure of the merozoite to a low K+ environment (that of blood plasma) causes a rise in parasite cytoplasmic Ca2+ concentration, which triggers release of the micronemes (Singh et al., 2010). After erythrocyte binding, cytosolic Ca2+ concentrations decrease towards the initial resting levels, and this triggers the subsequent release of the rhoptry proteins (Singh et al., 2010). It is clear that the temporal and spatial control of K+ and Ca2+ are important in parasite physiology, and this example highlights the necessity to understand cation regulation in the parasite. As the parasite enters the erythrocyte, the host membrane invaginates and incorporates rhoptry/micronemal membrane/contents to form a host- and parasite-derived parasitophorous vacuole membrane (PVM) surrounding the parasite (Eksi and Williamson, 2011).

In the initial hours after merozoite invasion of the erythrocyte, the parasite is metabolically and biosynthetically quiescent. This ‘ring’ stage is named because the ‘cup’-like structure adopted by the parasite, with a thicker rim of cytoplasm containing the nucleus and other organelles surrounding a thin, structure-free centre (Bannister et al., 2000) appears, in micrographs, like a signet ring. Endocytosis of the host cell cytoplasm, consisting mainly of haemoglobin, begins midway through the ring stage (Abu Bakar et al., 2010), and over the course of the intraerythrocytic cycle up to 75 % of the host erythrocyte haemoglobin is digested (Rudzinska et al., 1965; Loria et al., 1999). Multiple haemoglobin-containing endocytotic vesicles fuse to form the ‘digestive vacuole’, which is an acidic organelle, containing multiple classes of proteases which together mediate the degradation of the haemoglobin (Goldberg, 2005). Degradation of haemoglobin releases toxic ferriprotoporphyrin
IX, which is crystallised and stored in an inert haemozoin form in the digestive vacuole.

The trophozoite stage occurs approximately 12 - 36 hr after invasion, and this is the most metabolically active stage of the parasite when extensive protein synthesis and many physiological changes take place. The parasite grows rapidly within the host erythrocyte, and haemoglobin digestion allows the parasite to regulate the volume of the host cell to prevent premature host lysis (Lew et al., 2003; Allen and Kirk, 2004a; Lew et al., 2004). Only ~15% of the amino acids released from the digestion of haemoglobin are used for protein synthesis (Krugliak et al., 2002), and the rest are effluxed from the infected erythrocyte.

To power the metabolic and biosynthetic activity occurring during the trophozoite stage, the parasite is reliant solely on glycolysis for the generation of ATP. Glucose use by an infected erythrocyte is between 30 - 100 times higher than that in uninfected erythrocytes (Roth, 1990; Mehta et al., 2005). Despite having a full complement of tricarboxylic acid cycle enzymes encoded in the parasite genome (Gardner et al., 2002), there is no ATP production via oxidative phosphorylation. Instead, it was recently demonstrated that in the intraerythrocytic stage of *P. falciparum* the tricarboxylic acid cycle operates in a novel, bifurcated manner (Olszewski et al., 2010). From glutamine/glutamate the tricarboxylic acid cycle functions both in an oxidative direction to produce citrate (which is subsequently used for acetate/acetyl-CoA synthesis) and in a reductive direction to synthesise succinyl-CoA (which is subsequently used for haem biosynthesis; Ginsburg, 2010; Olszewski et al., 2010).

Another intriguing aspect of the *Plasmodium* parasite is the presence of a vestigial, non-photosynthetic plastid, called the apicoplast (McFadden, 2011). This organelle is found in all apicomplexan parasites (except *Cryptosporidium*). The apicoplast is thought to have originally been obtained by an ancestral cell through secondary endosymbiosis of a red alga (McFadden, 2011). The apicoplast was recently shown to serve an essential role in carrying out isoprenoid precursor synthesis in the intraerythrocytic stages (Yeh and DeRisi, 2011). Targeting the ‘plant-like’ pathways found in the apicoplast would seem an ideal chemotherapeutic strategy, as it
minimises the prospect of ‘off-target’ reactions with human proteins. Highlighting the potential of this strategy, inhibitors of acetyl-CoA carboxylase (an enzyme involved in ‘Type II’ fatty acid synthesis in plant chloroplasts and the parasite apicoplast) were synthesised and they demonstrated significant antimalarial activity (with IC\textsubscript{50} against parasite proliferation of 3 - 12 \textmu M) (Louie \textit{et al.}, 2010).

The parasite begins to replicate its DNA during the trophozoite stage, and at 40 - 44 hrs enters the schizont stage, during which it segments to form 16 - 32 new merozoites. Each of the new daughter merozoites can invade a new erythrocyte, and the intraerythrocytic life cycle continues. In response to unknown signals, a small percentage of infected erythrocytes proceed on an alternative development pathway to form the sexual stages of the parasites, the gametocytes, which are taken up by mosquitoes during the blood meal, continuing the life cycle.

1.2.6. Modification of the host erythrocyte by \textit{P. falciparum}

Living inside the erythrocytes of the host is of benefit to the parasite as it is ‘hidden’ from the host immune system. Additionally, erythrocytes do not express major histocompatibility complex class I proteins capable of presenting foreign antigens to T cells. Terminally differentiated erythrocytes are highly specialised for the carriage of \textit{O}_2, to the extent that they have lost their nucleus, mitochondria, Golgi and endoplasmic reticulum during development. As the erythrocyte lacks protein synthesis and protein trafficking machinery, the parasite must provide its own mechanisms to export proteins into the erythrocyte cytosol/onto the erythrocyte plasma membrane (reviewed in Cooke \textit{et al.}, 2004; Maier \textit{et al.}, 2009; Haase and de Koning-Ward, 2010). The exported proteins contribute to the virulence of \textit{P. falciparum}, by increasing the rigidity of the erythrocytes and allowing sequestration of infected erythrocytes on vascular endothelial cells (Maier \textit{et al.}, 2009) thus enabling the parasitised cells to avoid clearance by the spleen.

Proteins destined to be exported to the erythrocyte are first targeted to the parasite Golgi using an N-terminal hydrophobic signal sequence, and then are trafficked to the parasitophorous vacuole via a ‘classical’ brefeldin-A sensitive pathway (Baumgartner \textit{et al.}, 2001). To be transported across the PVM many (but not all)
exported proteins have an additional signal sequence, the *Plasmodium* Export Element ‘PEXEL’ motif (also known as the Vacuolar Transport Signal), with a consensus sequence of R/KxLxE/Q/D (Hiller *et al.*, 2004; Marti *et al.*, 2004). An endoplasmic reticulum localised protease, plasmepsin V, cleaves the PEXEL motif on the C-terminal side of the conserved L residue (Boddey *et al.*, 2010; Russo *et al.*, 2010), and the N-terminus is subsequently acetylated (Chang *et al.*, 2008; Boddey *et al.*, 2009). The protein is then transported to the parasitophorous vacuole. At the PVM, the protein is unfolded, aided by various chaperone proteins, and it is proposed to cross the PVM through the *Plasmodium* Translocon of Exported proteins, the ‘PTEX’ (de Koning-Ward *et al.*, 2009), although direct protein translocation has not been demonstrated.

In the cytosol of the *P. falciparum*-infected erythrocyte there are several parasite-induced membranous structures; these include the ‘tubulovesicular network’ (Behari and Haldar, 1994) and ‘Maurer’s clefts’ (reviewed in Tilley *et al.*, 2008). The Maurer’s clefts appear to function as secretory organelles that play a role in delivering parasite-exported proteins to the erythrocyte surface. Multiple proteins have been shown to associate with the Maurer’s clefts before reaching their final destination on, or associated with, the plasma membrane of the erythrocyte. These include the Erythrocyte Membrane Protein 1, PfEMP1 (Bhattacharjee *et al.*, 2008), PfEMP3 (Waterkeyn *et al.*, 2000) and the Knob-associated Histidine Rich Protein, KAHRP (Wickham *et al.*, 2001). It has been estimated that up to 8 % of the *P. falciparum* genome encodes exported proteins (Sargeant *et al.*, 2006; van Ooij *et al.*, 2008). The accumulation of exported proteins at the erythrocyte membrane causes the surface of infected erythrocytes to develop ‘knobs’. Within these structures KAHRP aggregates provide a scaffold for the display of the key virulence protein PfEMP1 (Maier *et al.*, 2009).

PfEMP1 binds to CD36, intracellular adhesion molecule 1 and chondroitin sulphate A receptors on host endothelial, placental and cerebral blood vessel cells (Kraemer and Smith, 2006), allowing the sequestration of infected erythrocytes. This accumulation of infected erythrocytes in the intravascular tissues accounts for the severe disease symptoms associated with *falciparum* malaria. Patients infected with *falciparum* malaria succumb to successive waves of parasitaemia due to antigenic
variation of the exposed surface PfEMP1 molecule (Scherf et al., 2008). Approximately 60 PfEMP1 gene variants are encoded by the var gene family, but only one variant is expressed at any one time (expression is 'mutually exclusive'; Scherf et al., 2008). The controlled ‘switching’ between antigenic variants means that the parasite can repeatedly modify the degree of PfEMP1 recognition by the immune system.

Disrupting the trafficking or function of exported proteins may prevent the extensive ‘remodelling’ of the erythrocyte host and it is thus a potential target for new antimalarials (Maier et al., 2009). Relevant to this thesis, exported proteins may play a role in parasite nutrient acquisition (Section 1.4.1.1; Alkhalil et al., 2009; Nguitragool et al., 2011).

1.3. An introduction to membrane transport proteins

Cellular contents are enclosed within a highly structured membrane, and intracellular organelles are also delineated by discrete membranes (or multiple membranes in the cases of the mitochondrion, the chloroplast and the relict plastid of apicomplexans, the apicoplast). These membranes serve as barriers to the free movement of ions and large solutes, and in order to regulate the intracellular (and intra-organelle) concentrations of these molecules the cell relies on membrane transport proteins. Highlighting the critical nature of transport proteins to cellular function, many diseases are caused by transport protein dysfunction/de-regulation; over 450 transporters linked to disease are listed on the Transporter Classification Database (Saier et al., 2006; Saier et al., 2009). There are a number of discrete classes of membrane transport systems: channels, uniporters, secondary active transporters (symporters and antiporters) and primary active transporters (pumps).

Channels are essentially aqueous pores that facilitate the diffusion of substrates down their electrochemical gradient. They are generally highly substrate specific (selecting between solutes on the basis of solute size and charge) and can be activated by a range of different mechanisms including voltage, temperature, and mechanical stress. A wide range of diseases and syndromes are associated with
mutations in channel proteins. These so-called channelopathies include epilepsy, myotonia (Jurkat-Rott et al., 2010), long-QT syndrome and other cardiac action potential conduction defects (Postema et al., 2010).

Uniporters also mediate the ‘facilitated diffusion’ of substrates down their electrochemical gradients. They move one solute across the membrane at a time. Transport by a uniporter is slower than flux through a channel because transport involves a series of conformational changes in the protein, whereby a solute binding site is exposed on one side of the membrane, a solute is bound, a conformational change occurs such that the binding site is exposed on the opposite side of the membrane and the solute is then released. This model of transport, with inward and outward facing conformations, also occurs in primary and secondary active transporters.

Primary active transporters (pumps) utilise an energy source (such as ATP hydrolysis or decarboxylation) to power a conformational change such that solutes can be transported against their electrochemical gradient. There are multiple types of ATP-hydrolysing transporters including: F- (‘coupling factor of oxidative phosphorylation’) and V-type ATPases (‘vacuolar’), both involving multi-subunit complexes with an immobile stator and spinning rotor, which can operate to hydrolyse or synthesise ATP, P-type ATPases (involving an aspartyl-phosphate intermediate), and ABC transporters (ATP-binding cassette; involving distinctive nucleotide-binding folds). In humans, ATPases are well developed drug targets, with examples including the use of Na\(^+\)/K\(^+\)ATPase inhibitors (cardiac glycosides) to treat heart dysfunction and H\(^+\)/K\(^+\)-ATPase inhibitors (omeprazole, lansoprazole) to treat acid reflux (Yatime et al., 2009). Current trials testing the inhibition of Ca\(^{2+}\)-ATPases in the treatment of prostate cancer (Christensen et al., 2009), and the inhibition of bone osteoclast V-type H\(^+\)-ATPases to treat osteoporosis (Yuan et al., 2010), provide further examples.

Secondary active transporters use the energy derived from the movement of one substrate down its electrochemical gradient to power the movement of another substrate up (or against) its electrochemical gradient. Symporters transport both solutes in the same direction; antiporters transport the solutes in opposite directions.
Two common ions used to energise secondary active transporters are H\(^+\) and Na\(^+\); for example in the small intestine Na\(^+\)-coupled symporters are used to absorb glucose (Wright et al., 2011), and H\(^+\)-coupled symporters are used to absorb amino acids and peptides (Anderson and Thwaites, 2010). Crucially, this requires that the Na\(^+\) and H\(^+\) gradients are tightly regulated. It has been argued that the use of Na\(^+\) as a coupling ion evolutionarily predates the use of H\(^+\), and that H\(^+\) coupling arose in multiple lineages independently (Mulkidjanian et al., 2008).

1.4. Membrane transport in the malaria parasite

1.4.1. Transport in a *P. falciparum* infected erythrocyte

One disadvantage of being an intracellular parasite is that the erythrocyte membrane represents a barrier to the uptake of nutrients and the efflux of parasite wastes. Erythrocytes possess only a basic transporter repertoire and it is likely that endogenous erythrocyte transporters are unable to meet the nutritional needs of the metabolically active parasite. The parasite requires at least some nutrients at a faster rate than the erythrocyte membrane can deliver them. For example, although the capacity of the erythrocyte Glut1 glucose transporter is more than sufficient to meet the parasite’s glucose requirements (Kirk et al., 1996), the endogenous erythrocyte transport of the essential amino acid isoleucine through the ‘L system’ (Tunnicliff, 1994) is not adequate for the parasite’s needs (Martin and Kirk, 2007) and the same may be true for methionine (Cobbold et al., 2011). The erythrocyte lacks a transporter capable of the uptake of the essential vitamin pantothenic acid, a vital precursor of coenzyme A (Saliba et al., 1998) and an essential nutrient for parasite growth.

1.4.1.1. Transport across the infected erythrocyte membrane

Around 12 - 16 hr after invasion of the erythrocyte the parasite induces an increase in the permeability of the erythrocyte membrane to a wide range of solutes, including sugars, amino acids, nucleosides, inorganic anions and to a lesser extent inorganic cations including Na\(^+\) and K\(^+\) (Ginsburg et al., 1985; Kirk et al., 1994; Staines et al.,
The ‘New Permeability Pathways’ (NPPs) are anion selective, and are inhibited by various anion transport inhibitors including furosemide, NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid), phloridzin and glibenclamide (Kutner et al., 1987; Kirk et al., 1993; Kirk et al., 1994).

The NPPs are thought to play an important role in the uptake of nutrients, as well as contributing to the efflux from the infected cell of metabolic wastes, such as lactate, the end-product of anaerobic glycolysis (Kanaani and Ginsburg, 1991; Cranmer et al., 1995). The NPPs have also been proposed to play an important role in volume regulation of the infected erythrocyte. As discussed in Section 1.2.5, the parasite digests a large proportion of the host erythrocyte haemoglobin, and unless the resulting amino acids are exported from the infected erythrocyte, this will impose a substantial osmotic burden on the parasitised erythrocyte. The NPPs contribute to the export of amino acids from the infected erythrocyte, thereby preventing osmotic swelling (Kirk, 2001). Additionally, the NPPs show some (2.3-fold) selectivity for K+ over Na+ (Staines et al., 2001); as a result, the NPP-mediated loss of KCl from the infected cell (down its chemical gradient, out of the cell) exceeds the NPP-mediated uptake of NaCl (down its chemical gradient, into the cell; Staines et al., 2001). There is, as a result, a small net efflux of water, and thus a transient decrease in erythrocyte volume after NPP induction during the initial stages of parasite growth (Staines et al., 2001; Lew et al., 2003; Lew et al., 2004). The influx of Na+ and efflux of K+ via the NPPs results in a transformation of the ionic composition of the erythrocyte cytosol (i.e., the extracellular environment of the intraerythrocytic parasite) from low [Na+] high [K+] to high [Na+] low [K+] (Staines et al., 2001). Further details of the physiological roles of Na+ and K+ flux through the NPP are provided in Section 1.6.3.5.

Despite the likely importance of the NPPs for parasite nutrient uptake, waste efflux, host cell volume regulation and changing the ionic composition of the erythrocyte, the molecular nature and origin of the NPPs are unknown. Based upon the anion selectivity and inhibitor-sensitivity observed, the NPPs were predicted to be anion-selective channels (Kirk et al., 1994; Kirk, 2001). Subsequent studies using electrophysiological techniques confirmed the presence of novel anion channel activity in the parasitised erythrocyte membrane. The first use of the patch clamping
technique to study transport at the erythrocyte plasma membrane was by Desai et al. (2000), who showed that infected erythrocytes (but not uninfected erythrocytes) had a voltage-dependent, low conductance, Cl− selective channel with pharmacological characteristics similar to those of the NPPs. They subsequently named this channel the Plasmodial Surface Anion Channel (PSAC; Alkhalil et al., 2004). Since the original patch-clamp study multiple research teams have confirmed the presence of anion-selective channels in the plasma membrane of infected erythrocytes (Egee et al., 2002; Huber et al., 2002), although there has been significant controversy over the electrophysiological characteristics of these channels, as well as their origin (Staines et al., 2003).

Egee et al. (2002) and Huber et al. (2002) demonstrated that anion-selective channels, with similar properties to those observed in infected erythrocytes, could be induced in uninfected erythrocytes by stimuli such as oxidation, phosphorylation or membrane stretch. This led to the hypothesis that the parasite induces the NPPs through activation of an endogenous erythrocyte channel. There is evidence that either the erythrocyte CIC2 channel (Huber et al., 2004) or the ‘cystic fibrosis transmembrane regulator’ channel (Verloo et al., 2004) may contribute to the observed anion conductance, though in neither case was the presence of the channel essential for parasite survival. Recently, Thomas and colleagues demonstrated that uninfected erythrocytes possess a dormant ‘maxi-anion’ channel (Glogowska et al., 2010), which can be part of a Peripheral-Type Benzodiazepine Receptor (PBR) complex comprised of a Voltage Dependent Anion Channel (VDAC; mediating the Cl− current), isoquinoline-binding protein and adenine nucleotide transporter (Bouyer et al., 2011). VDAC is selective for anions, but is able to transport sugars, purines, amino acids and organic/inorganic cations (Shoshan-Barmatz et al., 2010), similar to the NPP. Ligands that inhibit the PBR complex were shown to decrease the conductance observed across the infected erythrocyte membrane and to inhibit parasite growth, consistent with the hypothesis that the activation of the endogenous erythrocyte plasma membrane protein VDAC contributes to the parasite induced NPP activity (Bouyer et al., 2011).

However, there is also evidence for an alternative hypothesis: that the NPPs are parasite-encoded, and are exported from the parasite to the erythrocyte membrane.
Consistent with this hypothesis, Desai and colleagues showed that erythrocytes from a single donor infected with two different strains of *P. falciparum* showed significant differences in the voltage-dependent gating properties of the parasite-induced conductance (Alkhalil et al., 2004). They proposed that mutations in the gene encoding for PSAC could cause different channel properties in the different strains (Alkhalil et al., 2004). Further evidence to support this claim was presented in a second study by the same authors, who showed that the furosemide-sensitivity of the PSAC varied between 19 parasite strains that were the progeny of a *P. falciparum* genetic cross (Alkhalil et al., 2009). Other evidence for the NPPs being parasite-encoded includes the observation that chymotrypsin treatment of infected erythrocytes abolished NPP activity, but after washing away the protease NPP activity at the erythrocyte membrane returned over 3 hr in a brefeldin-A sensitive process (Baumeister et al., 2006). These data were interpreted in terms of NPP formation being dependent on the synthesis within the parasite and trafficking to the erythrocyte surface (via a brefeldin A-sensitive pathway) of new proteins (N.B. the erythrocyte has no protein synthesis machinery) (Baumeister et al., 2006). Lastly, a very recent study used quantitative trait locus analysis of the progeny of a *P. falciparum* genetic cross to link varying degrees of PSAC activity with the parasite Cytoadherence Linked Asexual Gene 3 (clag3; Nguitragool et al., 2011). Members of the clag gene family are exported to the erythrocyte membrane (Trenholme et al., 2000), and it is proposed that clag3 contributes to PSAC channel activity, either alone or in a complex with unidentified proteins (Nguitragool et al., 2011).

Just as it is uncertain whether the NPPs are induced by an activation of an endogenous erythrocyte protein or expression of a parasite-encoded protein, it is also unclear whether one transport pathway is capable of mediating the transport of such a wide range of solutes, or if multiple channels are involved (reviewed in Ginsburg and Stein, 2005). Elucidating the molecular nature of the NPPs is important, as blocking nutrient uptake in the parasite is an attractive strategy for chemotherapy (Staines et al., 2005; Pillai et al., 2010). Additionally, the NPPs are a pathway for drug entry into the parasite infected erythrocyte, as recently demonstrated for the antibiotic fosmidomycin (Baumeister et al., 2011).
In addition to clag3, several parasite transport proteins have been localised to the erythrocyte membrane; a sub-unit of the V-type H⁺-ATPase (Marchesini et al., 2005), a K⁺ channel, PfK1 (Waller et al., 2008) and a putative copper pump, PfCuP-ATPase (Rasoloson et al., 2004). The contribution of these proteins to inorganic cation regulation has not been determined in physiological studies, and it is unclear whether these proteins play a functional role, or whether their apparent localisation to the infected erythrocyte membrane might be an artefact of the immunolocalisation process.

1.4.1.2. Transport across the parasitophorous vacuole membrane

The parasitophorous vacuole membrane is not believed to be a significant permeability barrier, due to the presence in this membrane of high-capacity, non-selective pores that are highly permeable to low molecular weight solutes with a size less than 23 Å (Desai et al., 1993; Desai and Rosenberg, 1997; Desai, 1999). Solutes that enter the erythrocyte are believed to have unrestricted access to the parasite plasma membrane.

1.4.1.3. Transport across the parasite plasma membrane

Influx into the parasite, across the parasite plasma membrane, occurs through a multitude of transporters (see Section 1.4.2). This model of nutrient uptake, whereby solutes are transported across the erythrocyte membrane, then the parasitophorous vacuole membrane, and then the parasite plasma membrane is known as the ‘sequential’ model of nutrient uptake (Kirk, 2001). Fig. 1.5 shows a schematic representation of the membranes relevant to parasite nutrient uptake. Other ‘parallel’ pathways of nutrient uptake have been proposed including; (i) a ‘metabolic window’ whereby there is a specific region where the erythrocyte membrane, parasitophorous vacuole membrane and the parasite membranes are fused allowing the uptake of solutes across one membrane (Bodammer and Bahr, 1973), (ii) a ‘tubovesicular network’ in which extensions of the parasitophorous vacuole membrane fuse with the erythrocyte membrane and allow solutes to be taken up directly into the parasitophorous vacuole space (Lauer et al., 1997), and (iii) a ‘parasitophorous duct’ in which the extensions of the parasitophorous vacuole are directly open to the
extracellular solution, so that the parasite plasma membrane is exposed to the external environment (Pouvelle et al., 1991). Although at least some of these alternative hypotheses have not been ruled out, most of the physiological studies are consistent with the ‘sequential’ model of transport.

Figure 1.5. Membranes involved in nutrient uptake and waste efflux in a *Plasmodium* infected erythrocyte. Solutes must first cross the red blood cell (RBC) plasma membrane. This occurs through the parasite-induced channel-like New Permeability Pathways (NPP), endogenous erythrocyte transporters or parasite transporters exported to the erythrocyte membrane. The parasitophorous vacuole membrane (PVM) is thought not to present a barrier to solute transport as it contains large pores (labelled ‘PVM channel’). The parasite has many transporters on its plasma membrane, and also on internal organelles, such as the digestive vacuole (DV), for the uptake and efflux of nutrients and wastes. The figure is adapted from Landfear (2011).

1.4.2. Transport proteins of the malaria parasite

The transport of a range of solutes across the parasite plasma membrane has been characterised using a number of different techniques, including radioisotope flux measurements and fluorescent ion-sensitive probes. A number of the proteins
involved have been characterised using heterologous expression systems (e.g. *Xenopus laevis* oocytes or *Saccharomyces cerevisiae*). Bioinformatic analyses have yielded a comprehensive list of *P. falciparum* transport proteins (transporters, uniporters and channels), and it is estimated that approximately 2.5% of the parasite genome encodes transport proteins (Martin *et al.*, 2005; Martin *et al.*, 2009a).

Many transport proteins have been localised to the parasite plasma membrane. These include: an aquaglyceroporin PfAQP (Hansen *et al.*, 2002); the V-type H⁺-ATPase (Hayashi *et al.*, 2000; Marchesini *et al.*, 2005); a V-type K⁺-dependent H⁺-pyrophosphatase (PPase), PfVPl (McIntosh *et al.*, 2001); the P-type copper-ATPase PfCuP-ATPase (Rasoloson *et al.*, 2004); a putative cation-transporting ATPase, PfATP4 (Dyer *et al.*, 1996; Rottmann *et al.*, 2010), the multi-drug resistance (MDR) transporter 5, PfMDR5, and multi-drug resistance-associated proteins MRP1 and 2 (Kavishe *et al.*, 2009); the Na⁺-dependent inorganic phosphate transporter, PfPiT (Saliba *et al.*, 2006); an inorganic anion transporter, PfSulP (Henry *et al.*, 2007); the hexose transporter, PfHT1 (Woodrow *et al.*, 1999); and the nucleoside/nucleobase transporter, PfENT1 (Rager *et al.*, 2001). Additionally, there is physiological evidence for the presence on the parasite plasma membrane of: an ATP/ADP exchanger (Kanaani and Ginsburg, 1989; Hatin *et al.*, 1992), a monocarboxylate/ H⁺ symporters (Kanaani and Ginsburg, 1991; Cranmer *et al.*, 1995; Elliott *et al.*, 2001), a H⁺-folate symporter (Wang *et al.*, 2007), a neutral amino acid transporter (Martin and Kirk, 2007; Cobbeld *et al.*, 2011), a H⁺-pantothenate symporter (Saliba and Kirk, 2001) and a choline transporter (Biagini *et al.*, 2004; Lehane *et al.*, 2004).

The transport of solutes across intracellular membranes is less well studied. Three transporter proteins have been localised to the digestive vacuole membrane: the V-type H⁺-ATPase (Hayashi *et al.*, 2000), the ‘chloroquine resistance transporter’, PfCRT (Fidock *et al.*, 2000) and PfMDR1 (Cowman *et al.*, 1991). There is physiological evidence for a Ca²⁺-ATPase (Biagini *et al.*, 2003) and a V-type H⁺-PPase (Saliba *et al.*, 2003) on the digestive vacuole membrane. An ATP/ADP exchanger (Hatin *et al.*, 1992) and a Ca²⁺, Mn²⁺/H⁺ exchanger (Rottmann *et al.*, 2010) have been localised to the mitochondrial membrane, whilst two triose phosphate/ phosphoenolpyruvate antiporters have been characterised on the apicoplast membrane (Mullin *et al.*, 2006; Lim *et al.*, 2010).
Despite the known localisation of many membrane transport proteins, their physiological role/s are less well understood.

1.4.3. Transporters as drug targets and mediators of drug resistance

There are several membrane transport proteins that have been implicated in antimalarial drug resistance (recently reviewed by Sanchez et al., 2010; Petersen et al., 2011). Transporters may facilitate drug uptake and delivery to the target site. Conversely, they may facilitate drug efflux, decreasing the drug concentration at the target site.

One transporter involved in the efflux of drug from its site of action is the chloroquine resistance transporter, PfCRT. PfCRT is localised to the DV membrane (Fidock et al., 2000). Its physiological role and native substrate are unknown, but mutations in PfCRT are associated with chloroquine resistance (Wellems et al., 1991; Fidock et al., 2000). Using the Xenopus heterologous expression system, Martin et al. showed that mutant PfCRT (but not the wild-type form of the protein) is able to transport chloroquine across the membrane, consistent with the hypothesis that the mutant protein confers chloroquine resistance by transporting the drug out of the digestive vacuole where it has its antimalarial effect (Martin et al., 2009b).

A major mediator of multidrug resistance in mammalian cells is the ABC transporter P-glycoprotein, which facilitates the efflux of a wide range of drugs out of cells, away from their intracellular molecular targets. Mutations or copy number variations in the P. falciparum P-glycoprotein homologue (Pfmdrl) are associated with resistance to chloroquine, quinine, mefloquine and artemisinin (Reed et al., 2000; Sidhu et al., 2005). In Plasmodium, Pfmdrl is localised to the digestive vacuole where it is proposed to sequester drugs from the parasite cytosol. Mutant forms of Pfmdrl are unable to transport chloroquine and quinine (Sanchez et al., 2008).

A multidrug resistance-associated protein (PfMRP) has also been associated with drug efflux from the parasite. PfMRP is localised to the parasite plasma membrane, and PfMRP knock-out parasites have increased susceptibility to chloroquine, quinine and artemisinin (Raj et al., 2009).
Mutations and repeat variations in a putative Na⁺/H⁺ exchanger (NHE), PfNHE, have been linked to quinine resistance (reviewed in Okombo et al., 2011). PfNHE has been postulated previously to play a role in chloroquine resistance (Sanchez et al., 1997; Wunsch et al., 1998), but this was subsequently disputed (Bray et al., 1999a; Bray et al., 1999b) and some of the original findings underpinning the hypothesis were later reassessed (Kuhn et al., 2007). Using quantitative trait loci mapping of a parasite genetic cross, Ferdig et al. linked the inheritance of reduced quinine sensitivity to a 380 kb segment on chromosome 13 (Ferdig et al., 2004). There are about ~100 genes in this segment, including that encoding PfNHE. When the PfNHE genes of quinine resistant and sensitive isolates were sequenced there was found to be point mutations at three codons and microsatellite length variations at another three locations (Ferdig et al., 2004).

The microsatellite 'ms4760' of PfNHE is highly variable and the correlation between the number of repeats of DNNND and DDNNNDNHNDNND repeats sequences and quinine resistance has been investigated in many field and laboratory isolates (reviewed in Okombo et al., 2011). For the DNNND repeat, several studies found an increase in the quinine IC₅₀ (the quinine concentration at which parasite proliferation is reduced to half that seen in the absence of drug) with an increase in the number of DNNND repeats (Ferdig et al., 2004; Henry et al., 2009; Meng et al., 2010; Okombo et al., 2010; Pelleau et al., 2011). However, other studies have failed to find a correlation (Andriantsoanirina et al., 2009; Baliraine et al., 2010; Briolant et al., 2010; Parquet et al., 2010; Pradines et al., 2010; Briolant et al., 2011). Conflicting results were also obtained when looking at the DDNNNDNHNDNDNND repeats status; one study found an increase in quinine IC₅₀ with an increase in the number of DDNNNDNHNDNDNND repeats (Andriantsoanirina et al., 2010), three studies found a decrease in quinine IC₅₀ with an increase in the number of DDNNNDNHNDNDNND repeats (Henry et al., 2009; Meng et al., 2010; Pelleau et al., 2011), and other studies failed to find a correlation (Baliraine et al., 2010; Briolant et al., 2010; Okombo et al., 2010; Briolant et al., 2011). The role of PfNHE microsatellite variations in quinine resistance is therefore unclear.

The question of how quinine resistance may be related to the physiological function of PfNHE also remains unanswered. Bennett et al. claim to have demonstrated that
increased (plasma membrane) PfNHE activity correlates with increasing levels of quinine resistance (Bennett et al., 2007). However, the technique that they used to measure PfNHE activity has been called into question (Spillman et al., 2008; Nkrumah et al., 2009). In the study by Nkrumah et al. (2009) PfNHE knockdown lines were generated with a ~50% reduction in protein expression as determined by densitometric analysis of Western blots (Nkrumah et al., 2009). Knockdown parasite lines generated using chloroquine- and quinine-resistant parents showed a significant decrease in the quinine IC$_{50}$; however knockdown parasites generated using chloroquine- and quinine-sensitive parents showed no significant difference in the quinine IC$_{50}$ (Nkrumah et al., 2009).

Transporters may also be the target of antimalarial drugs. It has been postulated that the artemisinins target the putative Ca$^{2+}$-ATPase, PfATP6 (Eckstein-Ludwig et al., 2003; Krishna et al., 2010); however this has been controversial (Valderramos et al., 2010; Klonis et al., 2011). Both chloroquine and artemisinin have been shown to disrupt ion regulation in the murine parasite P. chabaudi (Gazarini et al., 2007).

This thesis is focussed on mechanisms of cation homeostasis in the parasite, particularly Na$^{+}$ regulation. The transport of Na$^{+}$ can affect the pH, volume and membrane potential of a cell. P. falciparum transporters involved in pH regulation and the maintenance of a low [Na$^{+}$], are discussed further in Sections 1.5.3.5 and 1.6.3.5.

### 1.5. Intracellular pH regulation

#### 1.5.1. The significance of intracellular pH regulation

The regulation of cytosolic pH (pH$_{i}$) is of crucial importance in all cells. The ionisation state of the cellular constituents is dependent on pH, and protein function is optimized for a particular, usually narrow, pH range. Fluctuations in pH$_{i}$ are therefore detrimental to cell function. Some membrane transport processes are H$^{+}$-coupled, using the transmembrane pH gradient (the ‘proton motive force’) as an energy source for solute transport; such processes are influenced directly by pH$_{i}$. 
The major mechanisms for cytosolic pH regulation are H\(^+\) binding by buffering groups, and the transport of ‘H\(^+\) equivalents’ into/out of the cytosol (Bethmann and Schonknecht, 2009). The term ‘H\(^+\) equivalents’ is used as the directional flux of acid (i.e., H\(^+\)) across a membrane has an equivalent effect on pHi to the flux of base (OH\(^-\) or HCO\(_3^-\)) in the opposite direction. To maintain pH at near-neutrality, the export of H\(^+\) equivalents (through the efflux of H\(^+\) or the influx of OH\(^-\) or HCO\(_3^-\)), referred to as ‘acid extrusion’, must equal the import of H\(^+\) equivalents (through the influx of H\(^+\) or the efflux of OH\(^-\) or HCO\(_3^-\)) referred to as ‘acid loading’. The activity of the transporters involved in pH regulation is finely controlled such that acid loading transporters tend to be activated at alkaline pH values and acid extruding transporters tend to be activated at acidic pH values.

A schematic of the transporters involved in pH\(_i\) regulation (referred to throughout Section 1.5), is shown in Fig. 1.6.

1.5.2. pH regulation in prokaryotes

Bacteria and archaea face an extraordinary range of environmental pH challenges, inhabiting niches with pH ranges from 0.5 – 5 (acidophiles), 5 – 9 (neutralophiles) and 9 – 12 (alkaliphiles) (Slonczewski et al., 2009). Both H\(^+\)-ATPases and cation/H\(^+\) exchangers are involved in pH regulation in prokaryotes (reviewed extensively by Slonczewski et al., 2009; Krulwich et al., 2011). Typically, an inwardly directed H\(^+\) gradient is generated through the action of respiratory chain H\(^+\)-pumping complexes (such as cytochrome bo and NADH-ubiquinone oxidoreductase in E. coli) or the F\(_1\)F\(_0\)-ATPase (in non-respiratory bacteria), both of which can be activated in response to an acid load (Krulwich et al., 2011). To respond to alkaline conditions the activity of cation/H\(^+\) antiporters (operating to import H\(^+\)) and F\(_1\)F\(_0\)-ATP synthases is increased leading to cytosolic H\(^+\) accumulation (Krulwich et al., 2011).

Bacteria use cation/H\(^+\) antiporters not only for pH regulation, but also for Na\(^+\) (or K\(^+\)) homeostasis (see Section 1.6.2) and volume regulation. Typically, extracellular bacteria have between 5 - 9 cation/H\(^+\) antiporters, whereas intracellular bacteria have
Fig. 1.6. Transport proteins involved in pH regulation in multiple cell types. Transporters included in this figure play key roles in the movement of $\text{H}^+$, and the specifics of their involvement are detailed in Sections 1.5.2 and 1.5.3. As indicated for the NHEs, Fig. 1.7 provides further details of their phylogenetic relationships.

0 - 1 and extremophiles have 11 - 14 such proteins (Krulwich et al., 2009). The *E. coli* $\text{Na}^+$/H$^+$ antiporter, NhaA has been studied extensively as a ‘model’ NHE (Padan et al., 2004; Padan et al., 2005). NhaA facilitates the electrogenic (2$\text{H}^+$: 1$\text{Na}^+$) influx of $\text{H}^+$, powered by the inwardly negative membrane potential (Padan et al., 2005). Structural information for NhaA has been determined at 3.45 Å in a ‘locked’ conformation at pH 4 where the transporter is inactive (Hunte et al., 2005) and also at pH 8 in an ion-translocating conformation (Appel et al., 2009). NhaA exists as a native homodimer (although the monomer is functional) and has 12-TMD with the N- and C- termini on the cytoplasmic side (Padan et al., 2004).

NhaA belongs to the cation proton antiporter 2 (CPA2) family of NHEs (Brett et al., 2005). Many members of the CPA superfamily are discussed throughout Sections...
1.5 and 1.6. Knowledge of the CPA classification scheme aids in understanding their specific function in cation homeostasis. Fig. 1.7 is an introductory guide to the CPA family structure and it includes the members referred to throughout the thesis, together with their functional roles.

Bacteria also express H⁺-translocating pyrophosphatases (H⁺-PPases), which couple the hydrolysis of pyrophosphate to the transport of H⁺, against an H⁺ gradient. These PPases are predicted to have 14 - 16 TMD, may function as a dimer, and have an absolute requirement for Mg²⁺ (Belogurov and Lahti, 2002; Malinen et al., 2007). There are two types of H⁺-PPases, a K⁺-dependent subfamily with a signature sequence GNXXA, and a K⁺-independent subfamily with a signature sequence GNXXXK (Belogurov and Lahti, 2002). There is recent evidence that in prokaryotes the K⁺-dependent subfamily may transport Na⁺ and not H⁺ (Luoto et al., 2011).

Aside from membrane transporters, bacteria have many other mechanisms to cope with pH fluctuations, including changes in metabolic pathways to produce different acidic/neutrotal end-products and activation of urease or carbonic anhydrase activity (Krulwich et al., 2011).

1.5.3. pH regulation in eukaryotes

1.5.3.1. pH regulation in animals

In animal cells several transporters play a role in pH regulation. Pre-eminent amongst these is the NHE, of which there are nine isoforms in humans, all of which are sensitive to the amiloride class of NHE inhibitors. These transporters belong to the CPA1 family (Fig. 1.7), and use the inward concentration gradient for Na⁺, established by the Na⁺/K⁺-ATPase (Section 1.6.3.1), to energise the efflux of H⁺ from the cell. Human isoform HsNHE1 is ubiquitously expressed in all cell types and mediates the electroneutral exchange of 1 Na⁺ for 1 H⁺. HsNHE1 is activated at acidic cytosolic pH values when a H⁺ binds at an intracellular allosteric binding site, and it mediates the pH changes associated with cell growth, proliferation,
Figure 1.7. Schematic representation of the organisation of the monovalent cation proton antiporter (CPA) superfamily. Abbreviations used include; NaT-DC; Na⁺-transporting carboxylic acid decarboxylase, NHE; Sodium proton exchanger, SOS; salty overly sensitive, NHA; Sodium proton exchanger, CHX; Cation proton exchanger, PM; plasma membrane. The coloured boxes distinguish the three CPA families and seven subfamilies. Members of the CPA superfamily mentioned in this thesis (with cross references) are included in this classification scheme. This diagram is based on a study of the evolutionary origins of eukaryotic NHE (Brett et al., 2005).
differentiation and apoptosis (Slepkov et al., 2007). HsNHE1 is also involved in cell volume regulation, facilitating an influx of Na⁺ in response to cell shrinkage (Alexander and Grinstein, 2006). Phosphorylation at multiple sites in the C-termini activates the transporter (Slepkov et al., 2007). Activity is also regulated by Ca²⁺-binding proteins; it is activated by calmodulin and calcineurin-homologous protein, and inhibited by tescalcin (Malo and Fliegel, 2006). Interactions of HsNHE1 with ezrin/radixin/moesin proteins anchor the C-termini to actin filaments and this localises NHE spatially within a cell to selective membrane domains (Meima et al., 2007).

Human NHE isoforms 2-5 are all plasma membrane localised, with restricted tissue distribution. For example HsNHE3 is in epithelia, kidney, intestine cells and HsNHE5 is in brain, spleen, testis and skeletal muscle (reviewed extensively in Slepkov et al., 2007). HsNHE6-9 are predominantly localised to intracellular exo/endocytic vesicles and organelles (Orlowski and Grinstein, 2007). Humans also have one sperm-specific NHE from the Na⁺-transporting carboxylic acid decarboxylase-like family of NHE (Fig. 1.7); this protein may have a role in pH regulation in sperm (Wang et al., 2003).

Under conditions of an intracellular alkalinisation, when the NHE is inactivated, anion exchangers such as the acid-loading Cl⁻/HCO₃⁻ exchanger are activated (Pushkin and Kurtz, 2006). There is evidence that the activities of the NHE and Cl⁻/HCO₃⁻ exchanger are functionally coupled, and it has been hypothesised that this is due to sub-membrane microdomains of H⁺ or HCO₃⁻ generated in the vicinity of the transporters (Hayashi et al., 2009). Along with the efflux of HCO₃⁻, which results in a decrease in pHᵢ, this transporter is also responsible for Cl⁻ accumulation in some cell types.

Animal cells also have a V-type H⁺-ATPase that is responsible for the acidification of intracellular vesicles/organelles (Jefferies et al., 2008). Both the exocytotic and endocytotic pathways require the V-type H⁺-ATPase-mediated acidification of vesicles. The maintenance of an acidic vesicle environment is essential for proper trafficking and functioning, for example neurotransmitter/hormone accumulation in secretory vesicles is H⁺-coupled (Marshansky and Futai, 2008). The V-type H⁺-
ATPase is also present on the plasma membrane of some cell types including kidney cells (the proximal tubular cells and intercalated cells of the distal tubule), osteoclasts, interdental inner ear cells, clear cells of the epididymis/vas deferens, neutrophils, macrophages and tumour cells (Breton and Brown, 2007; Jefferies et al., 2008). In these cells, the plasma membrane V-type H\(^{+}\)-ATPase plays a key role in H\(^{+}\) efflux, either to maintain resting pH\(_i\), or to acidify the extracellular space.

1.5.3.2. pH regulation in plants

In plants a P-type H\(^{+}\)-ATPase on the plant plasma membrane functions to efflux H\(^{+}\) and thereby prevent cytosolic acidification. In Arabidopsis there are 11 P-type H\(^{-}\)-ATPases (Axelsen and Palmgren, 2001) named AtAHA1-11 (for auto-inhibited H\(^{-}\)-ATPase); these generate a plasma membrane pH gradient that can subsequently be used for the secondary active transport of inorganic ions, metabolites and important nutrients. The active efflux of H\(^{+}\) also results in the generation of an inwardly negative membrane potential of -150 to -230 mV (Hirsch et al., 1998; Benito et al., 2002). P-type H\(^{-}\)-ATPase activity has been linked to multiple important plant processes including xylem and phloem loading, stomata aperture regulation, pathogen attack and cell elongation (Duby and Boutry, 2009; Liu et al., 2009).

The C-terminus of the P-type H\(^{-}\)-ATPase has an auto-inhibition domain and, on the basis of the crystal structure of AtAHA2, it has been proposed that auto-inhibition occurs when the C-terminus physically blocks the H\(^{+}\) entry site (Pedersen et al., 2007). All AtAHA proteins have a conserved penultimate threonine phosphorylation site in the C terminus, with phosphorylation required for pump activation. Additionally, pump activation also requires the binding of regulatory 14-3-3 proteins at these phosphorylated sites, which is believed to release the physical constraint of auto-inhibition (Speth et al., 2009).

Acidification of the plant cell’s internal vacuole is achieved by two other types of H\(^{+}\)-pumps; a V-type H\(^{+}\)-ATPase and a type I, K\(^{+}\)-dependent V-type H\(^{+}\)-PPase. These pumps generate a pH gradient across the vacuolar membrane and this is used for secondary active transport. They also maintain the acidic conditions necessary for macromolecule recycling in the vacuole (Muntz, 2007). The relative contribution
of the two pumps to vacuolar acidification, and the interactions between these two 
H⁺ proteins is unclear, although it was recently demonstrated that the *Arabidopsis* V-
type H⁺-PPase was capable of completely complementing (in terms of vacuole 
acidification level and endocytic pathway function) a yeast mutant lacking the V-
type H⁺-ATPase (Perez-Castineira *et al.*, 2011). This is consistent with some 
redundancy in the activities of the V-type H⁺-ATPase and V-type H⁺-PPase on the 
plant vacuole membrane.

The V-type H⁺-ATPase and a type II (K⁺-independent) V-type H⁺-PPase are 
localised to exocytic/secretory vesicles and the Golgi apparatus (Mitsuda *et al.*, 
2001; Segami *et al.*, 2010) where they play roles in vesicle acidification and 
trafficking. Additionally, in multiple plant species, V-type H⁺-PPases have also been 
localised to plant plasma membranes (Langhans *et al.*, 2001; Alexandersson *et al.*, 
2004; Paez-Valencia *et al.*, 2011). The contribution of the V-type H⁺-PPase to pH 
regulation across the plasma membrane is unclear. It has been proposed that the V-
type H⁺-PPase may function under conditions of low [ATP]. It has also been 
hypothesised that the plasma membrane V-type H⁺-PPases may operate in reverse to 
synthesise pyrophosphate (Paez-Valencia *et al.*, 2011).

### 1.5.3.3. pH regulation in fungi

Like plants, fungi have a plasma membrane P-type H⁺-ATPase that pumps H⁺ out of 
the cell, generating an inward H⁺-gradient. The yeast *S. cerevisiae* has been used as 
a model organism for the study of cation homeostasis in fungi due to its ease of 
growth and amenability to genetic manipulation. The major P-type H⁺-ATPase, 
Pma1, is the most abundant plasma membrane protein in *S. cerevisiae* and it 
consumes ~20 % of cellular ATP (Arino *et al.*, 2011). *S. cerevisiae* also encodes a 
second P-type H⁺-ATPase called Pma2. Yeast use the inwardly directed pH gradient 
across the plasma membrane for the secondary active transport of other inorganic 
ions and nutrients. The efflux of H⁺ via the P-type H⁺-ATPase also generates a very 
large inwardly negative membrane potential (the highest of all organisms) of up to 
~300 mV (Blatt *et al.*, 1987).
Yeast also have a V-type H⁺-ATPase (Vma), localised to the vacuole, endosomal and Golgi membranes, playing the role of acidifying these organelles (Arino et al., 2011). There is evidence that in yeast the functions of the P- and V-type H⁺-ATPases in the maintenance of cytosolic and vacuolar pH are coordinated, and that Pma1 can ‘mis’-localise to the vacuole membrane in Vma mutants without V-type H⁺-ATPase activity (Martinez-Munoz and Kane, 2008). Also, aspects of both cytosolic and vacuolar pH regulation are perturbed in the Vma mutants (Martinez-Munoz and Kane, 2008).

1.5.3.4. pH regulation in protozoan parasites

pH regulation has been investigated in multiple protozoan parasites. In T. gondii tachyzoites (the stage that invades nucleated cells of its host), a plasma membrane localised V-type H⁺-ATPase (sensitive to the V-type H⁺-ATPase inhibitor bafilomycin A₁) is responsible for pHᵢ maintenance (Moreno et al., 1998). The efflux of H⁺ also contributes to the generation of the membrane potential in tachyzoites, as V-type H⁺-ATPase inhibition led to a membrane depolarisation (Moreno et al., 1998). The V-type H⁺-ATPase (along with a K⁺-dependent H⁺-PPase) is also localised to an acidic Ca²⁺/polyphosphate enriched organelle, called the acidocalcisome where it is involved in organelle acidification (Rohloff et al., 2011). In T. gondii bradyzoites (the cyst-forming stage), a plasma membrane P-type H⁺-ATPase (TgPMA1) is expressed (Holpert et al., 2001), but although TgPMA1 knockout parasites have been generated, the role of TgPMA1 in bradyzoite pH regulation has not been investigated (Holpert et al., 2006).

T. gondii also encodes four NHE proteins (Gajria et al., 2008); however for only one of these, TgNHE2, is there evidence for a role in pH regulation. TgNHE2 is localised to invasion organelles, the rhoptries (Karasov et al., 2005). Rhoptries initially have a low pH of 3.5-5.5 however as they mature they become more neutral (Shaw 1998). It has been proposed that TgNHE2 plays a role in the acidification of the young rhoptries (Karasov et al., 2005).

In T. cruzi, P-type H⁺-ATPases have been localised to the plasma membrane of multiple parasite life stages (Vieira et al., 2005). This is consistent with
Physiological measurements of pH in amastigote and trypomastigote stages, which demonstrated that pH regulation was via an H^+-ATPase (of undetermined type; Van Der Heyden and Docampo, 2000). pH regulation was also found to involve H^+-ATPases in T. brucei although the bloodstream stage was found to be less able to cope with acid stresses than the procyclic stage (Fraser-L'Hostis et al., 1997). Further experiments demonstrated that this was because in bloodstream forms the major contributor to pH regulation is a monocarboxylate transporter (a pyruvate/H^+ symporter) with the H^+-ATPase playing only a minor role (Vanderheyden et al., 2000). Three P-type H^+-ATPases (TbHA1-3) have been identified in T. brucei and these are upregulated in procyclic stages (Luo et al., 2006). A knockdown of TbHA1 or 3 caused resting pH to be more acidic and cells were slower to recover from an acidification, confirming the role of the P-type H^+-ATPases in H^+ efflux (Luo et al., 2006). P-type H^+-ATPase activity has also been observed in Leishmania parasites (Marchesini and Docampo, 2002), and a regulatory autoinhibitory domain in the C-termini of the pump has been characterised (Grigore and Meade, 2006).

1.5.3.5. pH regulation in Plasmodium

In a parasitised erythrocyte, each of the compartments (see Fig. 1.5) has pH regulatory mechanisms. The normal pH of the blood plasma is ~7.4 (Boron and Boulpaep, 2009). However, one consequence of malaria is blood acidosis; thus in malaria-infected individuals blood pH may be lower, with values as low as ~7.1 reported in children with severe disease (English et al., 1997). Uninfected erythrocytes maintain a cytosolic pH of ~7.3 (Alonso et al., 1993). The cytosol of the infected erythrocyte also has a pH of ~7.3, however a zone close to the parasite surface pH is more acidic at ~6.9 (Hayashi et al., 2000). The parasite cytosol is maintained at a pH of ~7.3 (Saliba and Kirk, 1999; Hayashi et al., 2000; Lehane et al., 2004; Kuhn et al., 2007; Spillman et al., 2008). Organellar pH is also tightly controlled; for example the pH of the digestive vacuole (important in the digestion of erythrocyte haemoglobin) has a pH estimated to be between 4.5 - 6 (Yayon et al., 1984; Hayward et al., 2006; Klonis et al., 2007; Kuhn et al., 2007; Abu Bakar et al., 2010).
To maintain a stable, resting pH, parasite H⁺ efflux mechanisms must counter both the influx of H⁺ and the generation of H⁺ intracellularly via metabolic processes. *P. falciparum* is reliant on glycolysis for ATP generation. For every one glucose molecule utilised, two ATP are generated (with subsequent hydrolysis resulting in two H⁺) along with two pyruvate molecules which are converted to lactate by lactate dehydrogenase. The parasite uses a plasma membrane localised monocarboxylate symporter, with a stoichiometry of 1 H⁺ : 1 lactate to remove H⁺ derived from glycolysis (Elliott *et al.*, 2001). Another H⁺ efflux transport mechanism is needed to extrude H⁺ generated in other metabolic reactions and H⁺ that enter the parasite via membrane transport mechanisms.

Initial evidence suggested that, as in mammalian cells, the intraerythrocytic *Plasmodium* parasite uses an NHE to efflux H⁺ from the cytosol. Bosia *et al.* reported that the maintenance of pHᵢ was Na⁺-dependent and that the addition of an NHE inhibitor caused an acidification of the parasite (Bosia *et al.*, 1993). However, it was subsequently shown that parasite recovery from an acid load was Na⁺-independent and that recovery was inhibited by the V-type H⁺-ATPase inhibitor concanamycin A (Saliba and Kirk, 1999). V-type H⁺-ATPase inhibitors also caused a profound disruption of the resting pHᵢ (Saliba and Kirk, 1999). These data are consistent with the hypothesis that a plasma membrane V-type H⁺-ATPase plays a key role in parasite pHᵢ regulation. A number of other studies have provided further support for this hypothesis. Hayashi *et al.* confirmed the effects of V-type H⁺-ATPase inhibitors on parasite pH regulation, as well as demonstrating the presence of a subunit of the pump at the parasite surface and the digestive vacuole membrane (Hayashi *et al.*, 2000). A number of studies have shown an effect of V-type H⁺-ATPase inhibitors on the operation of transport processes at the parasite plasma membrane (Biagini *et al.*, 2004; Wang *et al.*, 2007) and evidence has also been presented for the plasma membrane V-type H⁺-ATPase being the primary source of the parasite’s large, inwardly negative membrane potential (Allen and Kirk, 2004b). Additionally, ATPase activity measured in purified membrane preparations of both the parasite plasma membrane, and the digestive vacuole membrane display sensitivity to the V-type H⁺-ATPase inhibitor bafilomycin A₁ (Elandalloussi *et al.*, 2005). These results are consistent with acid extrusion from asexual blood-stage *Plasmodium* parasites being via a V-type H⁺-ATPase and not via an NHE.
Previous studies demonstrated that pH regulation in the parasite is perturbed rapidly by removing extracellular glucose, or inhibiting ATP production (Saliba and Kirk, 1999; van Schalkwyk et al., 2008). A similar disruption of pH regulation in *P. falciparum* has been observed using a series of indole-based V-type H\(^+\)-ATPase inhibitors, which caused a rapid cytosolic acidification (van Schalkwyk et al., 2010). The degree of cytosolic acidification correlated with the antimalarial activity of the inhibitors, highlighting the potential of the V-type H\(^+\)-ATPase as an antimalarial drug target (van Schalkwyk et al., 2010).

In a recent study, Bennett et al. (2007) presented measurements of a Na\(^+\)-dependent pH-recovery of parasites that had been subjected to an intracellular acidification using the H\(^+\) ionophore nigericin (which catalyses the exchange of H\(^+\) and, nominally, K\(^+\), but also, to a lesser extent, other Group I cations). The authors of this study made no mention of the V-type H\(^+\)-ATPase in the interpretation of their results. The pH\(_r\) recovery observed following an intracellular acidification imposed using nigericin was attributed to a parasite NHE (Bennett et al., 2007). There is further discussion of the physiological role of the putative parasite NHE in Section 1.6.3.5.

*P. falciparum* also encodes two V-type H\(^+\)-PPases; the K\(^+\)-dependent PfVP1, and the K\(^+\)-independent PfVP2 (McIntosh et al., 2001). PfVP1 is localised to the parasite plasma membrane and also to punctuate intracellular inclusions (Luo et al., 1999; McIntosh et al., 2001). There was no evidence from the immunolocalisation studies of the localisation of PfVP1 to the parasite digestive vacuole (McIntosh et al., 2001); however there is functional evidence that the digestive vacuole is acidified by a V-type H\(^+\)-PPase (Saliba et al., 2003).

The relative contributions of the putative NHE, the V-type H\(^+\)-ATPase and the V-type H\(^+\)-PPases to pH regulation in the parasite is unclear. Multiple aspects of pH regulation in the malaria parasite have been investigated in this thesis (Chapters 3-6).
1.6. Na\(^+\) regulation

1.6.1. The significance of Na\(^+\) regulation

Of the Group IA alkali metals, only Na\(^+\) and K\(^+\) are essential for human health (Page and Di Cera, 2006). Na\(^+\) plays important roles in many biological processes such as action potential generation, muscle contraction and cell volume regulation. Additionally, hundreds of enzymes use monovalent cations as cofactors or allosteric modulators (Di Cera, 2006). Enzymes that are activated by K\(^+\) cannot be activated by Na\(^+\), and high [Na\(^+\)]\(_i\) can inhibit these enzymes through competitive inhibition. This has been investigated at an atomic level in dialkylglycine dehydrogenase and Hsc70, in which the binding of Na\(^+\) changes the geometry of the catalytic residues so that the substrates (pyridoxal phosphate or ATP respectively) cannot bind (Di Cera, 2006). Intracellular enzymes typically use K\(^+\) as a cofactor, whilst Na\(^+\) is more common in extracellular enzymes (Di Cera, 2006). Na\(^+\) is used as a cofactor in many enzymes including the well-studied \(\beta\)-galactosidase (encoded on the lacZ operon in \textit{E. coli}), fructose-1,6-bisphosphate aldolase and tagatose-1,6-bisphosphate aldolase, and Na\(^+\) binds allosterically to other enzymes including regulators of blood coagulation Trp synthase, thrombin (clotting protease) and factor Xa (Di Cera, 2006).

The maintenance of a low intracellular [Na\(^+\)]/[K\(^+\)] ratio is very important and is a key characteristic of all nucleated cells. Typically, eukaryotic cells maintain a low [Na\(^+\)]\(_i\) of 1 - 40 mM (Minta and Tsien, 1989; Page and Di Cera, 2006; Apse and Blumwald, 2007) despite a high [Na\(^+\)]\(_o\) of 120 - 450 mM (Minta and Tsien, 1989; Apse and Blumwald, 2007). Cells maintain a low [Na\(^+\)]\(_i\) by restricting Na\(^+\) influx, by compartmentalising Na\(^+\) and through Na\(^+\) efflux transporters.

A schematic of the transporters involved in [Na\(^+\)]\(_i\) regulation (referred to throughout Section 1.6), is shown in Fig. 1.8.
Fig. 1.8. Na\(^+\) transporters involved in [Na\(^+\)]\(_i\) regulation in multiple cell types.
Transporters included in this figure play key roles in the movement of Na\(^+\), and the specifics of their involvement are detailed in Section 1.6.2 and 1.6.3. As indicated, in the case of the NHEs Fig. 1.7 provides further details of their phylogenetic relationships.

1.6.2. Na\(^+\) regulation in prokaryotes

Prokaryotes employ multiple Na\(^+\) efflux mechanisms to maintain a low [Na\(^+\)]. NHE, using the energy derived from H\(^+\) entering the bacteria (down its electrochemical gradient) commonly play a key protein in Na\(^+\) extrusion. The NHE proteins involved are from the CPA1 NhaP clade (Fig. 1.7), named after the *Pseudomonas aeruginosa* exchanger discovered in 1998 (Utsugi *et al.*, 1998). Recently, the crystal structure of *Methanococcus jannaschii* NhaP was solved at 7 Å (Goswami *et al.*, 2010), and similarities and differences were observed with the crystal structure of NhaA (see Section 1.5.2). Like NhaA, NhaP functions as a dimer. However, unlike NhaA, NhaP is active at acidic pH values, and inactive under alkaline conditions. Some
NhaP members can transport K⁺ but, unlike other NHEs, they do not transport Li⁺ (although they can bind Li⁺) (Resch et al., 2011).

Anaerobic bacteria and archaea also use Na⁺ translocating decarboxylases to efflux Na⁺. These are biotin containing membrane enzymes that catalyse the decarboxylation of β-keto acids (such as oxaloacetate, malonate, methylmalonyl-CoA and glutaconyl-CoA) and that use the energy released from decarboxylation to translocate Na⁺. The stoichiometry of transport matches that of an NHE as the first step of decarboxylation requires an internal H⁺ (reviewed in Dimroth, 1987; Buckel, 2001). Novel, prokaryotic Na⁺ transporting NADH:quinone oxidoreductases are also used to pump Na⁺ out of the cell, using the energy released from the coupled oxidation of NADH and ubiquinone reduction (Juarez et al., 2009).

Bacteria also use multiple Na⁺ efflux ATPases of all types (F/V/P-type and ABC transporters). Most bacteria encode Na⁺-ATPases, and of those characterised to date only Mycoplasma pneumonia, M. genitalium, Borrelia burgdoferi, Helicobacter pylori and Mycobacterium tuberculosis do not have any recognisable Na⁺ pumps (Hase et al., 2001). Bacillus subtilis encodes a novel Na⁺ efflux ABC transporter, NatAB (Cheng et al., 1997).

Some bacteria, including Thermotoga maritima, Methanosarcina mazei and Moorella thermoacetica also have V-type Na⁺-PPases (Belogurov et al., 2005; Malinen et al., 2007). V-type Na⁺-PPases are highly specific for Na⁺, and in M. mazei the H⁺-PPase (see Section 1.5.2) and Na⁺-PPase are encoded next to each other in the genome suggesting a gene duplication event (Malinen et al., 2007). Of the two subfamilies of cation pumping-PPases, it has recently been suggested that the K⁺-dependent subfamily may exclusively transport Na⁺ and not H⁺ (Luoto et al., 2011).

In many bacteria, including T. maritima, the plasma membrane ATP synthase uses Na⁺ as a coupling ion, with the inwardly directed Na⁺ gradient providing the energy for the synthesis of ATP (Malinen et al., 2007). Aside from ATP synthesis, bacteria utilise the Na⁺ gradient for the secondary active transport of many solutes including amino acids, pantothenate, glucose and dicarboxylates, as well as for energising Na⁺-coupled multidrug efflux mechanisms (Hase et al., 2001).
1.6.3. Na⁺ regulation in eukaryotes

1.6.3.1. Na⁺ regulation in animal cells

Na⁺ enters animal cells through Na⁺ channels, Na⁺/Ca²⁺ exchangers, NHEs, Na⁺/HCO₃⁻ and Na⁺/K⁺/2Cl⁻ cotransporters, Na⁺/Mg²⁺ exchangers and other Na⁺ coupled metabolite/nutrient transporters (Murphy and Eisner, 2009). Na⁺ regulation in animals is achieved primarily through the action of the Na⁺/K⁺-ATPase, which is comprised of an α catalytic subunit and a β accessory subunit. Using the energy from ATP hydrolysis three Na⁺ are extruded from the cell concomitant with the entry of two K⁺ (reviewed by Aperia, 2007). Na⁺/K⁺-ATPase isoforms in various tissues exhibit different sensitivities to the Na⁺/K⁺-ATPase inhibitor ouabain, but all are inhibited by ouabain at a concentration of 1 mM (Blanco and Mercer, 1998). In animal cells Na⁺/K⁺-ATPase activity generates a high intracellular [K⁺]; K⁺ then diffuses out of the cell via K⁺ channels, creating an inwardly negative membrane potential. Loss-of-function mutations in the Na⁺/K⁺-ATPase, or disruption of the α subunit, cause embryonic and larval lethality in nematode, fruit fly and mouse studies (Davis et al., 1995; James et al., 1999; Palladino et al., 2003).

In the intestine and in kidney proximal tubule cells, a K⁺-independent, ouabain-insensitive Na⁺-ATPase is involved in Na⁺ regulation, in concert with the Na⁺/K⁺-ATPase (Proverbio et al., 1991). This Na⁺-ATPase activity has also been identified in the gill of trout and small mouth bass and in the brain cortex and liver of rats (Proverbio et al., 1991). This orthovanadate- and furosemide-sensitive Na⁺-ATPase is postulated to cotransport Cl⁻ and water (Proverbio et al., 1991; De Souza et al., 2007b). It has been postulated that the Na⁺-ATPase may compensate for the normal daily rhythmicity of Na⁺/K⁺-ATPase function that arises from changes in the levels of soluble hormonal inhibitor (Reyes et al., 2009). Na⁺/K⁺-ATPase activity oscillations are associated with circadian rhythms of urinary sodium secretion/flow rates, whereas the ouabain-insensitive Na⁺-ATPase did not show any variation of activity across a day (Reyes et al., 2009). Despite much physiological evidence, the gene for the human Na⁺-ATPase was only identified recently (Rocafiull et al., 2011). The Na⁺-ATPase and Na⁺/K⁺-ATPase share 13 exons (including transmembrane domains 2-5) but have distinct 5' and 3' untranslated regions and unique
transmembrane domains 6-10 (Rocafull et al., 2011). The differential ouabain sensitivity of the Na\(^+\)- and Na\(^+\)/K\(^+\)-ATPase are explained by the alternative splicing of transmembrane domain one and a mutation at threonine 774, both of which are implicated in ouabain-sensitivity (Rocafull et al., 2011).

Humans also have two homologues of the bacterial NhaA (belonging to the CPA2 family; Fig. 1.7; Xiang et al., 2007). The amiloride-insensitive HsNhaA2 is localised to the plasma membrane of MDCK cells (canine kidney distal tubule cell line), and when expressed in the *S. cerevisiae* it conferred Na\(^+\) resistance to the yeast (Xiang et al., 2007).

### 1.6.3.2. Na\(^+\) regulation in plants

Soil salinity, and the resulting osmotic and ionic stresses caused by Na\(^+\), is a growing agricultural problem (reviewed by Flowers and Colmer, 2008; Munns and Tester, 2008; Plett and Moller, 2009; Kronzucker and Britto, 2010). Na\(^+\) uptake in plant roots occurs via non-selective cation channels, though the involvement of glutamate receptors, cyclic-nucleotide gated channels, low-affinity cation transporters and several types of K\(^+\) transporters including K\(^+\)-uptake permeases, and high-affinity K\(^+\)-transporters has also been proposed (Plett and Moller, 2009; Kronzucker and Britto, 2010).

In vascular ('higher') plants there is no bioinformatic or physiological evidence for the presence of Na\(^+\)-ATPases (Axelsen and Palmgren, 2001; Benito and Rodriguez-Navarro, 2003). Plant cells use the inwardly directed H\(^+\) gradient (established by the P-type H\(^+\)-ATPase; Section 1.5.3.2) to energise the efflux of Na\(^+\) through an NHE. In *Arabidopsis*, the electroneutral efflux of Na\(^+\) is by the AtSOS1 ('Salty overly sensitive') exchanger, which is localised to the plasma membrane (Shi et al., 2000; Qiu et al., 2002). SOS1-like NHE proteins form a distinct clade of NHEs within the CPA1 family (Fig 1.7). AtSOS1 is activated in response to high [Na\(^+\)], when the calcineurin-like Ca\(^{2+}\)-binding protein AtSOS3 responds to a change in cytosolic [Ca\(^{2+}\)] which in turn activates the serine/threonine kinase AtSOS2, which phosphorylates AtSOS1 (Qiu et al., 2002; Quintero et al., 2002).
Plants can also sequester Na\(^+\) into the vacuole using another type of NHE designated NHX. Plant NHXs belong to the intracellular clade of NHEs, the same clade as intracellular human isoforms HsNHE6-9 (Fig. 1.7). NHXs mediate the amiloride-sensitive, electroneutral transport of Na\(^+\) into the vacuole, and there is some evidence that the SOS2/3 regulatory pathway also regulates NHX activity (Darley et al., 2000; Qiu et al., 2004). *Arabidopsis* encodes six vacuolar-type NHXs, which can also transport K\(^+\), and which are hence less specific for Na\(^+\) than are the SOS1-like NHEs (Rodriguez-Rosales et al., 2009).

The overexpression of AtSOS1 (or AtSOS3), results in an increase in plant salt tolerance (Shi et al., 2003; Yang et al., 2009). The overexpression or transgenic expression of AtNHX1 also confers salt resistance to many plant species, including *Arabidopsis* (Apse et al., 1999; Yang et al., 2009), tomato (Zhang and Blumwald, 2001), and rice (Ohta et al., 2002). Overexpression of the V-type H\(^+\)-PPase that generates the pH gradient for NHX sequestration of Na\(^+\) also leads to an increase in plant salt tolerance (Gaxiola et al., 2001). Recently salt tolerant tomatoes were generated by transgenically co-expressing AtNHX1 from millet and V-type H\(^+\)-PPase from *Arabidopsis* (Bhaskaran and Savithramma, 2011).

Na\(^+\) regulation has also been investigated in halophytes, which are defined as plants that can complete their natural life cycle in > 200 mM NaCl (Flowers and Colmer, 2008). The enzymes of halophytes have the same sensitivity to Na\(^+\)-inhibition as those of salt-sensitive plants, ‘glycophytes’ (Apse et al., 1999). Halophytes use plasma membrane SOS1-like NHEs and vacuole membrane NHXs to remove Na\(^+\) from the cytosol and, under saline conditions, the activity of the V-type H\(^+\)-ATPase or V-type H\(^+\)-PPase that provides the H\(^+\) gradient to power these transporters is increased (Wang et al., 2001; Vera-Estrella et al., 2005). To counter the osmotic effects resulting from accumulation of Na\(^+\) in the vacuole, halophytes synthesise multiple osmolytes in the cytoplasm to balance the osmotic potential, including sucrose, sorbitol, proline, glycinebetaine and dimethylsulphoniopropionate (Flowers and Colmer, 2008). Glycophytes also synthesise osmolytes, but to a lesser extent than halophytes (Munns and Tester, 2008).
Non-vascular ('lower') plants, for example moss and liverworts (bryophytes), use an ENA (*exitus natrii*)-type Na⁺-ATPase to efflux Na⁺ and maintain a low [Na⁺]; (reviewed in Rodriguez-Navarro and Benito, 2010). ENA-type Na⁺-ATPases are closely related to the SERCA-, plasma membrane- and Golgi PMR-type Ca²⁺-ATPases (Rodriguez-Navarro and Benito, 2010) and have 10 transmembrane domains. ENA-type Na⁺-ATPases have been identified in the moss *Physcomitrella patens* (Benito and Rodriguez-Navarro, 2003), and liverworts *Marchantia polymorpha* (Fraile-Escanciano et al., 2009) and *Riccia fluitans* (Rodriguez-Navarro and Benito, 2010). *P. patens* encodes three ENA Na⁺-ATPases, PpENA1-3 (Benito and Rodriguez-Navarro, 2003; Fraile-Escanciano et al., 2009). Only PpENA1 was found to have Na⁺-ATPase activity, demonstrated by its ability to complement a salt-sensitive yeast strain (Benito and Rodriguez-Navarro, 2003). A subsequent study showed that in *Physcomitrella*, PpENA1 expression levels increased in response to an increased extracellular concentration of NaCl and that a knockout of PpENA1 via homologous recombination led to a lower [K⁺]:[Na⁺] ratio compared to wild type plants (Lunde et al., 2007). Recently the cation binding site of PpENA1 was investigated by modelling the cation binding pocket between transmembranes four and six, using the SERCA Ca²⁺-ATPase as a scaffold (Drew et al., 2010). The most energetically favourable model predicts two bound Na⁺ in the cation binding pocket of the Na⁺-ATPase (Drew et al., 2010).

It has been proposed that the role of the ENA-type Na⁺-ATPase is to mediate Na⁺ efflux at high pH when the H⁺ gradient for Na⁺ efflux via an electroneutral NHE is insufficient (Fraile-Escanciano et al., 2009). *P. patens* also expresses two SOS-like NHEs, PpSOS1 and PpSOS1B; PpSOS1 was demonstrated to be involved in Na⁺ efflux, particularly at low pH values (Fraile-Escanciano et al., 2010). Understanding the relative contributions of, and interactions between, the ENA-type Na⁺-ATPase and the SOS-like NHE in lower plants awaits further investigation. Engineering a vascular plant to express an ENA-type Na⁺-ATPase has been proposed as a way to increase salt tolerance (Benito et al., 2002; Lunde et al., 2007). Consistent with this, when Ena1p from *S. cerevisiae* was expressed in a tobacco cell line (*Nicotiana tabacum*) the Na⁺-ATPase localised to plant plasma membrane and conferred Na⁺/Li⁺ tolerance (Nakayama et al., 2004). Similarly, when *Physcomitrella* PpENA1
was expressed in rice, the Na⁺-ATPase localised to rice plasma membrane and conferred Na⁺ tolerance (Jacobs et al., 2011).

1.6.3.3. Na⁺ regulation in fungi

In fungi, Na⁺ uptake is limited, but is known to occur via K⁺ uptake uniporters, non-selective cation channels of unknown molecular nature and also by secondary active transporters which use Na⁺ as a coupling ion (e.g. the Na⁺-coupled P₃ importer (Arino et al., 2011)). Na⁺-extruding ENA-type Na⁺-ATPases are found in all fungi (Benito et al., 2002) and are phylogenetically distinct from the bryophyte ENA-type Na⁺-ATPases (Rodriguez-Navarro and Benito, 2010). ENA-type Na⁺-ATPases have been identified in all yeast sequenced to date, including the pathogenic fungi Candida spp and Cryptococcus neoformans (Ramos et al., 2011). As ENA-type Na⁺-ATPases are not found in animals, they are a good antifungal chemotherapy target (Ramos et al., 2011).

ENA-type Na⁺-ATPases were first identified in S. cerevisiae; ScEna1p, was initially annotated as a Ca²⁺-ATPase and it was only the subsequent characterisation of its function that revealed its role in monovalent cation transport (Rudolph et al., 1989; Haro et al., 1991). It has been stated that fungal Na⁺ and Ca²⁺-ATPases “cannot be distinguished only on the basis of the protein sequences” (Gorjan and Plemenitas, 2006). ScEna1p is responsible for Na⁺/Li⁺ efflux during salt stress and K⁺ efflux at low [Na⁺], high pH and high [K⁺] (Haro et al., 1991; Benito et al., 2002).

Some yeast can survive with high cytoplasmic [Na⁺]; for example Ustilago maydis, S. cerevisiae and Debaryomices hansenii have been shown to survive with [Na⁺]:[K⁺] ratios as high as 4. These yeast are called ‘Na⁺ includers’ (Benito et al., 2009). However, for many yeasts, such as Neurospora crassa and Candida spp., the accumulation of high intracellular Na⁺ is toxic (Benito et al., 2009). This sensitivity to [Na⁺], is reflected in the ion-selectivity of the ENA-type Na⁺-ATPases found in these different organisms. In ‘Na⁺ includers’ such as U. maydis and S. cerevisiae, ENA-type Na⁺-ATPases facilitate Na⁺ and K⁺ transport, but in N. crassa the ENA-type Na⁺-ATPase is Na⁺ specific (Benito et al., 2009). In contrast, Schizosaccharomyces pombe does not encode any Na⁺-ATPases, and its ENA-type
ATPase is selective for $K^+$ (Benito et al., 2002). Many fungi express multiple ENA-type $Na^+$-ATPases, and not all have been identified to play a role in $Na^+$ efflux at the plasma membrane. In *U. maydis* a second ENA-type $Na^+$-ATPase, UmENA2 has been localised to the endoplasmic reticulum and in *N. crassa*, NcENA2 was localised to an endomembrane system (Benito et al., 2009). The roles of ENA-type $Na^+$-ATPases in internal $Na^+$ regulation are unknown.

Yeast also encode multiple NHEs involved in $Na^+$ homeostasis. *S. cerevisiae* encodes a plasma membrane NHE called Nhal that is expressed constitutively, mediating $Na^+$, $Li^+$ and $K^+$ efflux (Arino et al., 2011). *S. pombe* has a plasma membrane NHE Sod2p which mediates selective $Na^+$ and $Li^+$ efflux (Arino et al., 2011). Note that *S. cerevisiae* (which has an ENA-type $Na^+$-ATPase) has a non-specific NHE, whereas *S. pombe* (with no ENA-type $Na^+$-ATPase) has a $Na^+$-specific NHE. Yeast also have intracellular NHE, and in *S. cerevisiae*, the vacuolar NHE, Vnx1, mediates $Na^+$ or $K^+$ transport into the vacuole, and an endosomal NHE, Nhx1 in late endosomes, contributes to $Na^+$ sequestration (Arino et al., 2011).

There is some redundancy in function between the NHEs and ENA-type $Na^+$-ATPases. *S. pombe* Δsod2 mutants are salt sensitive, but transgenic expression of *S. cerevisiae* Ena1p complements the mutant line (Banuelos et al., 1995). The reverse has also been shown: the expression of *S. pombe* Sod2p can complement salt sensitive *S. cerevisiae* Δena mutants (Hahnenberger et al., 1996). It has been proposed that the NHE and ENA-type $Na^+$-ATPase systems are complementary and not redundant, as the NHE can operate under acidic conditions using the transmembrane pH gradient for energy, and the ENA-type $Na^+$-ATPase can function at more alkaline pH values using ATP hydrolysis for energy (Rodriguez-Navarro and Benito, 2010). This ensures the maintenance of low $[Na^+]$, under varying environmental conditions.

**1.6.3.4. $Na^+$ regulation in protozoan parasites**

ENA-type $Na^+$-ATPases have been identified in protozoan parasites, including *T. cruzi*, *T. brucei*, *Leishmania* spp. as well as in the non-apicomplexan parasite *Entamoeba histolytica* (reviewed in Rodriguez-Navarro and Benito, 2010).
Protozoal ENA-type Na\(^+\)-ATPases have been studied in the most detail in *T. cruzi*, where initially a ouabain-insensitive, furosemide-sensitive, Na\(^+\)-dependent ATPase activity was observed in membrane preparations (Caruso-Neves *et al.*, 1999). A putative ENA-type ATPase, TcENA, was cloned from *T. cruzi* (Stiles *et al.*, 2003), and overexpression of TcENA conferred salt resistance to insect-stage parasites (Iizumi *et al.*, 2006). When TcENA was expressed in a mammalian cell line it was found that it could transport both Na\(^+\) and K\(^+\), and that it was orthovanadate-sensitive, but ouabain-insensitive (Iizumi *et al.*, 2006). TcENA may be regulated by interaction with the Protein Kinase A regulatory sub unit (Bao *et al.*, 2009); however the effect of this interaction on Na\(^+\)-ATPase activity has not been investigated. In *T. cruzi*, the presence of a ouabain-sensitive Na\(^+\)/K\(^+\)-ATPase has also been proposed on the basis of membrane potential depolarisation upon addition of ouabain (Van Der Heyden and Docampo, 2002); however no molecular candidate for this phenomenon has been identified.

ENA-type ATPase activity has also been characterised in *E. histolytica*, *L. donovani* and *L. amazonensis* (Stiles *et al.*, 2003; De Souza *et al.*, 2007a; de Almeida-Amaral *et al.*, 2008). Using the same methods as were used in the Iizumi *et al.* (2006) study on *T. cruzi*, de Souza *et al.* (2007a) showed that there was a furosemide-sensitive, but ouabain-insensitive Na\(^+\)-dependent ATPase activity in *Entamoeba* membrane preparations. De Almeida-Amaral *et al.* (2008) demonstrated a similar activity in *Leishmania* membrane preparations. Overall, nine putative ENA-type ATPase genes from protozoan parasites have been identified (Rodriguez-Navarro and Benito, 2010).

[Na\(^+\)]\(_i\) regulation is poorly characterised in the close relative of *Plasmodium*, *T. gondii*. No ENA-type Na\(^+\)-ATPases have been annotated in the *T. gondii* genome, although the genome encodes four NHEs (Gajria *et al.*, 2008). The physiological roles of these NHEs are unclear; however when the vacuolar membrane protein TgNHE3 was knocked out, the lines were more sensitive to increased levels of extracellular Na\(^+\) (Francia *et al.*, 2011). Mechanisms of [Na\(^+\)]\(_i\) homeostasis in *Toxoplasma* require further investigation.
1.6.3.5. *Na*⁺ regulation in *Plasmodium*

The mechanism by which the malaria parasite regulates its [Na⁺]ᵢ is unknown. On entering an uninfected human erythrocyte, an invading *Plasmodium falciparum* malaria parasite passes from the high-[Na⁺]/low-[K⁺] environment of the blood plasma, to the low-[Na⁺]/high-[K⁺] environment of the host cell cytosol (Lee *et al.*, 1988). Some 12 hr after invasion the parasite induces NPPs in the host cell membrane and these mediate the influx of Na⁺ and the efflux of K⁺, down their respective concentration gradients (Section 1.4.1.1). There is a subsequent increase in [Na⁺] and decrease in [K⁺] in the erythrocyte cytosol, with both eventually reaching levels similar to those in the extra-erythrocytic plasma by the time the parasite is a mature trophozoite (Staines *et al.*, 2001; Lew *et al.*, 2003). The endogenous erythrocyte Na⁺/K⁺-ATPase initially increases its transport activity more than twofold in an effort to maintain a low erythrocytic [Na⁺] (Staines *et al.*, 2001); however this is not enough, and the pump is unable to stem the net influx of Na⁺ and the net efflux of K⁺. Furthermore, as the intraerythrocytic parasite matures the Na⁺/K⁺-ATPase activity actually decreases (Staines *et al.*, 2001).

The intraerythrocytic parasite itself maintains a low cytosolic [Na⁺] (Lee *et al.*, 1988; Wunsch *et al.*, 1998; Mauritz *et al.*, 2011), and there is therefore an inward [Na⁺] gradient across the plasma membrane of the mature parasite. This Na⁺ gradient (together with the inwardly negative membrane potential) is used to energise the uptake of the essential nutrient inorganic phosphate (Saliba *et al.*, 2006). There are other predicted Na⁺-dependent transporters in the parasite, including amino acid symporters (in the neurotransmitter:Na⁺ symporter family), a sugar transporter (in the glycoside-pentoside-hexuronide:cation symporter family), a multi-antimicrobial extrusion family transporter and major facilitator superfamily transporters (Martin *et al.*, 2005), but whether such proteins are active on the surface of the parasite is unclear.

One possibility is that the parasite uses a Na⁺-ATPase to extrude Na⁺ and maintain a low intracellular [Na⁺]. The *P. falciparum* genome encodes seven P-type cation-transporting ATPases and whilst two have been annotated as putative Ca²⁺-ATPases (PfATP4; PFL0590c and PfATP6; PFA310c) none have been specifically annotated
as a Na⁺-ATPase (Kirk et al., 2005; Martin et al., 2005). In *P. falciparum*, mutations in PfATP4 (PFL0590c), a P-type ATPase on the parasite plasma membrane (Dyer et al., 1996; Rottmann et al., 2010), confer resistance to a novel class of new antimalarials, the spiroindolones (Rottmann et al., 2010). The spiroindolones (Yeung et al., 2010) show a low nanomolar activity against blood-stage *P. falciparum* and *P. vivax* parasites (Rottmann et al., 2010) and are currently in Phase I clinical trials. PfATP4 has been annotated, on the basis of sequence homology (Trottein and Cowman, 1995; Trottein et al., 1995), as a Ca²⁺-transporting ATPase. There is some evidence for PfATP4 having an associated Ca²⁺-dependent ATPase activity (Krishna et al., 2001); however, this has not been confirmed (Rottmann et al., 2010) and a Ca²⁺ transport function has not been demonstrated directly.

Another candidate for involvement in Na⁺ regulation in the parasite is PfNHE (see Sections 1.4.3 and 1.5.3.5). PfNHE was identified in a phylogenetic study of NHEs to belong to the plant SOS1-like clade of transporters (Fig 1.7; Brett et al., 2005). It has been postulated that it may be involved in the maintenance of a low intracellular [Na⁺] (Ginsburg, 2002; Spillman et al., 2008), by using the energy provided by the inwardly directed pH gradient to extrude Na⁺, like in plant NHEs (see Section 1.6.3.2).

The transporter/s that the parasite uses to maintain a low [Na⁺]; are uncharacterised. Multiple aspects of [Na⁺] regulation in the malaria parasite have been investigated in this thesis (Chapters 3 - 6).
1.7. Aims of this thesis

The aim of the work reported in this thesis was to understand the mechanism/s that the trophozoite stage of the intraerythocytic malaria parasite, *P. falciparum*, uses to maintain a low [Na\(^+\)].

The specific aims of the study were:

i) To determine the contribution of a putative Na\(^+\)-ATPase and putative NHE to [Na\(^+\)]\(_j\) regulation (Chapter 3, 4 and 6);

ii) To investigate the possibility that Na\(^+\) transporters may be the targets of the novel class of antimalarial drug, the spiroindolones (Chapter 5).
Chapter 2:

Materials and Methods
2.1. Materials

Unless stated otherwise all chemicals were obtained from Sigma-Aldrich. The fluorescent pH indicator, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), the fluorescent Na$^+$-indicator, sodium-binding benzofuran phthalate (SBFI), and the fluorescent Ca$^{2+}$-indicator, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxo)ethoxy)-2-benzofuranyl) (Fura-2) were obtained, as the acetoxyethyl (AM) ester forms (BCECF-AM, SBFI-AM, and Fura-2-AM, respectively) from Molecular Probes (Invitrogen). Fluorescein-dextran was also from Molecular Probes (Invitrogen). RPMI-1640 (Roswell Park Media Institute, where the formulation 1640 was first defined) for routine culturing was obtained from the John Curtin School of Medical Research (The Australian National University). RPMI-1640 powder for preparation of bicarbonate-free RPMI, N-(2-hydroxyethyl)piperazine-$N'$-(2-ethanesulfonic acid) (HEPES) buffer solution, gentamicin sulphate and Albumax II were from Invitrogen. Microscint PS scintillation fluids was obtained from PerkinElmer, and $[^3]$H]hypoxanthine (30 Ci/mmol) was from GE Healthcare.

The four spiroindolones were synthesised as described previously (Yeung et al., 2010) and were obtained from the Novartis Institute for Tropical Diseases (NITD) under the terms of a Material Transfer Agreement.

Group O$^+$ erythrocytes were supplied by the Canberra Branch of the Australian Red Cross Service.

2.2. Solution Preparation

The composition of solutions used throughout the study is set out in Table 2.1. Unless stated otherwise, all solutions were prepared in deionised water and, when required, were sterilized by autoclaving. Solutions were pH-adjusted after preparation using a pH meter (Orion Instruments; calibrated before each use). The pH was altered using the dropwise addition of KOH/NaOH or HCl. Solutions required to be between pH 6.8 - 7.8 were buffered using HEPES (pK$_a$ = 7.5; useful
range 6.8 - 8.2); solutions < pH 6.8 were buffered using 2-(N-Morpholino)ethanesulfonic acid (MES; pKₐ = 6.1; useful range 5.5 - 6.7). The osmolarity of the solutions was measured using a freezing point osmometer (Advanced Instruments Inc.). The typical osmolarity of solutions used in this study was between 300 - 310 mOsm/L.

Ionophores, inhibitors and fluorescent dyes were dissolved in analytical grade dimethyl sulfoxide (DMSO), with several exceptions: orthovanadate, miltefosine and ouabain were dissolved in deionised water; bumetanide was dissolved in ethanol; aminomethylenediphosphonate (AMDP) was dissolved in standard saline and nigericin was prepared in 50 % v/v ethanol/DMSO.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard saline</td>
<td>125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH = 7.10</td>
</tr>
<tr>
<td>High K⁺ (pH calibration)</td>
<td>130 mM KCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH to 6.80, 7.10 and 7.80</td>
</tr>
<tr>
<td>Na⁺-free</td>
<td>125 mM NMDG-Cl or 125 mM Choline-Cl, 5 mM KCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH to 7.10</td>
</tr>
<tr>
<td>Na⁺-gluconate (Na⁺ calibration)</td>
<td>80 mM Na⁺-gluconate, 50 mM NaCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH = 7.10</td>
</tr>
<tr>
<td>K⁺-gluconate (Na⁺ calibration)</td>
<td>80 mM K⁺-gluconate, 50 mM KCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH = 7.10</td>
</tr>
<tr>
<td>K⁺-free Na⁺ loading</td>
<td>130 mM NaCl, 1 mM MgCl₂, 20 mM glucose, 25 mM HEPES; pH = 7.10</td>
</tr>
<tr>
<td>Cl⁻-free</td>
<td>125 mM Na⁺-gluconate, 5 mM K⁺-gluconate, 1 mM MgSO₄, 20 mM glucose, 25 mM HEPES; pH = 7.10 (with NaOH and H₂SO₄)</td>
</tr>
</tbody>
</table>

Table 2.1. Composition of solutions used in this study.
2.3. General Parasite Methods

2.3.1. Parasite Culture

*P. falciparum* parasites were routinely cultured in Group O (Rh+) erythrocytes, though fluctuations in availability occasionally meant that A/AB+ blood was used instead. This had no discernable effect on parasite growth (personal observation). Erythrocytes were typically received as buffy-coat (white blood cell) depleted, packed erythrocytes. However on several occasions the erythrocyte preparations contained the buffy-coat and this was removed before using the erythrocytes in culture. To buffy-coat deplete, the blood was washed three times in RPMI-1640 via centrifugation at 1000 x g (5 min). During each wash step the visible buffy-coat (top layer) was aspirated.

Parasites were cultured using aseptic techniques in 75 cm$^2$ Nunc culture flasks at a haematocrit of 4 - 5 % and a parasitaemia of ~10 % (as determined from a Giemsa-stained thin smear). Flasks were gassed with 1 % O$_2$, 3 % CO$_2$, 96 % N$_2$ gas mix (BOC), tightly sealed and placed in a temperature controlled 37 °C incubator. Flasks were orbitally shaken at 60 rpm. It has been reported (Allen and Kirk, 2010) that shaking not only eliminates cell settling, but also reduces the number of multiple infections and increases the parasite yield. Parasites were grown in bicarbonate-containing RPMI-1640 medium supplemented with 0.5 % w/v Albumax II, 20 mM D-glucose, 200 μM hypoxanthine, 25 mM HEPES and 25 mg/L gentamycin sulfate. When the parasites were in the ‘ring’ stage, the spent media was replaced with fresh media (by centrifugation; 500 x g for 5 min), the culture was synchronised if necessary (Section 2.3.2) and the flasks were gassed and returned to 37 °C. When the parasites were in the ‘trophozoite’ stage, ~5 - 10 % of the culture was used to propagate a new culture (the actual % varied according to the parasitaemia each cycle). Uninfected erythrocytes were added to the flask, along with fresh media, and the flask was gassed and returned to the 37 °C incubator.

Cell counts were performed using an Improved Neubauer haemocytometer.
2.3.2. Parasite Synchronisation

Ring stage cultures were synchronized (typically 24 and/or 72 hr before trophozoites were required) using sorbitol as described previously (Lambros and Vanderberg, 1979). Briefly, cells at ring stage were incubated in 5 % w/v sorbitol for 15 - 20 min. Sorbitol is a substrate for the NPPs that are induced in the erythrocyte membrane 12 - 16 hr after invasion (Section 1.4.1.1). On suspension of the parasitized erythrocytes in the (approximately isosmotic) sorbitol solution the sorbitol enters those cells expressing the NPPs (i.e., those cells older than 12-16 hr, causing them to undergo osmotic lysis. Younger (i.e., ring-stage) cells are unaffected. Where necessary, cells were synchronised up to 10 hr apart to reduce the age spread of the population.

2.3.3. *P. falciparum* parasite strains used in this study

Unless stated otherwise, the experiments described in Chapters 3-6 use *P. falciparum* strain 3D7, which is derived from isolate NF54 originating in the Netherlands (a case of airport contracted malaria; Walliker *et al.*, 1987). 3D7 was the first, complete *P. falciparum* strain to be sequenced (Gardner *et al.*, 2002), and is routinely used in malaria laboratories worldwide. Some experiments in Chapter 3 were performed in the FAF6 strain, derived from the ITG2 strain of Brazilian origin (Biggs *et al.*, 1991).

In Chapter 5, parasite lines with mutations in PfATP4 were used. These were a kind gift from Dr Elizabeth A. Winzeler (Scripps Institute), and were generated by Dr Marcus Lee in the laboratory of Dr David Fidock (Columbia University). The parent Dd2 line is of South East Asian origin (Joy *et al.*, 2003). As described previously (Rottmann *et al.*, 2010), the NITD609-RDd2-clone2 parasite line was generated by exposing Dd2 parasites to the potent spiroindolone NITD609, and the Dd2\textsuperscript{antB} CAM I398F/P990R parasite line was generated by transfecting the gene encoding a mutant PfATP4 protein from a resistant, NITD609-exposed line into spiroindolone-sensitive Dd2\textsuperscript{antB} parasites. The transfectant parasite line was maintained in the presence of 2.5 nM WR99210 and 5 μM blasticidin S. Parasites were cultured in the absence of drug selection for 48 hr (one complete intraerythrocytic cycle) prior to experimentation.
In Chapter 6, parasites with reduced expression of PfNHE were used. These were a kind gift from the laboratory of Dr David Fidock (Columbia University, New York) and were generated by Dr Louis Nkrumah (Nkrumah et al., 2009). The parent line, GC03, is derived from the genetic cross between HB3 (from Honduras) and Dd2 (from South East Asia) strains (Wellems et al., 1990). To generate parasites that under-express PfNHE an allelic exchange method was used (as described in Waller et al., 2003). Briefly, the 3' UTR of the PfNHE gene was replaced with a truncated 3' UTR from the PfCRT gene. In the transfectants, the shorter 3' UTR leads to an unstable mRNA product and this causes knockdown of the target gene. The transfectant parasite line was cultured in the presence of 5 μM blasticidin S. Parasites were cultured in the absence of drug selection for 48 hr (one complete intraerythrocytic cycle) prior to experimentation.

2.3.4. Mycoplasma Contamination

Periodically, cultures were tested for mycoplasma contamination using a PCR detection method as described previously (Uphoff and Drexler, 2002). A positive control (mycoplasma-positive culture media) was included. Cultures were never found to be contaminated with mycoplasma.

2.4. Trophozoite Isolation

Parasites were functionally isolated from their erythrocyte hosts using the plant-derived glycoside, saponin. Saponin consists of the active agent sapogenin bound to various sugars including glucose. Saponin disrupts the erythrocyte membrane (Dourmashkin et al., 1962) and the parasitophorous vacuole membranes which contain cholesterol, however leaves the cholesterol-free parasite plasma membrane intact. Parasites isolated using saponin are capable of maintaining large transmembrane gradients for H⁺ (Saliba and Kirk, 1999) and Ca²⁺ (Alleva and Kirk, 2001) as well as generating and maintaining a large membrane potential (Allen and Kirk, 2004b).

Saponin from the bark of the Quillaja tree with a sapogenin content of ≥ 10 % w/v was used throughout this study. Saponin was prepared at a working concentration of
1 % w/v in phosphate buffered saline (PBS). Typically a culture at ~4 % haematocrit and 10 % parasitaemia was exposed to 0.05 % w/v saponin (final concentration sapogenin ~0.005 % w/v) for 20 seconds (or less) until the solution lost its turbidity. The suspension was pelleted immediately at 1000 × g (5 min) and washed 2-3 × via centrifugation (12000 × g, 30 s) in 2 mL bicarbonate-free RPMI (BCF-RPMI; the same as the standard RPMI used for culturing, but without bicarbonate and Albumax II; pH 7.10). Using this protocol the parasite membrane remained intact as evidenced by the exclusion of the membrane impermeant dye trypan blue in > 98% of parasites (personal observation; data not shown). The results of an experiment highlighting the membrane integrity of saponin-isolated parasites are included in Section 6.3.2.1.

2.5. Measurement of Intracellular Na⁺ ([Na⁺]ᵢ) using SBFI-AM

SBFI, a Na⁺-sensitive, fluorescent dye, was used to measure [Na⁺]ᵢ (quantitatively) in saponin-isolated trophozoites. The structure of SBFI-AM, shown in Fig. 2.1, consists of a central crown ether with size selectivity for Na⁺, and fluorophores linked through the crown ether nitrogens. The acetoxymethyl (AM) ester derivative of SBFI was used, as in its uncharged form it can diffuse readily across the plasma membrane. Once inside the parasite, the ester groups are cleaved by non-specific esterases, resulting in a highly charged species that has a slow rate of leakage across the plasma membrane and out of the cell.

The [Na⁺]ᵢ of isolated trophozoites was measured at 37 °C using SBFI in conjunction with a Perkin Elmer LS-50B spectrofluorometer with a dual-excitation Fast Filter accessory. The Fast Filter wheel rotates an optical filter set rapidly through the excitation beam allowing ratio measurements to be recorded (as opposed to measurement with one filter at a time). Excitation was at 340 nm and 380 nm, and emission from both of these wavelengths was recorded at 515 nm. The benefits of using a ratiometric dye, such as SBFI (for which the ratio of fluorescence at 340 nm/380 nm is used) is that it allows the effects of photobleaching to be minimised, as the absolute intensity of the fluorescence signal does not change the ratio. The use of these excitation/emission wavelengths in the parasite system was validated in
Figure 2.1. Chemical structure of SBFI-AM (from Molecular Probes Product Information; Revised 4 June, 2003)

Chapter 3 (see Section 3.2.1). The FLWinLab program was used for real-time data display, for the collection of data at both excitation wavelengths, and for calculation of the 340 nm/380 nm fluorescence ratio.

To load the parasite cytosol with SBFI, saponin-isolated parasites were washed 2 x in BCF-RPMI (pH 7.10; 1000 x g, 5 mins) and resuspended to a cell concentration of 1.4 - 1.8 x 10^8 cells/mL in 4 mL BCF-RPMI. The SBFI-AM dye and 20 % (w/v) Pluronic-F127 (a detergent used to aid the solubilisation of the water-insoluble dye) were mixed in equal volumes (final concentrations 5.5 μM and 0.01 % (w/v), respectively) and the entire cell suspension (4 mL) was incubated with the dye mix for 20 mins at 37 °C. Tubes were wrapped in aluminium foil to avoid light exposure. The cells were washed twice in BCF-RPMI (12000 x g; 0.5 min) before another 20 min incubation at 37 °C, to allow for complete de-esterification of the dye. The dye-loaded cells were resuspended to a final cell concentration of 1.5 - 2.5 x 10^7 cells/mL for experimentation.
For measurements of [Na\textsuperscript{+}]\textsubscript{i}, a 1 mL sample of the SBFI-loaded cells was centrifuged for 0.5 min at 12000 \times g and resuspended (to 1 mL) in the appropriate solution (Table 2.1). This suspension was transferred to a UV-transparent, tapered, polystyrene 4 mL cuvette (Starna Pty Ltd).

Calibration of the relationship between the fluorescence ratio and [Na\textsuperscript{+}]\textsubscript{i} was performed using the method described by Harootunian and colleagues (1989). Prior to [Na\textsuperscript{+}]\textsubscript{i} measurements, 1 mL aliquots of cells were centrifuged (0.5 min; 12 000 \times g) and resuspended in Na\textsuperscript{+} gluconate / K gluconate solutions (pH 7.3) containing 0, 10, 20, 50 or 130 mM Na\textsuperscript{+} (with appropriate [K\textsuperscript{+}], such that that [Na\textsuperscript{+}] + [K\textsuperscript{+}] = 130 mM). An ionophore cocktail, containing nigericin (K\textsuperscript{+}/H\textsuperscript{+} ionophore), gramicidin (Na\textsuperscript{+}/K\textsuperscript{+} ionophore) and monensin (Na\textsuperscript{+}/H\textsuperscript{+} ionophore), was added to the cell solutions, giving final concentrations of 5 \mu M, 2.5 \mu M and 5 \mu M, respectively. Use of these ionophores quickly equilibrates the [Na\textsuperscript{+}]\textsubscript{i} with [Na\textsuperscript{+}]\textsubscript{o}.

Fluorescence values were converted to [Na\textsuperscript{+}]\textsubscript{i} using the standard Grynkiewicz equation (Grynkiewicz \textit{et al.}, 1985):

\[ [\text{Na}^+]_i = (K_d \times S_f/S_b) \times \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) \]

\textit{Equation 2.1}

where R is the emission fluorescence ratio (from excitation at 340 nm/380 nm) for the unknown [Na\textsuperscript{+}]\textsubscript{i}, R\textsubscript{min} is the emission ratio at 0 mM Na\textsuperscript{+}, R\textsubscript{max} is the emission ratio at saturating Na\textsuperscript{+} (estimated to occur at 130 mM Na\textsuperscript{+}), K\textsubscript{d} is the Na\textsuperscript{+} dissociation constant of the dye, S\textsubscript{f} is the fluorescence emission at 380 nm excitation for the free indicator (0 mM Na\textsuperscript{+}) and S\textsubscript{b} is the fluorescence value at 380 nm excitation for the Na\textsuperscript{+}-bound indicator (130 mM Na\textsuperscript{+}). Under physiological conditions K\textsubscript{d} for SBFI is approximately 17 - 19 mM (Minta and Tsien, 1989; Negulescu \textit{et al.}, 1990).

The calculation of the individual terms comprising K\textsubscript{d}S\textsubscript{f}/S\textsubscript{b} is difficult due to uncertainties in calculating a true value of S\textsubscript{b} (Harootunian \textit{et al.}, 1989; Diarra \textit{et al.}, 2001). Therefore an alternative calibration approach, established by Diarra and colleagues (2001), was used. In this method, the fluorescence ratios recorded at multiple [Na\textsuperscript{+}] were measured (as per Fig. 3.3A), and the data were fit to a three parameter hyperbolic curve that is a rearrangement of Eqn. 2.1:
\[ R = R_{\text{min}} \times \left( a \times [\text{Na}^+]_{i} \right) / \left( b + [\text{Na}^+]_{i} \right) \]

Equation 2.2

where \( R \) is the emission fluorescence ratio (from excitation at 340 nm/380 nm) for the unknown \([\text{Na}^+]_{i}\), \( R_{\text{min}} \) = emission ratio at 0 mM \( \text{Na}^+ \) and \( a \) and \( b \) are fitted constants \( (a = R_{\text{max}} - R_{\text{min}} \) and \( b = K_d \times S_f / S_b \) where the terms are defined in Eqn 2.1). From this equation, \([\text{Na}^+]_{i}\), for any \( R \) (unknown emission fluorescence ratio) can be calculated using the following rearrangement of Eqn. 2.2:

\[ [\text{Na}^+]_{i} = b \times (R - R_{\text{min}}) / (R_{\text{min}} + a - R) \]

Equation 2.3

where terms have been defined as per Eqn. 2.1 and 2.2.

In experiments in which the \( \text{pH}_{i} \) was changing with time, a correction factor was applied to distinguish between the changes in signal due to \( \text{pH} \) dependence of SBFI and the changes in the SBFI signal due to \([\text{Na}^+]_{i}\) changes. The correction required was calculated as described previously (Diarra et al., 2001):

\[ [\text{Na}^+]_{i \text{ (correction)}} = ([\text{Na}^+]_{i \text{ (measured)}} \times (7.3 - \text{pH}_{\text{exp}})) / \sigma \]

Equation 2.4

where \([\text{Na}^+]_{i \text{ (measured)}}\) is the \([\text{Na}^+]_{i}\) determined as per Eqn. 2.3, \([\text{Na}^+]_{i \text{ (correction)}}\) is the final value of \([\text{Na}^+]_{i}\) corrected for changes in \( \text{pH}_{i} \), \( \text{pH}_{\text{exp}} \) is the \( \text{pH}_{i} \) (measured using the pH-sensitive dye BCECF-AM as described in Section 2.5) and \( \sigma \) is an experimentally-determined constant. The ‘7.3’ included, in Eqn. 2.4 is the resting \( \text{pH}_{i} \) of the mature \( P. falciparum \) trophozoite (Saliba and Kirk, 1999; Hayashi et al., 2000; Kuhn et al., 2007). \( \sigma \) was calculated experimentally by performing the standard calibration at varying \( \text{pH}_{o} \) values (see Section 3.2.3). An average \( \sigma \) of 1.73 ± 0.12 (\( n = 6 \)) was calculated and used throughout the study.
2.6. Measurement of Intracellular pH (pH\textsubscript{j}) using BCECF-AM

BCECF, a pH-sensitive, fluorescent dye, was used to measure pH\textsubscript{j} (quantitatively) in saponin-isolated trophozoites. The structures of BCECF-AM are shown in Fig. 2.2, and all three species are present in the Molecular Probes (Invitrogen) preparation used here. Cleavage of all three forms of BCECF-AM by cytoplasmic esterases yields the same BCECF final compound, which is highly charged and thus cell-impermeable.

The pH\textsubscript{j} of isolated parasites was measured at 37 °C using BCECF in a Perkin Elmer LS-50B spectrofluorometer with the dual-excitation Fast Filter accessory. Fluorescence is sensitive to pH at the excitation wavelength of 495 nm and the isosbestic point (i.e., the wavelength at which fluorescence is pH-independent) occurs at 440 nm. Emission was recorded at 520 nm. The ratio of 495 nm/440 nm fluorescence intensities was determined, and used in the quantification of pH\textsubscript{j}.

Loading was performed as described previously (Saliba and Kirk, 1999). Briefly, isolated parasites at a density of 0.9-2.1 × 10\textsuperscript{8} cells/mL were suspended for 10 min at 37 °C in BCF-RPMI (pH = 7.10) containing 1 μM of BCECF-AM. The cells were then washed (12000 × g; 0.5 min) 3 × in BCF-RPMI (pH = 7.10) and allowed to rest for at least 10 min at 37 °C to allow the indicator to de-esterify fully before use. After another two washes (BCF-RPMI, 12000 × g for 0.5 min) the cells were resuspended in BCF-RPMI at a final experimental concentration of 1.0 - 2.0 × 10\textsuperscript{7} cells/mL.

For measurements of pH\textsubscript{j}, a 1 mL sample of the cells was centrifuged (0.5 min; 12 000 × g) and resuspended in the relevant solution. This suspension was transferred into a tapered, polystyrene 4 mL cuvette (Starna Pty Ltd). The FL WinLab program was used for real-time data display, for the collection of data at both excitation wavelengths, and for calculation of the 495 nm/440 nm fluorescence ratio.
Figure 2.2. Chemical structures of BCECF-AM (from Molecular Probes Product Information; Revised 24 April, 2006). All three compounds are formed in the commercial synthesis, but all are cleaved to produce a single BCECF charged product.

Calibration of the relationship between the fluorescence ratio and pH₁ was performed using the nigericin/high K⁺ method described by Saliba and Kirk (1999). Before any pH measurements were recorded, a 1 mL aliquot of cells was centrifuged (12000 × g; 0.5 min) and resuspended in a high-K⁺ solution at pH 6.8, 7.1 or 7.8. The K⁺/H⁺ ionophore, nigericin, was added at a concentration of 30 μM. For parasites suspended in the high-K⁺ solution there was no significant gradient for K⁺ movement and pH₁ quickly equilibrated with the pH₀. There was a linear relationship between the fluorescence measured by the spectrofluorometer and pH₁. Using the three calibration pH values, a linear regression was calculated for every experiment (typically R² > 0.98), and the resulting equation used to convert raw fluorescence data to pH.
2.7. Measurement of Intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) using Fura-2-AM

Fura-2, a Ca\textsuperscript{2+}-sensitive, fluorescent dye, was used to measure [Ca\textsuperscript{2+}]\textsubscript{i} (quantitatively) in saponin-isolated trophozoites. The structure of Fura-2-AM is shown in Fig. 2.3. As discussed in Section 2.4 and 2.5, the AM ester groups of Fura-2-AM are cleaved by esterases, resulting in a cell-impermeable charged product.

![Chemical structure of Fura-2-AM](from Molecular Probes Product Information; accessed online www.invitrogen.com; September, 2011).

The [Ca\textsuperscript{2+}]\textsubscript{i} of isolated trophozoites was measured at 37 °C using Fura-2 in conjunction with the Perkin Elmer LS-50B spectrofluorometer with the dual-excitation Fast Filter accessory. As was the case for the SBFI dye, excitation of Fura-2 was at 340 nm and 380 nm, and emission from both of these wavelengths was recorded at 515 nm. The ratio of fluorescence at 340 nm/380 nm was used to quantify [Ca\textsuperscript{2+}]\textsubscript{i}. The FLWinLab program was used for real-time data display, for the collection of data at both excitation wavelengths, and for calculation of the 340 nm/380 nm fluorescence ratio.

The loading protocol used was identical to that described in Section 2.4 for SBFI-AM, with the exception that the Fura-2-AM dye and 20 % (w/v) Pluronic-F127 were mixed in equal volumes (final concentrations 6 μM and 0.01 % (w/v), respectively). For measurements of [Ca\textsuperscript{2+}]\textsubscript{i}, a 1 mL sample of the cells was centrifuged (12000 x g; 0.5 min) and resuspended (to 1 mL) in the appropriate solution (Table 2.1). This
suspension was transferred to a UV-transparent, tapered, polystyrene 4 mL cuvette (Starna Pty Ltd).

Calibration was performed as described previously (Alleva and Kirk, 2001). Briefly, at the end of each trace CaCl₂ and the Ca²⁺-ionophore ionomycin (final concentrations 3 mM and 30 µM, respectively) were added to the cell suspension and incubated at 37 °C for 30 min. This provides an estimate of the maximum ratio value (Rₘₐₓ as described in Eqn. 2.1). To determine Rₘᵟᵣᵟ, 12.8 mM of the Ca²⁺-chelator Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and 91 mM Tris(hydroxymethyl)aminomethane was added, and Rₘᵟᵟ (at both wavelengths) was recorded once fluorescence had stabilised.

Conversion of fluorescence values to [Ca²⁺]ᵢ was calculated using the Grynkiewicz equation (Grynkiewicz et al., 1985):

\[
[Ca^{2+}]_i = \left( \frac{K_d \times S_f / S_b}{R - R_{min}} \right) \times \left( \frac{R_{max} - R}{R_{max} - R_{min}} \right)
\]

Equation 2.5

where R is the emission fluorescence ratio (from excitation at 340 nm/380 nm) for the unknown [Ca²⁺]ᵢ, Rₘᵟᵟ is the emission ratio at 0 mM Ca²⁺ (in the presence of EGTA), Rₘₐₓ is the emission ratio at saturating Ca²⁺ (estimated to occur at 3 mM Ca²⁺), K_d is the Ca²⁺ dissociation constant of the dye, S_f is the fluorescence emission at 380 nm excitation for the free indicator (0 mM Ca²⁺) and S_b is the fluorescence value at 380 nm excitation for the Ca²⁺-bound indicator (3 mM Ca²⁺). Under physiological conditions K_d for Fura-2 is approximately 120 nM (Molecular Probes product information; revised June 21, 2005).

2.8. Epifluorescence Microscopy

SBFI fluorescence was visualised in a wet-mount preparation (in standard saline) of saponin-isolated trophozoites using epi-fluorescence microscopy. An Olympus IX81 inverted epifluorescence microscope fitted with a 100 × (1.4 NA) objective lens and DAPI filter cube (340 - 380 nm excitation, 425 nm long pass emission) was used,
and images recorded using an FView II digital camera. Image analysis was performed using NIH ImageJ (version 1.40; http://rsb.info.nih.gov/ij/).

2.9. ATP measurements using a Firefly Luciferase-based assay

ATP measurements were made using a Firefly Luciferase-based assay as described previously (Saliba and Kirk, 1999), with modifications for measurement in a 96-well format (van Schalkwyk et al., 2008). Firefly Lantern Extract contains the substrate Luciferin and the enzyme Luciferase, which cause bioluminescence in firefly tails (McElroy, 1947). Luciferase (in the presence of Mg\(^{2+}\)) catalyses the reaction:

\[
\text{ATP} + \text{Luciferin} + O_2 \rightarrow \text{AMP} + \text{Oxyluciferin} + CO_2 + PP_i + \text{Light} \quad \text{Equation 2.6}
\]

Briefly, to measure intracellular [ATP] ([ATP]_i) parasites were suspended at a final concentration of 3.5 \( \times \) 10\(^7\) cells / mL in the experimental solution of interest (± inhibitor), and 50 \( \mu \)L aliquots were added to the required number of wells on a 96-well plate. For every experiment a calibration curve for ATP (using 0.04 – 4.5 \( \mu \)M ATP) was also included (in duplicate), and this was processed in the same manner as the samples. The ATP consuming/generating reactions of the cells were stopped (and cells lysed) by addition of 100 \( \mu \)L 0.1 M HCl at the required timepoints. Half the volume of each well (75 \( \mu \)L) was transferred to a duplicate plate, and was diluted in 200 \( \mu \)L water. Immediately before luminescence recording 15 \( \mu \)L of the ATP-containing parasite extract was added to 185 \( \mu \)L of reaction solution containing 1 % v/v Firefly Lantern Extract, suspended in a minimal solution containing 20 mM HEPES, 25 mM MgCl\(_2\), 5 mM Na\(_2\)HPO\(_4\) (pH 7.4). Luminescence was recorded in a FLUOStar Optima microplate reader (BMG Labtech). The ATP calibration curve was used to calculate [ATP]_i, assuming a parasite water volume of 28 fL (Saliba et al., 1998).
2.10. *In vitro* parasite proliferation assay

Hypoxanthine is a precursor for DNA/RNA synthesis and incorporation of radiolabelled $[^{3}H]$hypoxanthine can be used to measure parasite growth and the effect of drugs or altered growth conditions. Parasite proliferation assays were performed in 96-well plates over 48 hr as described previously (Desjardins et al., 1979). In experiments to test the effect of drugs (or, in one case, the effect of supraphysiological [Na$^+$]) on parasite proliferation, two-fold dilutions of the drug/Na$^+$ in low-hypoxanthine RPMI (culture RPMI containing only 2.4 μM hypoxanthine) were performed in triplicate across the plate (final volume 100 μL), with the top and bottom row of the plate filled with 200 μL RPMI to avoid ‘edge effects’ (Lundholt et al., 2003). Uninfected erythrocytes (1 % haematocrit; 100 μL) were used as a control for background radiolabel incorporation (DNA/RNA synthesis is absent in mature human erythrocytes). Aliquots (100 μL) of ring-stage parasite suspension in low-hypoxanthine RPMI, at a final parasitaemia of 1 % and final haematocrit of 1 %, were added to the drug solutions on the plate. A drug-free control was included in each experiment to calculate maximal parasite growth in the absence of drug. The plate was placed in an air-tight chamber, which was gassed with 1 % O$_2$, 3 % CO$_2$, 96 % N$_2$ gas mix (BOC), and placed in a 37 °C incubator.

After 24 hr, 0.4 μCi $[^{3}H]$hypoxanthine was added to each well and the cells were resuspended by pipetting before re-gassing and returning to the 37 °C incubator. At 48 hr the plates were frozen (causing termination of the growth assay, and cell lysis) at -20 °C, and thawed to harvest the DNA/RNA incorporated radiolabel onto glass fibre filters using a PerkinElmer Filtermate 196 harvester. The glass fibre filters were washed 5 x with H$_2$O and dried at 60 °C. The filter was then aligned in a 96-well microplate filter holders (PerkinElmer) such that the contents from each well of the growth assay were in a separate window in the filter holder. Microscint scintillation fluid (30 μL; PerkinElmer) was added to each well and the plate was covered with adhesive sealing film (PerkinElmer). The radioactivity in each well was recorded using a microplate scintillation counter (PerkinElmer).

The background incorporation from the uninfected erythrocyte wells was subtracted from the reading in experimental wells. Parasite proliferation was calculated as a
percentage of a drug-free control and graphed against the logarithm of the drug concentration. The IC$_{50}$ was calculated by fitting a 3-parameter, sigmoidal curve (Sigma Plot) to the data, typically:

\[
\text{Parasite proliferation (\%) = } \frac{a}{1 + ([\text{drug}] / \text{IC}_{50})^b} \]

Equation 2.7

where the Parasite proliferation (\%) is the $[\text{H}]$hypoxanthine incorporation under the condition of interest as a percentage of that measured under control conditions, [drug] is the concentration of drug (or the concentration of 'excess Na$^+$') present, IC$_{50}$ is the concentration of drug/excess Na$^+$ required to reduce parasite proliferation to half that seen under control conditions, and $a$ and $b$ are fitted constants.

2.11. Measurement of Cell Volume

Cell size (diameter) and volume were measured using a Millipore Scepter™ cell counter (version 2.0). A cell suspension is drawn into the sensor tip and as cells pass through a gap in the circuit they increase the circuit resistance, resulting in an increase in the recorded voltage (www.millipore.com; accessed September, 2011). This increase in voltage is converted to cell size and volume. Saponin-isolated parasites at a cell concentration of $0.5 - 2.5 \times 10^7$ cells/mL were incubated at $37^\circ C$ in the presence or absence of inhibitor. At the time of volume-recording, the parasites were diluted to a concentration of $\sim 3 \times 10^5$ cells/mL in standard saline, and 50 µL of the suspension was drawn into a 40 µm Scepter sensor (designed for a particle size range of 3 - 18 µM). The recorded volume and size distribution data were downloaded using the Scepter™ Application Software (version 2.0) and analysed in Microsoft Excel 2007.

Cell size (diameter) measurements were also made using a Mastersizer 2000 (Malvern) with Hydro 2000S (A) accessory, which works by measuring laser diffraction patterns as cells pass through a laser beam. The scattering pattern of the light is based upon the size of the particle, and the recorded pattern is converted to the size of a sphere of equivalent average cross-sectional area (www.malvern.com; accessed September, 2011). Saponin-isolated trophozoites ($\sim 2.4 \times 10^8$ cells) were
added to the instrument sample chamber containing PBS (pH 7.1), and run using a 'General purpose Analysis model' to detect particles with size range 0.02 - 2000 \( \mu \text{M} \). The Mastersizer 2000 software generated a Result Analysis Report which included histograms of particle size distribution.

### 2.12. Bioinformatics

Amino acid sequences (with accession numbers stated in the appropriate sections) were retrieved from EuPathDB (version 2), PlasmoDB (version 8) (Bahl et al., 2003; Aurrecoechea et al., 2007; Gajria et al., 2008) and GenBank (http://www.ncbi.nlm.nih.gov/). Predictions of protein topology were investigated using the MacVector program (Version 7.1). pBLAST (Altschul et al., 1990) was used to search for proteins sharing homology with PfATP4 and PfNHE. Sequence alignment was performed using ClustalW2 (Larkin et al., 2007). Further details on each of these methods are described in the relevant text/figure legends.

### 2.13. Statistics

Unless stated otherwise, all data is presented as mean ± standard error of the mean (S.E.M.). Data analysis was performed with Microsoft Excel 2003/2007 and Sigma Plot (Version 9.0 - 11.0). Regression modeling was also performed in Sigma Plot. Statistics were calculated using the program Graphpad Instat (Version 3.06). Unless specified otherwise, an unpaired t-test was used. In Chapter 5 (specifically in Fig. 5.3B, 5.8D) statistics were performed on fitted parameters from a regression fitted once to a data set. In this case a modified t-test was used, whereby the Standard Error of the Differences (S.E.-diff) was calculated:

\[
\text{S.E.-}\text{diff} = \sqrt{(S.E.M.}^2_{(x1)} + S.E.M.}^2_{(x2)})
\]

\textit{Equation 2.8}

where \( x1 \) and \( x2 \) are the two values being compared.
The t-test statistic is calculated using the equation:

\[ t \text{-statistic} = \frac{\text{mean } x_1 - \text{mean } x_2}{\text{S.E.}(\text{diff})} \]  \hspace{1cm} \text{Equation 2.9}

To calculate the p-value (reported in text) the probability of being in the tail (< -t or > t) of a t-distribution with between 8 - 16 degrees of freedom (based on the fitted regression) was calculated.
Chapter 3:

Na$^+$ regulation via a Na$^+$-ATPase in *P. falciparum* trophozoites
3.1. Introduction

The parasite maintains a low \([\text{Na}^+]_i/[\text{K}^+]_i\) ratio (Mauritz et al., 2011), estimated on the basis of X-ray microanalysis measurements to be \(\sim 0.06 - 0.12\) (Lee et al., 1988). Assuming that the parasite has a typical \([\text{K}^+]_i\), of the order of \(\sim 130\) mM, this corresponds to a \([\text{Na}^+]_i\) of \(-8.0 - 16.7\) mM. A previous estimate of \([\text{Na}^+]_i\) in the parasite, using the \(\text{Na}^+\)-sensitive, fluorescent dye sodium-binding benzofuran phthalate (SBFI), put the value at 21 mM (Wunsch et al., 1998). The high extracellular \([\text{Na}^+]\), together with the parasite's large inwardly negative membrane potential (\(\sim 95\) mV (Allen and Kirk, 2004b)), constitute a significant driving force for the influx of \(\text{Na}^+\) into the parasite. Many organisms rely on \(\text{Na}^+\)-coupled transporters, energised by the inward \(\text{Na}^+\) electrochemical gradient, for uptake of nutrients, and in \(P. falciparum\) it has been shown that the intraerythrocytic parasite uses a \(\text{Na}^+\)-coupled transporter for the uptake of the essential nutrient inorganic phosphate (Saliba et al., 2006).

The mechanism by which the \(Plasmodium\) parasite regulates its \([\text{Na}^+]_i\) is unknown. In animal cells, \(\text{Na}^+\) regulation typically involves the \(\text{Na}^+\)-extruding \(\text{Na}^+/\text{K}^+\)-ATPase (Aperia, 2007), with the resulting inward \(\text{Na}^+\) gradient utilised by a \(\text{Na}^+/\text{H}^+\) exchanger (NHE) to extrude \(\text{H}^+\) and thereby regulate pH\(_i\) (Slepkov et al., 2007; Sections 1.5.3.1 and 1.6.3.1). By contrast, in vascular plants a P-type \(\text{H}^+\)-ATPase extrudes \(\text{H}^+\), thereby generating an inward \(\text{H}^+\) gradient (Gaxiola et al., 2007) which is, in turn, used to energise the efflux of \(\text{Na}^+\) via NHEs (Plett and Moller, 2009; Sections 1.5.3.2 and 1.6.3.2). In lower plants (bryophytes), fungi and some protozoa, a P-type, ENA \(\text{Na}^+\)-ATPase operates to efflux \(\text{Na}^+\) (Rodriguez-Navarro and Benito, 2010; Section 1.6.3.2-4).

The \(P. falciparum\) genome encodes seven P-type cation-transporting ATPases (Kirk et al., 2005; Martin et al., 2005) and although none were annotated as \(\text{Na}^+\)-ATPases it is possible that one (or more) of these is involved in \(\text{Na}^+\) regulation. Another candidate for involvement in \(\text{Na}^+\) regulation in the parasite is PfNHE, the physiological role of which is unclear. PfNHE has previously been proposed to play a role in parasite pH\(_i\) regulation (Bosia et al., 1993; Bennett et al., 2007); however it has been demonstrated that pH regulation (specifically, \(\text{H}^+\) extrusion) is \(\text{Na}^+\)-
independent, inconsistent with the involvement of an NHE. Instead, H⁺ extrusion involves a plasma membrane V-type H⁺-ATPase (Section 1.5.3.5; Saliba and Kirk, 1999; Hayashi et al., 2000; Spillman et al., 2008) which, as well as playing a primary role in pHᵢ regulation is the primary source of the parasite’s membrane potential (Allen and Kirk, 2004b).

The aim of this chapter was to investigate Na⁺ regulation in *P. falciparum* and characterise the transport mechanism/s involved. Using pH- and Na⁺-sensitive dyes physiological evidence was obtained for involvement of a P-type ATPase in Na⁺ efflux in mature trophozoites.

### 3.2. Results

#### 3.2.1. Spectral Properties of SBFI in *P. falciparum*

[Na⁺], was measured using the Na⁺-sensitive dye, SBFI. SBFI does not exhibit the same spectral properties *in vivo* and *in vitro*, due to the dye binding to cytosolic “microviscosity” and to protein binding in the *in vivo* situation (Harootunian et al., 1989; Diarra et al., 2001; Birkedal and Shiels, 2007). To confirm the excitation wavelengths suitable for use in *P. falciparum*, an excitation spectrum was recorded, whereby fluorescence intensity was measured across different excitation wavelengths at a constant emission wavelength of 515 nm.

Fig. 3.1 shows excitation spectra of SBFI-loaded, saponin-isolated parasites suspended in a calibration solution (see Section 3.2.3 for further calibration details) containing 0 or 130 mM Na⁺, along with an ionophore mix of nigericin (5 μM), gramicidin (2.5 μM) and monensin (5 μM). Unloaded cells (suspended at 130 mM Na⁺ without ionophores) showed negligible changes in fluorescence, whereas SBFI-loaded cells were sensitive to changes in the excitation wavelength. The greatest variation in fluorescence intensity was close to excitation at 340 nm and the isosbestic point (i.e., the wavelength at which fluorescence intensity does not vary with [Na⁺]) was close to 380 nm. These results validate the use of an excitation filter wheel set at 340/380 nm in the parasite system.
Wavelength (nm)

Figure 3.1. In vivo excitation spectra of SBFI in *P. falciparum* isolated trophozoites across range of excitation wavelengths. Parasites loaded with SBFI were suspended in a calibration solution (with a composition defined in Table 2.1) containing either 0 or 130 mM Na⁺, and the calibration ionophores gramicidin (2.5 μM), monensin (5 μM) and nigericin (5 μM). Unloaded parasites were suspended in 130 mM Na⁺, without ionophores. Cells were excited from 300 - 430 nm at a rate of 750 nm/min, and emission at 515 nm was recorded. Traces are representative of those obtained in three independent experiments in different strains (the data shown here are from the PfNHEkd strain).

3.2.2. Localisation of SBFI in *P. falciparum*

Epifluorescence microscopy was used to investigate localization of SBFI in saponin-isolated trophozoites. SBFI fluorescence was distributed uniformly throughout the parasite cytoplasm (Fig. 3.2). There was no fluorescence arising from the digestive vacuole. Fragments of erythrocyte ghost membrane were observed around some isolated trophozoites using transmitted light, however no fluorescence was seen within the ghost.
3.2.3. Calibration of SBFI in \textit{P. falciparum}

[Na\textsuperscript{+}]\textsubscript{i} was calibrated in SBFI-loaded parasites using a previously described method (Harootunian \textit{et al.}, 1989). Calibration solutions contained appropriate ratios of the gluconate salts of Na\textsuperscript{+} and K\textsuperscript{+}, with 50 mM Cl\textsuperscript{−} (approximately [Cl\textsuperscript{−}]\textsubscript{i}) (Henry \textit{et al.}, 2010)). This [Cl\textsuperscript{−}]\textsubscript{o}, should minimise any Donnan effect, and lead to a true equilibration of intra- and extra-cellular [Na\textsuperscript{+}] (Harootunian \textit{et al.}, 1989). Fig. 3.3A shows a representative calibration, carried out using the ionophore combination of gramicidin (5 \textmu M) nigericin (2.5 \textmu M) and monensin (5 \textmu M). The fluorescence ratio increased with [Na\textsuperscript{+}] and was converted to [Na\textsuperscript{+}]\textsubscript{i}, using a hyperbolic rearrangement of the Grykiewicz equation (as outlined in Section 2.5).

In other systems, SBFI fluorescence is pH sensitive, and since H\textsuperscript{+} transport may be coupled to Na\textsuperscript{+} regulatory mechanisms (for example via an NHE) it was important to determine if pH changes altered SBFI fluorescence (Diarra \textit{et al.}, 2001). Fig. 3.3B shows SBFI calibration curves obtained at three different pH values: 6.0, 7.3 and 8.0. The curves were, in each case, hyperbolic, with the fluorescence ratio at any
Figure 3.3. Calibration and pH-sensitivity of the Na⁺-sensitive fluorescent dye SBFI in saponin-isolated *P. falciparum* trophozoites. (A) Representative calibration trace for SBFI-loaded parasites equilibrated to varying $[\text{Na}^+]_i$ using a combination of the ionophores gramicidin, monensin and nigericin (G, M, N). The traces, showing the fluorescence ratio increasing with $[\text{Na}^+]_i$, are representative of those obtained in at least 32 independent experiments. (B) Representative calibration curves showing the relationship between fluorescence ratio and $[\text{Na}^+]_i$ at three different pH values: 6.0, 7.3 and 8.0. The data obtained at each pH were fitted to a hyperbolic function ($R^2 > 0.95$) which was used subsequently to convert the measured fluorescence ratio to $[\text{Na}^+]_i$. The curves are representative of those obtained in at least five independent experiments. (C) Representative calibration trace for SBFI-loaded parasites equilibrated to varying $[\text{Na}^+]_i$ using a G, M, N ionophore mix or gramicidin alone. The traces, showing no difference between calibrations with gramicidin alone compared to the G, M, N mix, are representative of those obtained in at least three independent experiments.
given [Na\(^+\)], increasing with increasing pH. In experiments in which [Na\(^+\)], was estimated under conditions of altered pH, (one example being the removal of extracellular Cl\(^-\), Fig. 4.2. (Henry et al., 2010)), it was necessary to calculate a correction factor based on the time-dependent change in pH, (see Section 2.5).

Although there was no dye compartmentalisation observed within the parasite cytoplasm using epifluorescence microscopy (Fig. 3.2) it was important to determine whether part of the SBFI signal might have arisen from acidic intracellular compartments; this could potentially have decreased the SBFI ratio, resulting in an underestimate of [Na\(^+\)]. Gramicidin interacts primarily with the plasma membrane (Rink et al., 1980), whereas monensin and nigericin can distribute into intracellular membranes (Harootunian et al., 1989). Therefore, if the dye were significantly localised in acidic organelles, then the calibration using gramicidin only will have differed from calibration using the gramicidin/monensin/nigericin (G, M, N) combination. As shown in Fig. 3.3C there was no difference between calibration with gramicidin alone versus calibration with the G, M, N mix. Thus, consistent with the localisation results, calibration results confirm the predominant cytoplasmic localisation of SBFI.

3.2.4. Inner Filter Effects

As SBFI is excited by UV wavelengths, inner filter effects (also known as concentration quenching) affect the SBFI signal. Inner filter effects occur when a compound present in the cell preparation (e.g. an added inhibitor) interferes with the excitation or emission light (Srinivas and Mutharasan, 1987). Many of the inhibitors/ionophores used in this study, including the P-type ATPase inhibitor orthovanadate, the NHE inhibitors amiloride and 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), the protozoal Na\(^+\)-ATPase inhibitor furosemide and the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), absorb light in the UV range. These inhibitor/ionophores were found to affect the calibration curve significantly (Fig. 3.4A), therefore separate calibrations were performed with the compound present. Notably, the NHE inhibitor amiloride (500 \(\mu\)M) could not be used in experiments because the fluorescence ratio (340 nm/380 nm) was no longer Na\(^+\)-sensitive in its presence (Fig. 3.4A).
Figure 3.4. Inner filter effects of inhibitors/ionophores (A) and cell density (B) on SBFI fluorescence. (A) Representative calibration traces for SBFI-loaded parasites equilibrated to varying [Na⁺], using the G, M, N ionophore mix in the presence of varying concentrations of several inhibitors/ionophores used in the course of this study. The traces are representative of those obtained in at least three independent experiments per inhibitor/ionophore. (B) Representative calibration traces for SBFI-loaded parasites equilibrated to varying [Na⁺], using the G, M, N ionophore mix at varying cell densities. The traces, showing the fluorescence ratio increasing with cell density are representative of those obtained in at least three independent experiments.
The effect of cell density on SBFI fluorescence was also investigated. As the SBFI dye is ratiometric, it was expected that a change in cell number should not cause the fluorescence ratio to change. However as demonstrated in Fig 3.4B, cell density did cause an inner filter effect. As the cell density increased, the fluorescence ratio also increased. This highlights the necessity to perform a calibration for every experiment, as differences in cell number can cause a shift in the ratio values recorded.

3.2.5. Resting [Na\(^+\)]\textsubscript{i} under physiological conditions

In saponin-isolated, 3D7-strain, trophozoite-stage parasites suspended in standard saline, the resting [Na\(^+\)]\textsubscript{i} was estimated to be 11.0 ± 0.6 mM (n = 34). During the course of a typical 1 - 2 hr experiment, resting [Na\(^+\)]\textsubscript{i} increased by 3.8 ± 1.0 mM (n = 12). A similar value for resting [Na\(^+\)]\textsubscript{i} (10.3 ± 0.8 mM; n = 8) was estimated in another P. falciparum strain, FAF6. The resting [Na\(^+\)]\textsubscript{i} of several other strains (including genetically modified strains), was also examined (see Chapter 5 and 6 for detailed evaluation of Na\(^+\) regulation in these strains).

3.2.6. Decreasing or increasing [Na\(^+\)]\textsubscript{o}

On replacement of extracellular Na\(^+\) with an alternative cation (choline\(^+\), N-methyl-D-glucamine\(^+\) (NMDG\(^+\)) or K\(^-\)), [Na\(^+\)]\textsubscript{i} decreased towards 0 mM (Fig. 3.5A), indicative of a net Na\(^+\) efflux. The initial rate of decrease of [Na\(^+\)]\textsubscript{i} for cells suspended in a Na\(^+\)-free solution was 2.8 ± 0.7 mM/min (n = 3) in a solution in which Na\(^-\) was replaced with K\(^+\), 2.8 ± 0.1 mM/min (n = 3) in a solution in which Na\(^-\) was replaced with choline\(^-\) and 3.1 ± 0.7 mM/min (n = 3) in a solution in which Na\(^-\) was replaced with NMDG\(^+\). These values were not significantly different from one another (p = 0.9; one-way analysis of variance (ANOVA)). The final values of [Na\(^+\)]\textsubscript{i} attained (after 10 min) using each of the three different replacement cations were 1.1 ± 0.5 mM (n = 10) with K\(^+\), 0.5 ± 0.6 mM (n = 9) with choline\(^+\) and 0.3 ± 0.6 mM (n = 9) with NMDG\(^+\). These values were not significantly different from one another (p = 0.54; one-way ANOVA). Altering pH\textsubscript{o} or addition of transport inhibitors (EIPA; 20 μM, or orthovanadate; 100 μM) caused no change in the efflux characteristics (data not shown).
Figure 3.5. Effect of decreasing (A) or increasing (B, C) [Na\(^+\)]\(_o\) on [Na\(^+\)]\(_i\) in SBFI-loaded, saponin-isolated P. falciparum trophozoites. (A) Extracellular Na\(^+\) (125 mM in standard saline) was replaced isosmotically with choline\(^+\) at the timepoint indicated by the closed triangle. The extracellular Na\(^+\) concentration over the course of the experiment is shown above the trace. The trace is representative of those obtained in at least three independent cell preparations. (B) [Na\(^+\)]\(_o\) was increased (above 125 mM) by the addition of 75 mM NaCl at the timepoint indicated by the closed triangle (black trace). Isosmotic quantities of choline Cl (75 mM; red trace), KCl (75 mM; blue trace) or sucrose (150 mM; gray trace) were added to separate cell aliquots to allow an assessment of the degree of [Na\(^+\)]\(_i\) increase attributable to osmotic shrinkage. The traces are representative of those obtained in at least five independent cell preparations. (C) [Na\(^+\)]\(_o\) was increased above 125 mM as per (B), with [Na\(^+\)]\(_o\), determined 10 min later. At each [Na\(^+\)]\(_o\), the increase in [Na\(^+\)]\(_i\), measured following the addition of an equimolar concentration choline Cl was subtracted from that measured following addition of NaCl to correct for osmotic shrinkage of the parasite. Where not shown, the error bars lie within the data point. The line was drawn using a sigmoidal curve fitted to the data (R\(^2\) = 0.98). Each data point is averaged from between three and five independent experiments (mean ± S.E.M.).
The effect of increasing the extracellular Na\(^+\) concentration ([Na\(^+\)]\(_o\)) on [Na\(^+\)]\(_i\) was also investigated. In order to account for changes in [Na\(^-\)]\(_i\), due to osmotic effects, the effect of addition of other monovalent cations (‘osmotic controls’) was also investigated. Fig 3.5B shows the effect on [Na\(^+\)]\(_i\) of addition of 75 mM Na\(^+\), K\(^+\), choline\(^+\) (as Cl\(^-\) salts) or 150 mM sucrose to cells in standard saline. When excess [Na\(^+\)]\(_o\) was added (above the 125 mM in standard saline), [Na\(^+\)]\(_i\) increased above the level of the osmotic controls. Addition of 75 mM K\(^+\) resulted in a sudden increase in [Na\(^+\)]\(_i\) (as was seen for the other two osmotic controls), however [Na\(^+\)]\(_i\) then decreased and quickly recovered toward resting [Na\(^+\)]\(_i\). This behaviour was not observed for the other two osmotic controls.

Fig. 3.5C shows the relationship between [Na\(^+\)]\(_o\), and [Na\(^+\)]\(_i\), as [Na\(^+\)]\(_o\) was increased. At each [Na\(^+\)]\(_o\), the increase in [Na\(^-\)]\(_i\), measured following the addition of an equimolar concentration choline Cl was subtracted from that measured following addition of NaCl, in order to correct for the increase in concentration arising from osmotic shrinkage of the parasite and to provide, thereby, an estimate of the magnitude of the ‘shrinkage-independent’ increase in [Na\(^-\)]\(_i\). At the maximum concentration tested ([Na\(^-\)]\(_o\) = 300 mM, an increase of 175 mM above that in standard saline), the increase in [Na\(^+\)]\(_i\) (corrected for osmotic cell shrinkage) was less than 20 mM above its normal resting value (Fig. 3.5C). The parasite is therefore capable of maintaining a low [Na\(^+\)]\(_i\), even when exposed to a very high [Na\(^+\)]\(_o\).

### 3.2.7. A novel Na\(^+\) loading mechanism in P. falciparum

Standard saline in which isolated parasites were suspended contains 5 mM KCl (Table 2.1). In experiments investigating the relationship between Na\(^+\) and K\(^+\) regulation it was found that on removal of K\(^+\) from the medium (by isosmolar replacement with Na\(^-\)) there was a steady increase in [Na\(^+\)]\(_i\). (Fig. 3.6A). The increase was linear with time, occurring at a rate of 0.6 ± 0.2 mM/min (n = 7). When K\(^+\) was restored to the extracellular solution, by the addition of 10 mM KCl, [Na\(^+\)]\(_i\) recovered to a level not significantly different from the initial resting [Na\(^+\)]\(_i\), (recovery to 9.5 ± 2.7 mM; n = 15, p = 0.147, paired t-test). The decrease in [Na\(^+\)]\(_i\), following the restoration of K\(^+\) to the extracellular solution provides a direct demonstration of the ability of the parasite to extrude Na\(^+\) against an inward electrochemical gradient.
Figure 3.6. The effect of removal and restoration of extracellular K⁺ on [Na⁺]ᵢ and pHᵢ in saponin-isolated P. falciparum trophozoites. (A) SBFI trace showing the effect of removal of [K⁺]₀ on [Na⁺]ᵢ. At the time-point indicated by the open triangle the cells (in standard saline) were washed twice by centrifugation and resuspension in a K⁺-free saline (in which K⁺ was replaced isosmotically with Na⁺). At the timepoint indicated by the closed triangle, 10 mM K⁺ (as KCl) was added to the suspension. The trace is representative of those obtained from at least twenty independent cell preparations. (B) Trace showing the effect of the same manoeuvers (i.e., removal at the point indicated by the open triangle then restoration at the point indicated by the closed triangle, of extracellular K⁺) on the pHᵢ in BCECF-loaded parasite suspension. The trace is representative of those obtained from at least seven independent cell preparations. (C) Cells were loaded to varying [Na⁺]ᵢ between 11 mM (resting) and 70 mM using the method described in (A), before adding 10 mM KCl to the cell suspensions, thereby initiating the net efflux of Na⁺. The initial rate of recovery was calculated and plotted against the [Na⁺]ᵢ at the point of addition of KCl. The data points are collated from six independent cell preparations. A three-parameter sigmoidal curve was fitted to the data, as described in the text (R² = 0.66).
The time-course for the efflux of Na\(^+\) from the cells, following the addition of KCl, was fitted to an exponential function:

\[
[Na^+]_{i} = [Na^+]_{i}^{t=0} + \Delta[Na^+]_{i}^{max} \times e^{-at}
\]

Equation 3.1.

where \([Na^+]_{i}^{t=0}\) is the initial \([Na^+]_{i}\) at the point of addition of K\(^+\), \(\Delta[Na^+]_{i}^{max}\) is the increase in \([Na^+]_{i}\) (i.e., the additional Na\(^+\) load from the initial resting value), \(t\) is the time after KCl addition, and \(a\) is a fitted constant.

The initial Na\(^+\) efflux rate (at \(t = 0\)) = \(a \times \Delta[Na^+]_{i}^{max}\), and the half time to complete recovery is \(t_{1/2} = \ln(0.5)/-a\). These parameters have been used throughout this study to compare the activity of the Na\(^+\) efflux mechanism under varying conditions.

When extracellular K\(^+\) was removed and \([Na^+]_{i}\) allowed to reach a level approximately double the normal resting value (i.e., \([Na^+]_{i}\) between 18 - 25 mM) before the addition of 10 mM KCl to the extracellular solution, \([Na^+]_{i}\) recovered at an initial rate of 2.7 ± 1.2 mM/min (\(n = 15\)) with a half time to complete recovery of 184 ± 22 s (\(n = 15\)).

The effect of removal of \([K^+]_{o}\) on pH\(_i\) was also investigated. The increase in \([Na^+]_{i}\) seen on removal of extracellular K\(^+\) was accompanied by a time-dependent decrease in pH\(_i\) (0.026 ± 0.003 pH units/min; \(n = 9\); Fig. 3.6B). When K\(^+\) was restored to the extracellular solution, by the addition of 10 mM KCl, pH\(_i\) increased, albeit not quite to the initial resting value.

The manoeuvre described above (i.e., the removal then restoration of \([K^+]_{o}\)) provides a means of imposing a 'Na\(^+\) load' on the parasite, and the subsequent net efflux of Na\(^+\) from the parasite can be measured. The kinetic parameters of the transporter involved in Na\(^+\) efflux were investigated by loading the cell to various \([Na^+]_{i}\) (above the normal resting value) and determining the initial Na\(^+\) efflux rate upon restoration of extracellular K\(^+\). The initial rate of Na\(^+\)-efflux was calculated using Eqn. 3.1. The Na\(^+\) efflux rate increased with increasing \([Na^+]_{i}\) load (Fig. 3.6C), but at higher concentrations reached a maximal rate of 4.1 ± 0.5 mM/min (\(R^2 = 0.66\)).
A sigmoidal function was used to calculate a $K_m$ and Hill coefficient for the transport process:

$$V = \frac{V_{max} \times [Na^+]_i^n}{(K_m^n + [Na^+]_i^n)} \quad Equation \ 3.2.$$ 

where $V$ is the initial $Na^+$ efflux rate, $V_{max}$ is the maximum initial rate of $Na^+$ efflux and $n$ is the Hill coefficient.

Using this equation the Hill coefficient was estimated to be $3.6 \pm 1.3$, and the $K_m$ was estimated as $19.4 \pm 1.9$ mM ($R^2 = 0.66$).

### 3.2.8. The effect of ionophores and inhibitors on resting $[Na^+]_i$

Various ionophores and ion transport inhibitors were tested for their effect on $[Na^+]_i$ in isolated parasites. Gramicidin (5 μM), induced a rapid increase in $[Na^+]_i$, with $[Na^+]_i$ approaching $[Na^+]_o$ (125 mM; Fig. 3.7A). The addition of monensin (2.5 μM) and nigericin (5 μM) also led to a rapid increase in $[Na^+]_i$ (data not shown).

The NHE inhibitor EIPA (20 μM) did not have a significant effect on resting $[Na^+]_i$ (Fig 3.7B). Two other inhibitors of human NHE isoforms, N-(diaminomethylidene)-2-methyl-5-methylsulfonyl-4-pyrrol-1-ylbenzamide (EMD96785; 500 μM) and [5-(2-Methyl-5-fluorophenyl)furan-2-ylcarbonyl]guanidine (KR-32568; 500 μM), also had no effect on $[Na^+]_i$ (data not shown). An inner filter effect precluded the inclusion of amiloride as an NHE inhibitor suitable to use in this study (Fig. 3.4A).

Ouabain (2 mM), an inhibitor of the $Na^+$/K$^+$-ATPase of animal cells, had no significant effect on resting $[Na^+]_i$, (Fig. 3.7C). Neither did the pyrophosphatase inhibitor AMDP (20 μM) nor bumetanide (10 μM), an inhibitor of NKCC ($Na^+$/K$^+$/2Cl$^-$) cotransporters (data not shown).

Furosemide (100 μM), an inhibitor of some protozoal $Na^+$-ATPases (Iizumi et al., 2006; De Souza et al., 2007a) caused resting $[Na^+]_i$ to increase significantly by $2.3 \pm 0.7$ mM ($n = 4; p = 0.009$; Fig. 3.7D). Sodium orthovanadate (100 μM), an inorganic
Figure 3.7. Effect of an ionophore and ion transport inhibitors on $[\text{Na}^+]_i$ in saponin-isolated, SBFI-loaded *P. falciparum* trophozoites. $[\text{Na}^+]_i$ traces showing the effect of addition (at the time-point indicated by the closed triangle) of (A) gramicidin (5 μM), (B) EIPA (20 μM), (C) ouabain (2 mM), (D) furosemide (100 μM), and (E) orthovanadate (100 μM). For all additions other than that of ouabain, the compounds were added as a concentrated stock. Cells were exposed to 2 mM ouabain by being sedimented by centrifugation then resuspended at the timepoint indicated in an equivalent saline containing the inhibitor. The traces shown are, in each case, representative of those obtained from at least three independent cell preparations.
phosphate analogue that inhibits P-type ATPases (Cantley et al., 1978a; Cantley et al., 1978b), caused \([\text{Na}^+]_i\) to undergo a prolonged time-dependent increase (Fig. 3.7E), consistent with a P-type ATPase playing a role in \(\text{Na}^+\) regulation in the intraerythrocytic parasite. The maximum rate of increase of \([\text{Na}^+]_i\) seen following the addition of 500 \(\mu\text{M}\) orthovanadate was 7.8 ± 1.8 mM/min (n = 4).

3.2.9. Effect of transport inhibitors on the net efflux of \(\text{Na}^+\) following an imposed \(\text{Na}^+\) load

The \(\text{Na}^+\) loading manoeuvre described in Section 3.2.7 (i.e., the removal then restoration of \(\text{K}^+\)) provides a means of imposing a ‘\(\text{Na}^+\) load’ on the parasite, and of testing the effect of inhibitors on the net efflux of \(\text{Na}^+\) from the parasite. EIPA (20 \(\mu\text{M}\)) had no effect on the timecourse for the recovery of \([\text{Na}^+]_i\) seen following the restoration of extracellular \(\text{K}^+\) (Fig. 3.8A). Ouabain (2 mM) also had no effect on the \(\text{Na}^+\) recovery timecourse (data not shown).

Furosemide (100 \(\mu\text{M}\)) slowed the rate of \(\text{Na}^+\) efflux, whilst still allowing \([\text{Na}^+]_i\) to recover to a normal resting value over an extended period (Fig. 3.8B). The \(t_{1/2}\) for recovery in the presence of 100 \(\mu\text{M}\) furosemide was 587 ± 17 s (n = 3), which was significantly longer than that seen under control conditions (described in Section 3.2.7; \(p = 0.0004\)). Orthovanadate (100 \(\mu\text{M}\)), also inhibited \(\text{Na}^+\) efflux; in the presence of this compound the restoration of extracellular \(\text{K}^+\) had no effect, with \(\text{Na}^+\) influx continuing unabated (Fig. 3.8C).

3.2.10. The energy dependence of \(\text{Na}^+\) regulation

The energy dependence of the maintenance of resting \([\text{Na}^+]_i\), was investigated using two methods to deplete the parasite of intracellular ATP ([ATP]_i): removal of extracellular glucose and addition of the glycolysis inhibitor NaF. Fig. 3.9A shows the effect of these two methods on parasite [ATP]_i, measured using the Firefly Luciferase ATP assay. In isolated parasites in standard, glucose-containing saline, [ATP]_i was constant over the 1 hr timecourse (data not shown). In glucose-free standard saline, [ATP]_i was reduced below detectable levels by the first timepoint (approximately one minute after glucose removal by a rapid centrifugation and resuspension in glucose free standard saline). On addition of NaF (to cells in
Figure 3.8. Effect of ion transport inhibitors on the recovery of $[\text{Na}^+]_i$ from an imposed Na$^+$ load in saponin-isolated *P. falciparum* trophozoites. SBFI-loaded parasites were subjected to a Na$^+$ load (by suspension in K$^+$-free medium), as illustrated in Fig. 3.6A. The traces commenced with the addition to the cell suspension of 10 mM KCl either with (light traces) or without (control, dark traces) (A) EIPA (20 μM), (B) furosemide (100 μM) or (C) orthovanadate (100 μM). The traces shown are representative of those obtained from at least three independent cell preparations.
Figure 3.9. Effect of ATP-depletion on the maintenance of resting [Na$^+$]$_i$ in saponin-isolated trophozoites. (A) Intracellular [ATP] was measured using the firefly luciferase assay and results were standardised to the resting intracellular [ATP] in parasites suspended in glucose-containing, inhibitor-free medium. At time-zero parasites were suspended in glucose-free medium (○) or medium containing 2 mM NaF (●). The data are averaged from three independent experiments (mean ± S.E.M.). Where not shown error bars lie within the symbol. Lines were drawn using either an exponential or linear function fitted to the data ($R^2 > 0.89$). (B) [Na$^+$]$_i$ traces showing the increase in [Na$^+$]$_i$ of SBFI-loaded, saponin-isolated parasites seen in response to ATP-depletion using the two different methods. At the timepoint represented by the closed triangle, cells were resuspended in glucose-free saline (in which the glucose was replace isosmotically with NMDG$^+$; light trace) or standard saline with 2 mM NaF (an inhibitor of several glycolytic enzymes; dark trace). Traces are representative of those obtained in at least four independent experiments.
glucose-containing standard saline) there was an initial rapid decrease in ATP levels (with [ATP]ᵢ decreasing to approximately 30 % of its initial value within 5 min), then a subsequent slower decline, with [ATP]ᵢ decreasing to 11 ± 6 % (n = 3) of its initial value after 50 min.

Both methods caused [Na⁺]ᵢ to undergo a time-dependent increase (Fig. 3.9B). Although glucose removal caused a faster depletion of [ATP]ᵢ than NaF, NaF induced a more rapid increase in [Na⁺]ᵢ than glucose-depletion. The maximal rate of Na⁺ influx upon F⁻ addition was 1.0 ± 0.3 mM/min (n = 5), whilst the maximal rate of Na⁺ influx upon glucose removal was slower at 0.4 ± 0.1 mM/min (n = 4), though this difference was not significant (p = 0.18).

3.3. Discussion

In this chapter, the mechanism of Na⁺ regulation in saponin-isolated trophozoites was investigated using the Na⁺-sensitive, fluorescent dye SBFI. The suitability of SBFI to report [Na⁺]ᵢ accurately was investigated in detail (Fig. 3.1-4). Although the dye showed some pH-sensitivity, and many of the key inhibitors exhibited inner filter effects, these difficulties were overcome using a multiple calibration approach, whereby separate calibrations were performed under the different conditions tested.

3.3.1 Na⁺ regulation in the intraerythrocytic parasite involves a P-type ATPase.

Using SBFI, the resting [Na⁺]ᵢ of trophozoite-stage 3D7 and FAF6 *P. falciparum* parasites was estimated here to be ~11 mM. This is well within the range typical of eukaryote cells (1 - 40 mM (Minta and Tsien, 1989; Page and Di Cera, 2006; Apse and Blumwald, 2007)), and somewhat lower than the only previous quantitative estimate of [Na⁺]ᵢ in blood stage *P. falciparum* parasites (21 mM; (Wunsch et al., 1998)). The low [Na⁺]ᵢ (and the large, inwardly negative membrane potential (Allen and Kirk, 2004b)) means that there is a large inward electrochemical gradient, both under the conditions used in this study (in which isolated parasites were suspended in medium containing 125 mM Na⁺) and in intact parasitised erythrocytes, in which the [Na⁺]ᵢ in the erythrocyte cytosol approaches that in the extra-erythrocytic plasma
(−130 mM (Page and Di Cera, 2006)). The parasite’s ability to generate and maintain a large inward Na⁺ electrochemical gradient under normal resting conditions (as well as in the presence of supra-physiological [Na⁺]₀; Fig. 3.5C), is consistent with the parasite having an effective Na⁺ extrusion mechanism. The presence of such a mechanism was confirmed by the demonstration of the ability of the parasite to extrude Na⁺ from Na⁺ loaded parasites, against a steep inward Na⁺ gradient (Fig. 3.6A).

Addition of the P-type ATPase inhibitor orthovanadate (Cantley et al., 1977; Cantley et al., 1978b) resulted in a prolonged time-dependent increase in [Na⁺], (Fig. 3.7E), consistent with the involvement of a P-type ATPase in the maintenance of a low [Na⁺]ᵢ. Orthovanadate also inhibits phosphotyrosine phosphatases, through its action as a phosphate analogue (Gordon, 1991), and the possibility of the increase in [Na⁺]ᵢ being secondary to inhibition of a phosphatase (or indeed any other protein) cannot be excluded. Nevertheless, disruption of [Na⁺]; regulation by orthovanadate (and prevention of the recovery from a Na⁺ load; Fig. 3.8C) is consistent with inhibition of a P-type Na⁺-ATPase.

Addition of furosemide also caused a small increase in the resting [Na⁺], (Fig. 3.7D), and a significant slowing of the recovery from a Na⁺ load (Fig. 3.8B). The 100 μM concentration used here was lower than the IC₅₀ for inhibition of Na⁺-dependent ATPase activity observed in two previous studies on ENA-type Na⁺-ATPase activity in protozoans of ~120 μM and 270 μM (De Souza et al., 2007a; de Almeida-Amaral et al., 2008); greater inhibition may be observed at higher concentrations not tested here. Notably, the maintenance of [Na⁺]ᵢ in the parasite was insensitive to ouabain which, at the concentration tested here (2 mM), inhibits all known Na⁺/K⁺-ATPases, including those that are relatively ‘ouabain-insensitive’ (De Souza et al., 2007b). [Na⁺] regulation was also insensitive to all inhibitors of NHEs tested (Fig. 3.7B), though it should be noted here that this does not rule out NHE involvement in parasite Na⁺ regulation because, as revealed in the bioinformatics analysis presented in Section 6.3.1.3, the P. falciparum isoform might be expected to be insensitive to amiloride and its derivatives.
This inhibitor sensitivity profile (sensitivity to orthovanadate and furosemide, and insensitivity to ouabain) is the same as that obtained previously in other protozoa and fungi, in which Na\(^+\) is extruded via an ENA class, orthovanadate- and furosemide-sensitive Na\(^+\)-ATPase (Iizumi et al., 2006; De Souza et al., 2007a). The ENA class of ATPases was first identified in *S. cerevisiae* in 1991 (Haro et al., 1991) and members of this class have been identified in fungi, lower plants (bryophytes and moss) and several protozoa including *Trypanosoma* spp. (Meade et al., 2000; Stiles et al., 2003; Iizumi et al., 2006), *Leishmania* spp. (Stiles et al., 2003; de Almeida-Amaral et al., 2008) and *E. histolytica* (De Souza et al., 2007a). Notably no animal or higher (vasculated) plant members have been described.

From the kinetic analysis performed in this study, the parasite Na\(^+\) extrusion mechanism was estimated to have a \(V_{\text{max}}\) of 4.1 mM/min and a \(K_m\) of 19.4 mM. This \(K_m\) value is within the range of the half-maximal stimulation of Na\(^+\)-dependent ATPase activities reported for *E. histolytica* (13.3 mM; De Souza et al., 2007a) and for *L. amazonensis* (28.9 mM; de Almeida-Amaral et al., 2008). The Hill coefficient was estimated to be 3.6, indicating that there is cooperative ion binding in this transporter. Consistent with this finding, ENA-type Na\(^+\)-ATPases are predicted (based on their similarity to SERCA Ca\(^{2+}\)-ATPases) to contain two Na\(^+\) binding pockets (Drew et al., 2010) though no structural information is available to date.

### 3.3.2. Effects of altering K\(^+\) on [Na\(^+\): A novel Na\(^+\) loading pathway reveals a Na\(^+\)-linked acidification

The mechanistic basis of the progressive increase in [Na\(^+\)], seen on removal of K\(^+\) from the extracellular solution is unclear. In this study there was no further investigation of the pathway(s) involved; the phenomenon was simply used as a means of imposing an intracellular Na\(^+\) load on the parasite. The net Na\(^+\) influx induced upon K\(^+\) removal may be due to a Na\(^+\) loading pathway being *activated* upon removal of K\(^+\) and/or to a Na\(^+\) efflux pathway being *inhibited*. One possibility is that removal of K\(^+\) leads to *inhibition* of a Na\(^+\) extruding/K\(^+\) importing Na\(^+\)/K\(^+\)-ATPase; in the absence of extracellular K\(^+\) such a transport system could not function. However, as discussed above, there is no evidence for a Na\(^+\)/K\(^+\)-ATPase in the parasite. Another possibility is that when the parasite is suspended in the absence of
plasma membrane K⁺ channels become permeable to Na⁺, allowing it to enter the cell, down its electrochemical gradient. It has been shown that when normally highly K⁺-selective channels (for example, human Kv1.5 and rat Kv3) were expressed in a human embryonic kidney cell line, exposure to K⁺-free conditions rendered the channels permeable to other monovalent cations (Kiss et al., 1999; Wang et al., 2000). Both Na⁺ and NMDG⁺ have been shown to be permeable under certain experimental conditions, a phenomenon that is thought to be due to a change in structure of the channel’s K⁺ selectivity filter (Lockless et al., 2007; Wang et al., 2009). It is possible that a similar structural change occurs in parasite plasma membrane K⁺ channels when K⁺ is removed from the extracellular solution. It is important to note that although the SBFI dye is 20 x more selective for Na⁺ than K⁺ (Minta and Tsien, 1989), when [K⁺]ᵢ decreases considerably (as may occur during the Na⁺ loading procedure used here) there may be a slight underestimate of [Na⁺]ᵢ (Zhang et al., 1993).

In parasites subjected to the Na⁺- loading manoeuvre there was a cytosolic acidification (Fig. 3.65B) consistent with a net influx of H⁺. This could be due to an increase in the rate of H⁺ influx, and/or a decrease in the rate of H⁺ efflux. Under conditions of excess [Na⁺], the activity of the Na⁺-ATPase might be expected to increase in an attempt to maintain [Na⁺]ᵢ at the resting value of ~11 mM. The concomitant increased efflux of Na⁺ and net influx of H⁺ is consistent with Na⁺ transport being coupled to H⁺, a characteristic of NHEs and a proposed property of ENA-type Na⁺-ATPases (Rodriguez-Navarro and Benito, 2010). Another possibility is that the activity of the V-type H⁺-ATPase is reduced under the conditions of Na⁺ loading, such that it is unable to efflux sufficient H⁺ to maintain resting pHᵢ. A similar acidification on exposure to K⁺-free media has also been seen in T. gondii tachyzoites (in which there was a decrease in pHᵢ from ~7.07 under standard conditions to 7.02 pH units in K⁺-free solution (Moreno et al., 1998)) and in T. brucei (in which the pHᵢ was ~0.04 - 0.05 pH units lower in K⁺-free conditions in both bloodstream trypomastigotes and procyclic forms (Vanderheyden et al., 2000)). A proposed H⁺ coupling of the Na⁺-ATPase activity is the subject of Chapter 4.

When the effect of increasing [Na⁺]₀ above 125 mM was investigated (Fig. 3.5B), the response of [Na⁺]ᵢ to an increase in extracellular [KCl] was different from that seen
when the extracellular osmolarity was increased by the addition of either choline Cl or sucrose. In all three cases there was a fast initial increase in $[\text{Na}^+]_i$. However, whereas in the case of choline Cl or sucrose the increased $[\text{Na}^+]_i$ was maintained for the duration of the experiment, when KCl was added there was a rapid recovery of $[\text{Na}^+]_i$ toward the resting value (Fig. 3.5B). The initial increase in osmolarity is expected to have resulted in cell shrinkage, and this is the likely explanation for the initial increase in $[\text{Na}^+]_i$. The fact that the elevated $[\text{Na}^+]_i$ is maintained in the medium containing choline Cl or sucrose is consistent with there being little if any cell volume recovery under these conditions. The fact that $[\text{Na}^+]_i$ recovered rapidly in the presence of excess extracellular K$^+$ is consistent with the parasite either undergoing a rapid ‘Regulatory Volume Increase’ under these conditions and/or with the parasite facilitating the rapid efflux of Na$^+$ in the high [K$^+$] medium. One possibility is that the parasite has a Na$^+$/K$^+$-ATPase that is activated in response to the raised [K$^+$]o; however there is no obvious candidate for a type IIC, P-type Na$^+$/K$^+$-ATPase (Gardner et al., 2002) and the ouabain-insensitivity of $[\text{Na}^+]_i$ regulation under normal, resting conditions implies that under these conditions at least a Na$^+$/K$^+$-ATPase is unlikely to be involved. Alternatively, an increased efflux of Na$^+$ might be secondary to an effect of extracellular K$^+$ on the parasite membrane potential. The increase in [K$^+$]o will have caused a membrane depolarization (Allen and Kirk, 2004b), which may allow increased activity of a proposed Na$^+$ efflux pathway (providing it conducts a net efflux of positive charge). Both electrogenic and electroneutral P-type ATPases exist (examples including the SERCA Ca$^{2+}$-ATPase (2 Ca$^{2+}$ in exchange with 2-3 H$^+$ per ATP) and H$^+$/K$^+$-ATPase (2 H$^+$ in exchange with 2 K$^+$ per ATP) respectively) (Niggli and Sigel, 2008). The membrane potential sensitivity of Na$^+$ regulation has been investigated in Chapter 4.

3.3.3. A pump-leak balance for Na$^+$

The observation that, on inhibition of Na$^+$ extrusion with orthovanadate, $[\text{Na}^+]_i$ increased significantly, on a timescale of minutes is consistent with the intraerythrocytic malaria parasite having a substantial Na$^+$ influx pathway (‘leak’) which, under normal conditions, is countered by Na$^+$ efflux via the Na$^+$-ATPase. The identity of the influx pathways is unknown. The rate of Na$^+$ influx observed upon inhibition of the Na$^+$-ATPase using orthovanadate (~7.8 mM/min) was higher
than the rate of Na\(^+\) influx upon glucose deprivation or F\(^-\) addition (~0.4 and 1 mM/min respectively). The higher net influx of [Na\(^+\)] when the cells were exposed to F\(^-\) compared to when they were glucose deprived (Fig. 3.9B; though not significant), may be due to F\(^-\) having a direct inhibitory effect on the Na\(^+\) extrusion mechanism. F\(^-\) has over 70 reported enzymatic targets (Adamek \textit{et al.}, 2005), not just within the glycolytic pathway. In other systems F\(^-\) has been shown to inhibit several pumps, including Mg\(^{2+}\)-ATPases (Auland \textit{et al.}, 1994; Vani and Reddy, 2000), Na\(^+\)/K\(^+\)-ATPases (Murphy and Hoover, 1992; Vani and Reddy, 2000) and Ca\(^{2+}\)-ATPases (Missiaen \textit{et al.}, 1988).

The question remains of why the net Na\(^+\) influx measured in ATP-depleted parasites (i.e., parasites deprived of glucose or treated with F\(^-\)) was lower than that measured in cells treated with orthovanadate. One likely explanation is that the Na\(^+\) influx pathways are conductive and that the loss of the membrane potential in the ATP-depleted parasites (Allen and Kirk, 2004b) results in a lower rate of influx of Na\(^+\). Alternatively the influx pathways themselves might have some dependence on the ATP status of the parasite. It is possible that there may be an ATP-independent Na\(^+\) efflux mechanism in operation, or that there may be membrane-associated microdomains of ATP that could energise the Na\(^+\)-ATPase. ATP ‘compartmentalisation’ has been characterised in (uninfected) erythrocytes, in which there is an ATP/ADP pool “diffusonally restricted from bulk cell nucleotides” associated with membrane tethered glycolytic enzymes (Hoffman \textit{et al.}, 2009; Tiffert and Lew, 2011). This membrane-associated ATP pool is used to power the erythrocyte Ca\(^{2+}\)-ATPase and Na\(^+\)/K\(^+\)-ATPase (Hoffman \textit{et al.}, 2009). Therefore it is at least a theoretical possibility that the decreased Na\(^+\) influx upon glucose deprivation or F\(^-\) addition compared to orthovanadate addition may be due to the Na\(^+\) efflux mechanism still operating, albeit at a reduced rate.

The extrusion of Na\(^+\) which has entered the parasite via a Na\(^+\) leak represents a significant energy investment by the parasite. What, if any, purpose might be served by the parasite having a high background Na\(^+\) leak, and therefore having to utilise significant amounts of ATP in pumping Na\(^+\) out of the parasite is unclear. In many organisms ATPase activity associated with ion regulation accounts for a very high proportion of energy use. For example it has been estimated that 17 - 20 % of human...
daily whole body ATP expenditure is due to SERCA Ca\(^{2+}\)-ATPase activity (Norris et al., 2009), and in neurons, up to 70 % of the ATP usage is for the maintenance of ion gradients (Ivannikov et al., 2010). High Na\(^{+}\)-ATPase activity would be a substantial metabolic burden for the parasite, which generates ATP through glycolysis and not via the high-return of the tricarboxylic acid cycle coupled to oxidative phosphorylation (Olszewski et al., 2010).

One possibility is that the Na\(^{+}\) cycling is futile to keep the Na\(^{+}\)-ATPase in an active state. Futile cycling in biological systems (an overall net ATP consumption with the accompanying reactions appearing to ‘cancel out’) is common. Examples include the simultaneous operation of the key glycolytic enzyme phosphofructokinase and the opposing hexosediphosphatase (Nelson and Cox, 2008) or the simultaneous polymerization and degradation of polyhydroxyalkanoates (Ren et al., 2009). One hypothesis is that these futile cycles are used to regulate metabolic pathways rapidly, so that the cell can adapt very quickly to changes without the time constraints of potentially complex cascades of transcriptional regulation (Ren et al., 2009). Futile Na\(^{+}\) cycling has been observed previously across the rice root plasma membrane, under both resting and in salt-stressed conditions (Malagoli et al., 2008). In the parasite, a basal Na\(^{+}\)-ATPase activity may mean the transporter could respond quickly to changes in [Na\(^{+}\)], and alter its activity accordingly (an analogy being that it is easier to change a car from first to second gear rather than starting the car in the first place).

3.3.4. Molecular candidates for the Na\(^{+}\)-ATPase in *Plasmodium*

There has been limited functional characterisation of the P-type ATPase repertoire in *Plasmodium*, and although 16 P-type ATPases were originally annotated in the *P. falciparum* genome (Gardner et al., 2002) this was later re-evaluated to 13 members (Martin et al., 2005; Martin et al., 2009a). Whilst several of the P-type ATPase members are predicted to act as phospholipid or Cu\(^{2+}\) transporters, a number of candidates remain for the plasma membrane Na\(^{+}\)-ATPase activity observed in this study, including PfATPase1 (PFE0805w), PfATPase3 (PFE0195w), PfATP4 (PFL0590c) and two putative ‘cation-transporting P-ATPases’ (MAL13P1.246, PF07_0115). Bioinformatic information available suggests that not all are ideal
candidates; two (PF07_0115, PFE0805w) contain putative apicoplast targeting motifs (Martin et al., 2005), and PfATPase 3 and MAL13P1.246 are putative DV P-type ATPases (Martin et al., 2009a).

No ENA Na\(^+\)-ATPases were identified in the *Plasmodium* (or *Toxoplasma*) genome in a recent assessment of protozoal ENA transporters (Rodriguez-Navarro and Benito, 2010). However, as the ENA family shares sequence/structural similarities with both SERCA (class IIA) and PMCA (class IIB) Ca\(^{2+}\)-ATPases, in some cases ENA Na\(^+\)-ATPases have been initially misannotated as Ca\(^{2+}\)-ATPases (Benito et al., 2002; Rodriguez-Navarro and Benito, 2010). There are two candidate Ca\(^{2+}\)-ATPases annotated in the *P. falciparum* genome: non-SERCA-like PfATP4 (PFL0590c) and SERCA-like PfATP6 (PFA0310c). Although PfATP6 has not been directly localised, fluorescent inhibitor studies are consistent with it being localised to a "tubo-vesicular membranous network" homologous to the mammalian endoplasmic reticulum (Eckstein-Ludwig et al., 2003). It has recently been demonstrated that PfATP6 heterologously expressed in yeast binds Ca\(^{2+}\) and has Ca\(^{2+}\)-dependent ATPase activity (Arnou et al., 2011). Although there is also some evidence that the plasma membrane localised (Dyer et al., 1996; Rottmann et al., 2010) PfATP4 has Ca\(^{2+}\)-dependent ATPase activity (Krishna et al., 2001), this has not been confirmed (Rottmann et al., 2010). Therefore, of these two possibilities, PfATP4 is the stronger candidate as the putative ENA-type ATPase. The molecular nature of the Na\(^+\)-ATPase is explored in Chapter 5.
3.4. Conclusion

The extrusion of Na\(^+\) and the maintenance, thereby, of a low [Na\(^+\)], is a fundamental property of all cells. Together, the evidence presented in the chapter is consistent with the major mechanism of [Na\(^+\)]\(e\) extrusion from isolated *P. falciparum* parasites being a P-type Na\(^+\)-ATPase. This putative Na\(^+\)-ATPase is orthovanadate- and furosemide-sensitive, but insensitive to ouabain, a pharmacological profile shared by ENA type Na\(^+\)-ATPases of other protozoa. Additionally, a novel Na\(^+\) loading pathway (revealed on removal of extracellular K\(^+\)) was identified and utilised to load parasites with Na\(^+\) and thereby make direct measurements of net Na\(^+\) efflux from the parasite. The identity of this Na\(^+\) influx pathway remains unknown.
Chapter 4:

$H^+$-coupling and membrane potential dependence of the *P. falciparum* $Na^+$-ATPase
4.1. Declaration

The work presented in this chapter is all my own work. There are two instances (Fig. 4.5.A and 4.6.A) where the data presented is based on preliminary results included in my own Honours thesis (Spillman, 2007). However, the data shown here were generated as part of this current study.

4.2. Introduction

In Chapter 3, evidence was presented consistent with a P-type Na\(^+\)-ATPase playing the major role in Na\(^+\) regulation in *P. falciparum*. This ATPase was proposed to be of the ENA class, similar to that in operation in bryophytes, fungi and other protozoa (Rodriguez-Navarro and Benito, 2010). The ENA class of transporters has not been characterised in great detail. Typically, identification of an ENA-type Na\(^+\)-ATPase is based on bioinformatic sequence analysis, Na\(^+\)-dependent ATPase activity of membrane preparations showing sensitivity to orthovanadate and furosemide (but not ouabain), and the ability of the gene to complement the B31 mutant yeast strain deficient in Na\(^+\) efflux transporters (For examples see Benito and Rodriguez-Navarro, 2003; Iizumi *et al.*, 2006; Lunde *et al.*, 2007; Rodriguez-Navarro and Benito, 2010). ENA-type Na\(^+\)-ATPases are postulated to extrude Na\(^+\) (or K\(^+\) with varying specificities (Benito *et al.*, 2002)) in exchange for H\(^+\) entry (Rodriguez-Navarro and Benito, 2010), although this antiport has not been directly demonstrated. Acid-loading mechanisms are poorly characterised in *P. falciparum*, the one exception being the recent characterisation of a Cl\(^-\)-dependent acid-loading mechanism on the parasite plasma membrane, important for the accumulation of Cl\(^-\) (Henry *et al.*, 2010). The maintenance of resting pH\(_i\) is a balance between acid-loading transporters, and acid-extruders. The major acid-extruder of *P. falciparum* is the V-type H\(^+\)-ATPase on the parasite plasma membrane, which is essential for pH\(_i\) regulation (Saliba and Kirk, 1999) and which generates the large inwardly negative Δψ (Allen and Kirk, 2004b). Metabolically-generated H\(^+\) are removed in symport with lactate (Elliott *et al.*, 2001); thus the postulated role of the V-type H\(^+\)-ATPase is to remove H\(^-\) originating from other sources. These are likely to include H\(^+\)
produced metabolically (e.g. when acetate/succinate are produced as the end by-products of glycolysis and are not removed by the lactate/H+ symporter) H+ ions which enter the cell via H+-linked transporters (e.g. perhaps the Ca2+/H+ exchangers and/or the NHE (Ginsburg, 2002)). If the Na+ efflux of the ENA-type ATPase is coupled to H+ import, then these incoming H+ ions would have to be removed by the V-type H+-ATPase.

The transport stoichiometry of an ENA-type Na+-ATPase has not been determined, though modelling of the Physcomitrella PpENA onto the structure of rabbit SERCA Ca2+-ATPase suggested that binding two Na+ is energetically favourable (Drew et al., 2010). This is consistent with the model for transport in SERCA Ca2+-ATPases, in which two Ca2+ ions bind on the site facing the cytoplasm, and two/three H+ ions bind on the site facing the sarcoplasmic reticulum lumen. The bound H+ ions play a key role in the pump mechanism by partially neutralising the negative charge imbalance (from four carboxyl groups in the Ca2+ binding site) revealed after Ca2+ dissociation (Niggli and Sigel, 2008). This stabilises the protein structure and enables the transporter to return to the conformation in which the Ca2+-binding site faces the cytosol (Moller et al., 2010). For the SERCA Ca2+-ATPase, this stoichiometry means transport is always electrogenic (Niggli and Sigel, 2008), though as Ca2+ is divalent, whereas Na+ is only monovalent this would not always be true of ENA-type ATPases with the same stoichiometry (transport would be electroneutral if two H+ and two Na+ are transported).

In the work described in this chapter, pH- and Na+-sensitive dyes were used to investigate the H+ coupling and Δψ dependence of the Na+-ATPase postulated to be the primary Na+ extrusion mechanism in the parasite. The data are consistent with the activity of the Na+-ATPase imposing a significant acid load on the parasite, which is balanced by the activity of the V-type H+-ATPase. Additionally, resting [Na+], was shown to be Δψ dependent, though whether this was due to sensitivity of the Na+ influx or efflux pathways remains to be elucidated.
4.3. Results

4.3.1. Effect of orthovanadate on pH\(_i\)

As was shown in the preceding chapter, the P-type ATPase inhibitor orthovanadate caused a disruption of [Na\(^+\)]\(_i\) (Fig. 3.7E). Fig. 4.1 shows the effect of orthovanadate on resting pH\(_i\). In the presence of Na\(^+\), the addition of orthovanadate (100 \(\mu\)M) caused a significant alkalinisation from an average resting pH\(_i\) of 7.28 ± 0.03 to 7.36 ± 0.03 (n = 6; \(p = 0.024\)). By contrast, there was no alkalinisation on addition of orthovanadate to cells washed and suspended in Na\(^+\)-free medium (Fig. 4.1). It is important to note that this alkalinisation represents pH\(_i\) moving \textit{away} from the pH\(_o\) of 7.1 (i.e., the transmembrane pH gradient \textit{increases}).

![Figure 4.1](image_url)

**Figure 4.1.** Effect of orthovanadate on pH\(_i\) in saponin-isolated, BCECF loaded \(P. falciparum\) trophozoites suspended in the presence or absence of Na\(^+\). Parasites were washed and suspended in either standard saline (dark trace) or Na\(^+\)-free solution (in which Na\(^+\) was replaced isosmotically with choline\(^+\); light trace); orthovanadate (100 \(\mu\)M) was added at the timepoint indicated by the black triangle. The addition of orthovanadate induced an alkalinisation in the presence, but not the absence of extracellular Na\(^+\). The traces shown are representative of those obtained from at least three independent cell preparations.
4.3.2. Effect of altering $pH_0$ and $pH_i$ on $[Na^+]_i$

In light of the finding that the P-type ATPase inhibitor orthovanadate caused an increase in $pH_i$, experiments were carried out to investigate the relationship between the intra- and extracellular $pH$, and $[Na^+]_i$, in isolated parasites. On increasing $pH_0$ from 7.1 to 8.0, $[Na^+]_i$ increased within a few seconds by an average of $4.4 \pm 1.3$ mM ($n = 3$), followed by a slow recovery, with $[Na^+]_i$ decreasing to its initial resting value after approximately 10 min (Fig. 4.2A). When $pH_0$ was decreased from 7.1 to 6.0, $[Na^+]_i$ decreased by an average maximum of $4.3 \pm 0.5$ mM ($n = 3$) then, over time, recovered towards resting $[Na^+]_i$ (Fig. 4.2A). The concomitant perturbation of $pH_i$ when $pH_0$ was changed from 7.1 to 6.0/8.0 is shown in Fig. 4.2B. The initial changes in $[Na^+]_i$ and $pH_i$ both occurred rapidly when $pH_0$ was altered, however whereas $[Na^+]_i$ recovered towards resting $[Na^+]_i$, there was no recovery of $pH_i$.

The effect on $[Na^+]_i$ of changing $pH_i$ under conditions of constant $pH_0$ was also investigated, using NH$_4$Cl to elicit a transient intracellular alkalinisation (Saliba and Kirk, 1999), and lactate to elicit a transient intracellular acidification (Saliba and Kirk, 1999; Elliott et al., 2001). The addition of 40 mM NH$_4$Cl (sufficient to cause $pH_i$ to increase by $\sim$0.25 pH units; Fig. 4.2D) caused $[Na^+]_i$ to decrease by $1.7 \pm 1.1$ mM ($n = 3$) after 15 min, with little recovery back towards resting $[Na^+]_i$ on the timescale of the experiment (Fig. 4.2C). By contrast, upon addition of 40 mM lactate (sufficient to cause $pH_i$ to decrease by $\sim$0.3 pH units; Fig. 4.2D), $[Na^+]_i$ increased by a maximum of $10.5 \pm 1.4$ mM ($n = 3$) above the initial resting value, peaking $377 \pm 32$ s ($n = 3$) after lactate addition (Fig. 4.2C). After reaching this maximum value, $[Na^+]_i$ slowly recovered back towards the initial resting $[Na^+]_i$. Although the addition of both lactate and NH$_4^+$ caused a rapid change in $pH_i$, the effect on $[Na^+]_i$ was slower. In the case of lactate in particular, the maximum increase in $[Na^+]_i$ occurred at a time by which $pH_i$ had almost recovered to resting $pH_i$ (N.B. the timescales on panels Fig. 4.2C and D are identical).
Figure 4.2. Effect of altering pH\textsubscript{e} and pH\textsubscript{i} on [Na\textsuperscript{+}]\textsubscript{i} in saponin-isolated trophozoites. (A) and (B) show the effects on [Na\textsuperscript{+}]\textsubscript{i} (A) and pH\textsubscript{i} (B) of altering the extracellular pH. (C) and (D) show the effects on [Na\textsuperscript{+}]\textsubscript{i} (C) and pH\textsubscript{i} (D) of altering the intracellular pH, under conditions of constant extracellular pH. For the experiment represented in (A) and (B) SBFI-loaded (A) or BCECF-loaded (B) parasites in standard saline (pH = 7.1) were resuspended at the point indicated by the closed triangle in medium at pH = 6.0 (light traces) or 8.0 (black traces). For the experiment represented in (C) and (D) SBFI-loaded (C) or BCECF-loaded (D) parasites in standard saline were exposed at the point indicated by the closed arrow to either 40 mM NH\textsubscript{4}Cl (to elicit a transient alkalinisation; light traces) or 40 mM lactate (to elicit a transient acidification; black traces). The fluorescence data were corrected to account for the pH dependence of the SBFI dye as described previously (Diarra et al., 2001). The traces are, in each case, representative of those obtained from at least three independent cell preparations.
4.3.3. Effect of removal of Cl\(^-\) on [Na\(^+\)]\(_i\)

As has been reported previously (Henry et al., 2010), when extracellular Cl\(^-\) (132 mM in standard saline) was removed (replaced isosmotically with gluconate), there was a rapid alkalisation of the parasite cytosol (Fig. 4.3A). Restoration of Cl\(^-\) resulted in an acidification with pH\(_i\) returning to close to its resting value (Henry et al., 2010). As shown in Fig. 4.3A, upon removal of Cl\(^-\) from the medium there was a decrease in [Na\(^+\)]\(_i\) of 7.8 ± 2.2 mM (n = 3). The decrease in [Na\(^+\)]\(_i\) occurred over a similar timecourse to the alkalisation of pH\(_i\). When Cl\(^-\) was restored to the extracellular solution, by the addition of 40 mM NaCl, [Na\(^+\)]\(_i\) underwent a partial recovery towards the initial resting [Na\(^+\)]\(_i\).

![Graphs showing [Na\(^+\)]\(_i\) and pH\(_i\) changes](image)

**Figure 4.3.** The effect of [Cl\(^-\)]\(_o\) removal on [Na\(^+\)]\(_i\) in saponin-isolated *P. falciparum* trophozoites. (A) shows a [Na\(^+\)]\(_i\) trace from SBFI-loaded parasites and (B) shows a pH\(_i\) trace from BCECF-loaded parasites. In both panels, at the time-point indicated by the open triangle parasites (in standard saline) were resuspended in a Cl\(^-\)-free saline (in which Cl\(^-\) was replaced isosmotically with gluconate) which causes a rapid cytosolic alkalisation (Henry et al., 2010). At the timepoint indicated by the closed triangle, 40 mM Cl\(^-\) (as NaCl) was added to the suspension. Removal of extracellular Cl\(^-\) resulted in a decrease in [Na\(^+\)]\(_i\). The fluorescence data were corrected to account for the pH dependence of the SBFI dye as described previously (Diarra et al., 2001). The traces are representative of those obtained from three independent cell preparations.
4.3.4. Effect of pH$_0$ on recovery from a Na$^+$ load

The pH-sensitivity of Na$^+$ efflux from the parasite was investigated using the Na$^+$ loading technique described in Section 3.2.7. In the experiments represented in Fig. 4.4A cells were suspended in K$^+$-free medium, leading to an increase in [Na$^+$]$_i$. They were then resuspended (at time-zero) in media containing 10 mM KCl and with a pH of either 6.0, 7.1 or 8.0. The maximal rates of Na$^+$ efflux were calculated using Eqn. 3.1 (Section 3.2.7). At pH$_0$ = 6.0, the maximal rate of Na$^+$ efflux (6.9 ± 1.3 mM/min; n = 4) was significantly faster than at pH$_0$ 7.1 (3.6 ± 0.9 mM/min; n = 4; p = 0.011, paired t-test). Recovery at pH$_0$ = 8.0 had an initial ‘lag phase’ before the parasites recovered with a maximal rate of 2.9 ± 0.6 mM/min (n = 4), not significantly different from the rate at pH$_0$ 7.1 (p = 0.54, paired t-test). Regardless of pH$_0$, recovery still trended towards the initial resting [Na$^+$]. The concomitant perturbation of pH$_i$ when pH$_0$ was changed during the Na$^+$ efflux phase is shown in Fig.4.4B. The ‘lag phase’ of [Na$^+$]$_i$ recovery at pH$_0$ of 8.0 corresponds approximately to the time when there was a net efflux of H$^+$ from the parasite.

Figure 4.4. Effect of altering pH$_0$ on the efflux of Na$^+$ from saponin-isolated trophozoites following an imposed Na$^+$ load. Cells were loaded with Na$^+$ by suspension in K$^+$-free medium as illustrated in Fig. 3.6A. (A) and (B) show the effects on [Na$^+$]$_i$ (A) and pH$_i$ (B) of altering the extracellular pH during the efflux of Na$^+$ from Na$^+$ loaded trophozoites. For the experiment represented in (A) and (B) SBFI-loaded (A) or BCECF-loaded (B) parasites were suspended at time-zero in medium containing 10 mM KCl at pH$_0$ 6.0 (light trace), 7.1 (black trace) or 8.0 (red trace). The fluorescence data were corrected to account for the pH dependence of the SBFI dye as described previously (Diarra et al., 2001). The traces are, in each case, representative of those obtained from at least four independent cell preparations.
4.3.5. Identification of a Na\(^+\)-dependent acidification mechanism

Inhibition of the plasma membrane V-type H\(^+\)-ATPase with concanamycin A in cells suspended at pH\(_o\) 7.1 results in a cytosolic acidification to pH \(\sim 6.8\) (Saliba and Kirk, 1999). In earlier experiments, pre-dating the work on this thesis, it was shown that when Na\(^+\) was removed (replaced isosmotically with choline\(^+/\)K\(^+\) or NMDG\(^+\)) and the V-type H\(^+\)-ATPase was inhibited, the acidification was not as pronounced (Spillman, 2007). These data are shown in Fig. 4.5.1. In parasites suspended in Na\(^+\)-containing solution, the addition of concanamycin A caused the parasite cytosol to acidify to a final value of 6.75 ± 0.02 pH units (n = 4). By contrast, for parasites suspended in Na\(^+\)-free solution there was significantly less acidification, with the addition of concanamycin A causing pH\(_i\) to decrease to a value of 7.09 ± 0.03 (n=4; p = 0.0001), essentially the same as the extracellular pH (7.1). The initial rate of acidification upon addition of concanamycin A was significantly faster in the presence of Na\(^+\) (2.8 ± 0.5 \(\times\) \(10^-3\) pH units/s; n = 4) than in its absence (1.0 ± 0.3 \(\times\) \(10^-3\) pH units/s; n = 4; p = 0.02).

One hypothesis that might explain the Na\(^+\)-dependent acidification observed in Fig. 4.5.1 is that the efflux of Na\(^+\) by the putative Na\(^+\)-ATPase is coupled to an influx of H\(^+\) and it is this that drives pH\(_i\) below pH\(_o\) following inhibition of the H\(^+\)-extruding V-type H\(^-\)-ATPase. According to this hypothesis, under Na\(^+\)-free conditions (when [Na\(^+\)] ~ 0 mM; Section 3.2.6) the Na\(^+\)-ATPase is not operating and inhibition of the plasma membrane V-type H\(^+\)-ATPase results in pH\(_i\) simply equilibrating with pH\(_o\) (i.e., pH 7.1).

To investigate this hypothesis, the effect of concanamycin A on pH\(_i\) was tested in parasites suspended in Na\(^+\)-containing medium in the presence of orthovanadate, an inhibitor of the putative Na\(^+\)-ATPase. As seen previously (Fig. 4.1), on addition of orthovanadate to parasites suspended in the presence of Na\(^+\) there was a cytosolic alkalisation (Fig. 4.5B). Subsequent addition of concanamycin A to the orthovanadate-treated parasites (in the presence of Na\(^+\)) resulted in a decrease in pH\(_i\) to 7.09 ± 0.03 pH units (n = 3); this was not significantly different from the acidification seen in response to the addition of concanamycin A to parasites suspended in the absence of Na\(^+\) (p = 0.97). The initial rate of acidification observed
Figure 4.5. Acidification of isolated parasites via a Na⁺-dependent, orthovanadate-inhibited mechanism, revealed on inhibition of the plasma membrane V-type H⁺-ATPase with concanamycin A. pHᵢ was measured in saponin-isolated, BCECF loaded *P. falciparum* trophozoites. (A) Parasites were suspended in standard saline (Na⁺-containing, dark trace) or in a Na⁺-free solution (in which Na⁺ was replaced isosmotically with choline⁺; light trace). Concanamycin A (75 nM) was added at the timepoint indicated by the black triangle. The magnitude and the rate of the subsequent acidification was greater in the Na⁺-containing medium than in the Na⁺-free medium. The pHᵢ of 7.1 is indicated by the dashed reference line. (B) Orthovanadate (100 μM) was added to parasites suspended in standard (Na⁺-containing) saline at the timepoint indicated by the open triangle. The V-type H⁺-ATPase inhibitor concanamycin A (75 nM) was added at the timepoint indicated by the black triangle. The extracellular pH of 7.1 is indicated by the dashed reference line. The traces shown are representative of those obtained from at least three independent cell preparations.
following the addition of concanamycin A to orthovanadate-treated parasites was $1.6 \pm 0.2 \times 10^3$ pH units/s ($n = 3$). This was significantly slower than the initial rate of acidification seen for parasites suspended in Na$^+$-containing medium in the absence of orthovanadate (paired t-test; $p = 0.02$), but not significantly different from the initial rate of acidification in Na$^+$-free conditions (paired t-test; $p = 0.35$). These results are consistent with the Na$^+$-dependent acidification being due to a substantial H$^+$ load being imposed on the cell by the putative Na$^+$-ATPase. This load is countered by the plasma membrane V-type H$^+$-ATPase under physiological conditions, but results in a marked acidification of the parasite cytosol when the V-type H$^+$-ATPase is inhibited by concanamycin A.

### 4.3.6. The Na$^+$-dependent acidification mechanism operates across a wide range of pH values

In other experiments pre-dating this study, it was shown that when isolated parasites were acidified using an NH$_4^+$-pre-pulse (the addition then subsequent removal of NH$_4$Cl) and then treated with the V-type H$^+$-ATPase concanamycin A the pH$_i$ response varied with the ionic composition of the extracellular medium. For parasites in a Na$^+$-containing medium, pH$_i$ remained stable (at the imposed acidic value); by contrast, for parasites in a Na$^+$-free medium pH$_i$ underwent a slow recovery, towards the pH$_o$ of 7.1. Fig. 4.6A illustrates this phenomenon. The data are consistent with there being a Na$^+$-dependent acidification mechanism which operates in the acidified parasite. It maintains the acidic pH$_i$ in parasites in Na$^+$-containing medium but is inactive (allowing pH$_i$ to recover) in parasites in Na$^+$-free medium.

Evidence for the operation of a Na$^+$-dependent acidification mechanism was also observed at alkaline pH values. Fig. 4.6B shows the alkalinisation of the parasite cytosol after removal of extracellular Cl$^-$ (by replacement with gluconate; see Section 4.3.3). In the presence of Na$^+$, the parasites began to acidify back towards resting pH$_i$, with a rate of $6.8 \pm 0.6 \times 10^3$ pH units/min ($n = 3$). In the absence of Na$^+$, pH$_i$ recovered at a significantly lower rate: $2.8 \pm 0.7 \times 10^3$ pH units/min ($n = 3$; $p = 0.046$).
Figure 4.6. Evidence for a Na⁺-dependent acidification mechanism that operates at both low (A) and high (B) pHᵢ values. (A) Saponin-isolated, BCECF-loaded parasites were acidified by the addition (black triangle) then removal (white triangle) of 40 mM NH₄Cl (i.e., an “NH₄⁺ pre-pulse”) then resuspended in either standard (Na⁺-containing) saline (pH 7.1; dark trace) or Na⁺-free medium (in which Na⁺ was replaced isosmotically with NMDG⁺; pH 7.1; light trace), both containing the V-type H⁺-pump inhibitor concanamycin A (75 nM). For parasites in Na⁺-containing medium the acidic pHᵢ persisted, whereas for parasites in Na⁺-free medium the parasites underwent a gradual alkalinisation, with pHᵢ increasing towards pH₀. (B) Saponin isolated, BCECF-loaded parasites were suspended in standard saline and at the timepoint indicated by the red triangle the parasites were resuspended in Cl⁻-free medium in which Cl⁻ was replaced with gluconate, added as either the Na⁺ (dark trace) or K⁺ (light trace) salt. On replacement of Cl⁻ with gluconate there was a rapid cytosolic acidification, followed by a recovery towards resting pHᵢ, with the recovery occurring more rapidly in the presence of Na⁺ than in its absence. Traces are representative of those obtained in at least three independent cell preparations.
Thus, there is evidence for a Na\(^+\)-dependent acid-loading mechanism that operates in the parasite under conditions of physiological pH\(_i\), and under both acidic and alkaline (pH\(_i\)) conditions.

### 4.3.7. The \(\Delta \Psi\) sensitivity of resting [Na\(^+\)]\(_i\)

The membrane potential (\(\Delta \Psi\)) of isolated malaria parasites can be modulated by varying \([K^+]_o\); increasing \([K^+]_o\) causes a membrane depolarisation (i.e., \(\Delta \Psi\) becomes more positive) whereas decreasing \([K^+]_o\) causes a membrane hyperpolarisation (i.e., \(\Delta \Psi\) becomes more negative) (Allen and Kirk, 2004b). The effect of \(\Delta \Psi\) on Na\(^+\) regulation in the parasite was investigated by replacing 25 %, 50 %, 75 % and 100 % of the 125 mM Na\(^+\) present in standard saline (Table 2.1) with either K\(^+\) (which depolarises the membrane) or the inert monovalent cations choline\(^+\) or NMDG\(^+\) (which do not) to give a final [Na\(^+\)]\(_o\) of 94, 63, 31 or 0 mM, respectively.

As [Na\(^+\)]\(_o\) decreased, so too did resting [Na\(^+\)]\(_i\); (Fig. 4.7). At each of the different Na\(^+\) concentrations tested, the [Na\(^+\)]\(_i\); when choline\(^+\) was used as the replacement cation was the same as that when NMDG\(^+\) was used as the replacement cation (Fig. 4.7; \(p > 0.44\)). By contrast, when Na\(^+\) was replaced by K\(^+\), the [Na\(^+\)]\(_i\); was lower than in cells at the same [Na\(^+\)]\(_o\), but in which either choline\(^+\) or NMDG\(^+\) was the replacement cation. For cells in which 25 % and 50 % of the Na\(^+\) was replaced this difference was statistically significant (repeated measures one-way ANOVA using Tukey post test; \(p = 0.003\) and 0.005 respectively), whereas for cells in which 75 % of the Na\(^+\) was replaced the [Na\(^+\)]\(_i\); was again lower in the K\(^+\) medium than in either the choline\(^+\) or NMDG\(^+\) medium, but not significantly so. In the cells exposed to the high extracellular K\(^+\) concentrations the cells were depolarised relative to the cells in the choline\(^+\) or NMDG\(^+\) media. It should be noted that in cells suspended in medium in which Na\(^+\) was replaced with either choline or K\(^+\) the pH\(_i\) was not significantly different (data not shown); the differences shown in Fig. 4.7 are therefore not due to difference in pH\(_i\) but are consistent with resting [Na\(^+\)]\(_i\); being \(\Delta \Psi\)-dependent, decreasing as \(\Delta \Psi\) becomes less negative.
As is illustrated in Fig. 4.7, and as described in Section 3.2.6, there was no statistically significant difference between the $[\text{Na}^+]_i$ in the three different Na$^+$-replacement solutions under Na$^-$-free conditions (100 % replacement).

Figure 4.7. Effect on $[\text{Na}^+]_i$ of replacing extracellular Na$^+$ with either K$^+$ (black bars), choline$^+$ (grey bars) or NMDG$^+$ (red bars). Saponin-isolated, SBFI-loaded trophozoites were suspended for 15 min in the solution of interest before $[\text{Na}^+]_i$ was measured. The extracellular $[\text{Na}^-]$ was as indicated in brackets. The asterisks indicate statistical significance (using a repeated measures one-way ANOVA with Tukey post test) between $[\text{Na}^+]_i$ measured in cells in the K$^+$ solution and $[\text{Na}^+]_i$ measured in cells in the NMDG$^+$ or choline$^+$ solutions. Each data point is the average $[\text{Na}^+]_i$ obtained in at least four independent cell preparations.
4.3.8. The effect on $[\text{Na}^+]_i$ of compounds that cause a membrane depolarisation

Concanamycin A and CCCP both cause a depolarisation of $\Delta \Psi$ (Allen and Kirk, 2004b); concanamycin A does so through inhibition of the V-type H$^+$-ATPase and CCCP, a protonophore, does so by dissipating the transmembrane H$^+$-gradient. In addition to its effect on $\Delta \Psi$, concanamycin A causes pH$_i$ to decrease to a final value of ~6.8 (Fig. 4.5A). CCCP is a H$^+$ protonophore and following its addition pH$_i$ equilibrates with pH$_o$ (i.e., 7.1; see Fig. 6.4).

On addition of concanamycin A there was a small, transient decrease in $[\text{Na}^+]_i$ followed by a net, time-dependent increase in $[\text{Na}^+]_i$ (Fig. 4.8A). The maximal decrease in $[\text{Na}^+]_i$, occurring 312 ± 34 s (n = 4) after the addition of concanamycin A was 2.4 ± 0.4 mM (n = 8). The ensuing Na$^+$ influx (following the transient efflux) was approximately linear with time and occurred at a rate of 0.4 ± 0.1 mM/ min (n = 8). A similar small, transient decrease in $[\text{Na}^+]_i$, followed by a net Na$^+$ influx, was observed for CCCP (Fig. 4.8B).

A third compound found to have a very similar effect on $[\text{Na}^+]_i$, as concanamycin A and CCCP was clotrimazole (Fig. 4.8C). Clotrimazole is a potent antifungal agent that has been shown previously to inhibit the in vitro growth of blood-stage $P. falciparum$ parasites (Saliba and Kirk, 1998; Tiffert et al., 2000) and which, in unpublished experiments by Dr R.J.W. Allen, has been shown to cause a depolarisation of mature trophozoite-stage $P. falciparum$ parasites (R.J.W. Allen and K. Kirk, unpublished). The effect of clotrimazole (50 μM) on pH$_i$ was investigated, and the compound was shown to cause pH$_i$ to decrease from its normal resting value (of pH 7.3) to 6.76 ± 0.05 (n = 6) under the conditions of the experiment illustrated in Fig. 4.8C.

In summary, three compounds, all known to cause a depolarisation of the parasite plasma membrane were shown to cause a transient decrease in $[\text{Na}^+]_i$ (i.e., to cause the transmembrane $[\text{Na}^+]$ gradient to increase) which was followed by a time-dependent increase in $[\text{Na}^+]_i$.
Figure 4.8. Effect on $[\text{Na}^+]_i$ of three compounds known to cause a membrane depolarisation in saponin-isolated, SBFI-loaded *P. falciparum* trophozoites. The compounds - (A) concanamycin A (75 nM), (B) CCCP (10 μM) and (C) clotrimazole (50 μM) - were added at the time-points indicated by the closed triangle. The traces shown are, in each case, representative of those obtained from at least three independent cell preparations.
4.3.9. The effect of compounds that cause a membrane depolarisation on the net efflux of Na⁺ following an imposed Na⁺ load

The Na⁺ loading manoeuvre described in Section 3.2.7. (i.e., the removal then restoration of extracellular K⁺) was used to impose a ‘Na⁺ load’ on the parasite, and thereby test the effect on Na⁺ extrusion of compounds known to cause a membrane depolarisation. When concanamycin A was present during recovery from a Na⁺ load (Fig. 4.9A), there was a rapid recovery to resting levels with an initial rate of 11.6 ± 2.8 mM/min (n = 4), significantly faster than that observed in the absence of the inhibitor (3.9 ± 0.2 mM/min; n = 4; p = 0.035). In the concanamycin A treated cells there was, following the initial net efflux, a progressive increase in [Na⁺]ᵢ at a rate of 1.7 ± 1.1 mM/min (n = 5). This was not significantly different from the rate of influx of Na⁺ when concanamycin A was added to parasites under resting conditions (Section 4.3.8; p = 0.15).

Similar results were observed for CCCP (Fig. 4.9B). When CCCP was present during the recovery, the rate of efflux of Na⁺ (11.4 ± 8.3 mM/min; n = 3) was significantly higher than that under control conditions, and not significantly different from the efflux rate observed in the presence of concanamycin A (p = 0.98). As was seen in the presence of concanamycin A, following the initial net efflux, there was a progressive increase in [Na⁺]ᵢ at a rate of 0.4 ± 0.04 mM/min (n = 3), not significantly different from the rate of increase seen in the presence of concanamycin A (p = 0.41).
Figure 4.9. Effect on the recovery of $[\text{Na}^+]_i$ from an imposed $\text{Na}^+$ load of compounds known to cause a membrane depolarisation in saponin-isolated $P. falciparum$ trophozoites. SBFI-loaded parasites were subjected to a $\text{Na}^+$ load (by suspension in $\text{K}^+$-free medium), as illustrated in Fig. 3.6A. The traces commenced with the addition to the suspension of 10 mM KCl either with (light traces) or without (dark traces) (A) concanamycin A (75 nM) or (B) CCCP (10 μM). The traces shown are representative of those obtained from at least three independent cell preparations.
4.4. Discussion

Work presented in the previous chapter led to the hypothesis that Na⁺ extrusion from the intraerythrocytic *P. falciparum* trophozoite is via an ENA-type Na⁺-ATPase. It has been predicted previously that ENA Na⁺-ATPases mediate the exchange of one cation for another (Rodriguez-Navarro and Benito). Ion counter-transport has been proposed to be mandatory in all P-type ATPases (Niggli and Sigel, 2008), and only one exception to this has been identified so far, in a H⁺-ATPase from *A. thaliana* (Pedersen et al., 2007). The results presented in this chapter are consistent with the hypothesis that the putative *P. falciparum* ENA-type Na⁺-ATPase mediates the efflux of Na⁺, with the counter-transport of H⁺.

4.4.1. Orthovanadate-induced, Na⁺-dependent alkalinisation of the parasite

The increase in pHᵢ seen on addition of orthovanadate to parasites suspended in the presence (but not in the absence) of extracellular Na⁺ is consistent with the presence in the parasite plasma membrane of a Na⁺-ATPase that extrudes Na⁺ in exchange for H⁺. The putative influx of H⁺ via the Na⁺-ATPase would constitute a significant ‘acid load’ on the parasite; inhibition of the Na⁺-ATPase and the consequent elimination of this acid load would result in a cytosolic alkalinisation, as was observed (Fig. 4.1). In cells suspended in Na⁺ free medium [Na⁺]ᵢ is reduced to close to zero (Fig. 3.5), the Na⁺-ATPase no longer functions, there is therefore no Na⁺-ATPase-associate H⁺ load, and no alkalinisation on addition of orthovanadate (Fig. 4.1).

4.4.2. Na⁺ efflux from *P. falciparum* trophozoites is pH-dependent

Resting [Na⁺]ᵢ in the parasite was shown to be pH-sensitive (Figs 4.2 and 4.3). Decreasing pHₒ caused [Na⁺]ᵢ to decrease whereas decreasing pHᵢ whilst leaving pHₒ unchanged caused [Na⁺]ᵢ to increase. Conversely increasing pHₒ caused [Na⁺]ᵢ to increase whereas increasing pHᵢ whilst leaving pHₒ unchanged caused [Na⁺]ᵢ to decrease.

The effects of these different manoeuvres on [Na⁺]ᵢ may be understood in terms of their effect on the transmembrane H⁺ gradient and, consequently, on the overall
driving forces for transport via a Na\(^+\)/H\(^+\) counter-transport mechanism (as the parasite’s putative ENA-type Na\(^+\)-ATPase is proposed to be). However, there are also small changes in ΔΨ when the pH is altered; an extracellular alkalisation results in an increase in ΔΨ (hyperpolarisation), an extracellular acidification results in a decrease in ΔΨ (depolarisation) and vice versa for intracellular pH changes (Allen and Kirk, 2004b). Additionally, it is possible that the effect of changing the H\(^+\) gradient directly alters the ATPase activity (for example modifies H\(^+\) loading onto the ATPase).

Under normal resting conditions there was a modest inward H\(^+\) gradient across the parasite plasma membrane (pH\(_o\) = 7.1 and pH\(_i\) ~ 7.3). Decreasing pH\(_i\) with lactate reversed this gradient (pH\(_o\) = 7.1 and pH\(_i\) ~ 6.9); there was now an outward H\(^+\) gradient and therefore reduced driving force for the overall Na\(^+\) efflux/H\(^+\) influx counter-transport process. The ATPase-mediated Na\(^+\)/H\(^+\) counter-transport mechanism was now less effective at extruding Na\(^+\) and [Na\(^+\)]\(_i\), therefore increased. Conversely increasing pH\(_i\), either with NH\(_4\)\(^+\) (Fig. 4.2) or by replacing extracellular Cl\(^-\) with gluconate (Fig. 4.3) increased the inward H\(^+\) gradient (immediately following the addition of NH\(_4\)\(^+\) pH\(_o\) = 7.1 and pH\(_i\) ~ 7.4 (Fig. 4.2D); on replacement of extracellular Cl\(^-\) with gluconate pH\(_o\) = 7.1 and pH\(_i\) ~ 7.8 (Fig. 4.3B and Henry et al., 2010)) there was now an increased inward H\(^+\) gradient and therefore increased driving force for the overall Na\(^+\) efflux/H\(^+\) influx counter-transport process. The ATPase-mediated Na\(^+\)/H\(^+\) counter-transport mechanism was now more effective at extruding Na\(^+\) and [Na\(^+\)]\(_i\), therefore decreased. Note that the degree of alkalinisation, and hence the magnitude of the increase in the inward H\(^+\) gradient, was greater in the Cl\(^-\) replacement experiment than in the NH\(_4\)\(^+\) addition experiment, and this was reflected in the observation that the decrease in [Na\(^+\)]\(_i\) was greater following Cl\(^-\) removal than following NH\(_4\)\(^+\) addition (~2 vs 8 mM, respectively).

When pH\(_o\) was decreased and the inward H\(^+\) gradient thereby increased, the efficiency of the ATPase-mediated Na\(^+\)/H\(^+\) counter-transport mechanism was increased, resulting in a decrease in [Na\(^+\)]\(_i\) (Fig. 4.2). When pH\(_o\) was increased the inward H\(^+\) gradient was decreased, the efficiency of the ATPase-mediated Na\(^+\)/H\(^+\) counter-transport mechanism was decreased, resulting in a decrease in [Na\(^+\)]\(_i\).
Just as \([\text{Na}^+]_i\) varied in a systematic manner with the transmembrane \(H^+\) gradient, the rate of recovery of \([\text{Na}^+]_i\) following an imposed \(\text{Na}^+\) load varied with \(pH_0\), increasing significantly when \(pH_0\) was decreased from 7.1 to 6.0, as might be expected for a transport process involving transport of \(H^+\) from the extracellular solution, coupled to the transport of \(\text{Na}^+\) from the parasite cytosol. The basis of the somewhat complex \(\text{Na}^+\) efflux profile seen for cells suspended in media at \(pH\) 8.0 (i.e., a lag phase, followed by a net \(\text{Na}^+\) efflux that occurred at a similar rate to that seen under control conditions) was not investigated further here.

Although the data presented here may be accounted for by the hypothesis that \(\text{Na}^+\) extrusion from the parasite is via an ENA-type \(\text{Na}^+\)-ATPase that effluxes \(\text{Na}^+\) and counter-transport \(H^+\) ions the possibility should also be considered that the \(pH\) sensitivity of \([\text{Na}^+]_i\), regulation observed here may be due, at least in part, to the operation of a plasma membrane \(\text{NHE}\) acting to extrude \(\text{Na}^+\) using the energy provided by an influx of \(H^+\) down its electrochemical gradient. The candidate \(P. falciparum\) \(\text{NHE}\), PfNHE is a member of the NhaP1/SOS family of \(\text{NHE}\) (Section 1.6.3.5 and Brett et al., 2005). \(\text{NHE}\) of the NhaP1/SOS family are active at acidic extracellular \(pH\) values, and inactive at alkaline extracellular \(pH\) values (Goswami et al., 2010). Although this would explain the \(\text{Na}^+\)-dependent acidification in operation upon cytosolic acidification after V-type \(H^+\)-ATPase inhibition (Fig. 4.5A and 4.6A), it is not consistent with the \(\text{Na}^+\)-dependent acidification seen under alkaline conditions (Fig. 4.6B). By contrast, an ATP-driven primary active transport \(\text{Na}^+\)-ATPase might be expected to be operational under both acidic and alkaline conditions.

### 4.4.3. An answer to the enigma of ‘abundant \(H^+\)-pumping’ in \(P. falciparum\)

In 2002, Hagai Ginsburg posed the question: if all of the \(H^+\) generated by glycolysis are removed by the lactate/\(H^+\) symporter, then why does the V-type \(H^+\)-ATPase have such a high activity (Ginsburg, 2002)? This question was summarised in the title of his paper ‘Abundant proton pumping in \(Plasmodium falciparum\), but why?’. As evident in Fig. 4.5 and 4.6, there is a significant \(\text{Na}^+\)-dependent acidification (i.e., \(H^+\) influx), imposing a large \(H^+\) burden on the parasite, and it is the role of the V-type \(H^+\)-ATPase to counter the influx of \(H^+\) via this route and thereby maintain a steady
resting pH. The finding that this Na\(^+\)-dependent acidification was inhibited by orthovanadate (Fig. 4.5B) is consistent with it being due to the putative Na\(^+\)-ATPase. Thus, there is ‘abundant proton pumping in *P. falciparum*’ in order to efflux H\(^+\) that have entered the cell via the H\(^+\) counter-transporting Na\(^+\)-ATPase activity.

The possibility of the futile cycling of Na\(^+\) was introduced in Chapter 3, and the energetic burden of any such cycling on the parasite was noted. The fact that (energy-requiring) Na\(^+\) efflux is accompanied by the influx of H\(^+\) ions, which then themselves have to be extruded via an energy-requiring process (the V-type H\(^+\)-ATPase) means that the parasite needs to expend substantial amounts of ATP on controlling [Na\(^+\)].

### 4.4.4. [Na\(^+\)]\(_i\) regulation is ΔΨ-sensitive in *P. falciparum*

Fluorescent [Na\(^+\)], indicators such as SBFI allow the detection of net increases or decreases in [Na\(^+\)]. A net decrease in [Na\(^+\)]\(_i\) can be due to either an increase in Na\(^+\) efflux, or a decrease in Na\(^+\) influx. Conversely, a net increase in [Na\(^+\)]\(_i\) can be due to either a decrease in Na\(^+\) efflux, or an increase in Na\(^+\) influx.

The net (transient) decrease in [Na\(^+\)]\(_i\), seen to occur in response to agents that cause a plasma membrane depolarisation (Fig. 4.8) could be due to either an increase in Na\(^+\) efflux, or a decrease in Na\(^+\) influx. An increase in Na\(^+\) efflux with membrane depolarisation might arise if the Na\(^+\) efflux process is electrogenic, with the Na\(^+\)-ATPase carrying a net outward positive charge, such that in each transport cycle the number of Na\(^+\) ions extruded exceeds the number of H\(^+\) ions imported. Considering the previous report that two Na\(^+\) may bind to the *Physcomitrella* ENA-type ATPase (Drew *et al.*, 2010), this would lead to a predicted stoichiometry here of 2 Na\(^+\)/ 1 H\(^+\).

An alternative stoichiometry of 3 Na\(^+\)/ 2 H\(^+\) is also plausible, bearing in mind that the related electrogenic Na\(^+\)/K\(^+\)-ATPase operates with a stoichiometry of 3 Na\(^+\)/ 2 K\(^+\).

Alternatively, or perhaps additionally, the reduction in [Na\(^+\)]\(_i\), seen on addition of compounds that cause a membrane depolarisation might be due to reduced Na\(^+\) influx. With ΔΨ decreased from its normal resting value of -95 mV (Allen & Kirk) there is a reduced driving force for the entry of Na\(^+\) via conductive pathways.
Whether such pathways are present in the parasite, and form a significant part of the (large) inward Na⁺ leak, is not clear.

Similar considerations apply both to the observed variation of [Na⁺] that was seen to occur when Δψ was altered by changing the extracellular [K⁺], and to the Δψ-sensitivity of net Na⁺ efflux, as revealed using the K⁺-free Na⁺ loading manoeuvre to load the cells with Na⁺, and then observe Na⁺ recovery in the presence and absence of the depolarising agents concanamycin A and CCCP. Both agents caused the rate of recovery from a Na⁺ load to increase. This is consistent with greater Na⁺-ATPase activity under depolarising conditions, as might be expected of an electrogenic transporter. However, a reduced influx of Na⁺ via conductive pathways could also have caused this result.

The net Na⁺ influx that was seen to follow the transient decrease in [Na⁺], following the addition of membrane depolarising agents (Fig. 4.8) might be due to the concomitant cytosolic acidification (observed for concanamycin A and CCCP in Fig. 4.5A and 6.4 respectively, and measured for clotrimazole in Section 4.3.8). The changes in pH; result in a reduction, or reversal, of the inward H⁺ gradient which would be expected to lead to an increase in [Na⁺]; (see Section 4.3.2).

The H⁺ coupling of the ENA-type ATPase is likely to vary based on the available ions and temperature. For PMCA Ca²⁺-ATPases the H⁺-antiport stoichiometry varies between 1-2 (Niggli et al., 1982; Hao et al., 1994), and for SERCA Ca²⁺-ATPase varies between 2-3 (Yu et al., 1993; Yu et al., 1994). Increasing the temperature decreases the H⁺/Ca²⁺ stoichiometry, and this is proposed to be due to ATPase slippage, a phenomenon whereby ATP hydrolysis occurs in the absence of ion translocation (Hao et al., 1994). It remains to be tested whether environmental stimuli, like temperature changes, could alter the H⁺ coupling in the putative P. falciparum ENA-type Na⁺-ATPase.
4.5. Conclusion

The data presented in this chapter are consistent with the efflux of Na\(^+\) from saponin-isolated *P. falciparum* parasites being via a H\(^+\) coupled P-type Na\(^+\)-ATPase. The observation of a Na\(^+\)-dependent alkalinisation of the parasite cytosol following the addition of the P-type ATPase inhibitor orthovanadate provides support for this hypothesis. In experiments in which pH\(_i\) and pH\(_o\) were altered, [Na\(^+\)]\(_i\) varied systematically with the transmembrane H\(^+\) gradient, as expected for a H\(^+\) coupled Na\(^+\) transport mechanism.

The experiments reported here revealed the presence in the parasite of a large Na\(^+\)-dependent acid loading mechanism, postulated to be the H\(^+\) coupled Na\(^+\)-ATPase. The influx of H\(^+\) via this mechanism is normally countered by the V-type H\(^+\)-ATPase which thereby maintains a steady resting pH\(_i\). However on inhibition of the V-type H\(^+\)-ATPase with concanamycin A the acid loading mechanism is revealed. The H\(^+\) coupling of the Na\(^+\)-ATPase explains the need for ‘abundant H\(^+\) pumping’ by the V-type H\(^+\)-ATPase (Ginsburg, 2002), but does mean that the energy cost of [Na\(^+\)]\(_i\) regulation in the parasite is likely to be very high.

Finally, the resting [Na\(^+\)]\(_i\), as well as the net efflux of Na\(^+\) from Na\(^+\) loaded parasites, was shown to vary in response to manoeuvres that caused a change in Δψ. Whether this was due to the Δψ-sensitivity of Na\(^+\) influx or Na\(^+\) efflux was not determined, though the data are consistent with the *P. falciparum* Na\(^+\)-ATPase mediating a net efflux of positive charge.
Chapter 5:

PfATP4 as a candidate Na\(^+\)-ATPase and emerging drug target
5.1. Introduction

Antimalarial drug resistance is widespread and there is an urgent need to develop new antimalarial therapies. The spiroindolones (Yeung et al., 2010) are a new class of antimalarial with a low-nanomolar activity against blood-stage \( P. falciparum \) and \( P. vivax \) (Rottmann et al., 2010) parasites. Mutations in a P-type cation ATPase (PfATP4; PFL0590c) were shown to confer resistance to the spiroindolones (Rottmann et al., 2010); however the physiological role of PfATP4 is not well characterised and although there is some evidence that it has \( \text{Ca}^{2+} \)-dependent ATPase activity (Krishna et al., 2001) this has not been confirmed (Rottmann et al., 2010). PfATP4 is one of seven putative ion-transporting P-type ATPases encoded by \( P. falciparum \) (Martin et al., 2005; Martin et al., 2009a). It is expressed throughout the intraerythrocytic lifecycle and is localized to the parasite plasma membrane (Dyer et al., 1996; Rottmann et al., 2010).

In Chapter 3, evidence was presented that a P-type, \( \text{Na}^{+} \)-ATPase plays a major role in \( \text{Na}^{+} \) regulation in \( P. falciparum \). Evidence presented in Chapter 4 indicates that the flux of \( \text{Na}^{+} \) via this transporter is membrane potential sensitive, and that the translocation of \( \text{Na}^{+} \) is coupled to the antiport of \( \text{H}^{+} \). Such a \( \text{Na}^{+}/\text{H}^{+} \) antiport mechanism has been proposed for the ENA (IID) class of \( \text{Na}^{+} \)-ATPases (Rodriguez-Navarro and Benito, 2010), employed by some lower eukaryotes, including other protozoa, to extrude \( \text{Na}^{+} \) and thereby maintain a low \( [\text{Na}^{+}] \). (Stiles et al., 2003; Iizumi et al., 2006; De Souza et al., 2007a). The ENA family is closely related to SERCA (class IIA) and PMCA (class IIB) \( \text{Ca}^{2+} \)-ATPases, leading in some cases to \( \text{Na}^{+} \)-ATPases of this family being misannotated as \( \text{Ca}^{2+} \)-ATPases (Benito et al., 2002).

Based on the work presented in the preceding chapters, it was hypothesised that PfATP4 may be the plasma membrane \( \text{Na}^{+} \)-ATPase that extrudes \( \text{Na}^{+} \) from the parasite, thereby maintaining a low \( [\text{Na}^{+}] \). This raises the possibility that the antimalarial effect of the spiroindolones is due to their inhibiting \( \text{Na}^{+} \) extrusion via PfATP4. In this chapter, the effect of the spiroindolones on ion regulation in the parasite was examined. The results are consistent with the hypothesis that PfATP4 is
a plasma membrane Na\(^+\)-ATPase, similar to the ENA Na\(^+\)-ATPases of other lower eukaryotes, and the target of the spiroindolone antimalarials.

### 5.2. Results

#### 5.2.1. PfATP4 has conserved amino acid residues found in ENA-type Na\(^+\)-ATPases

P-type ATPases have nine highly conserved motifs (described in Moller et al., 1996; Thever and Saier, 2009). Analysis of the PfATP4 sequence showed that it possessed these nine motifs. The PfATP4 sequences at these motifs are listed in Table 5.1, and where the PfATP4 sequence differed from the consensus sequences for single-cell eukaryote ENA-type Na\(^+\)-ATPases (reported in Thever and Saier, 2009) the amino acid changes are noted.

<table>
<thead>
<tr>
<th>Motif #</th>
<th>PfATP4 Sequence</th>
<th>Comparison to consensus motif sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VGD</td>
<td>Identical</td>
</tr>
<tr>
<td>2</td>
<td>SAD</td>
<td>Typically a P in position 1, S is the fourth least frequent residue</td>
</tr>
<tr>
<td>3</td>
<td>TGES</td>
<td>Identical</td>
</tr>
<tr>
<td>4</td>
<td>PEGL</td>
<td>Identical</td>
</tr>
<tr>
<td>5</td>
<td>DKTGTLT</td>
<td>Identical</td>
</tr>
<tr>
<td>6</td>
<td>KGAPD</td>
<td>Contains residues from the two consensus motifs reported (KGACD/KGAPE)</td>
</tr>
<tr>
<td>7</td>
<td>DPPR</td>
<td>Identical</td>
</tr>
<tr>
<td>8</td>
<td>MITGD</td>
<td>Contains residues from the two consensus motifs reported (IITGD/MLTGD)</td>
</tr>
<tr>
<td>9</td>
<td>VAMTDGDNADA</td>
<td>Contains conserved DGVND Mg(^{2+})-binding submotif; contains residues from the two consensus motifs reported (SSMTGDNADAPAIKSSNGIAM/VAMTDGFPNDSPSIKADVGCAM)</td>
</tr>
</tbody>
</table>

Table 5.1. PfATP4 sequences at highly conserved motifs (1-9) found in P-type ATPases. The sequence residue number corresponds to the PfATP4 amino acid position. Where noted, the sequence is identical to that of the consensus motif. Grey shaded residues differ from the single-cell eukaryote consensus motif (reported in Thever and Saier, 2009).
Alignments of the amino acid sequences of ENA family members from *Saccharomyces* (ScENA1), *Leishmania* (LdCA1), *Trypanosoma* (TcENA1), and *Entamoeba* (Enthist1) against PfATP4, reveals significant homology between these proteins in key catalytic sites. A comparison of known fungal, bryophyte and protozoal ENA-type Na\(^+\)-ATPases identified some highly conserved residues in the ‘P-domain’, a region of the protein involved in nucleotide binding and phosphorylation (Chan *et al.*, 2010; Rodriguez-Navarro and Benito, 2010). Fig. 5.1 shows the alignment between conserved amino acid residues in this ‘ENA’ domain of P-type ATPases, and the same region of PfATP4. Importantly, the IVQSLKRK\(^{856}\) sequence in PfATP4 (MIEALHRK\(^{748}\) in ScENA1) is functionally conserved. The \(^{854}\)KRK triple-basic motif in PfATP4 is characteristic of the ENA-type Na\(^+\)-ATPases (and not Na\(^+/K\(^+\)) ATPase or SERCA Ca\(^{2+}\)-ATPases; (Rodriguez-Navarro and Benito, 2010)) and was not found in any other annotated P-type ATPase of *P. falciparum* (including PfATPase1 (PFE0805w), PfATPase3 (PFE0195w), PfATP6 (PFA0310c), and two putative ‘cation-transporting P-ATPases’ (MAL13P1.246, PF07_0115)). A double-basic \(^{1424}\)KNK was found to be present in the putative cation-transporting P-ATPase PF07_0115; yet this protein was found to lack P-type ATPase motifs 2,4,6,7 and 9, making it unlikely to be capable of hydrolysing ATP (data not shown). Most notably the SERCA-like PfATP6 (which was found to contain all nine P-type ATPase motifs and which, like PfATP4, showed significant homology to the ENA-type Na\(^+\)-ATPases) contained the sequence \(^{902}\)KDL in the corresponding position (data not shown), making PfATP6 a less likely candidate than PfATP4 for an ENA-type Na\(^+\)-ATPase.

**Figure 5.1.** Amino acid alignment of the P-domain of various ENA-type ATPases and PfATP4. This region has been found to be conserved in fungal, bryophyte and protozoal ENA-type ATPases (Rodriguez-Navarro and Benito, 2010). Accession numbers are ScENA1; P13587, LdCA1; AAC19126, TcENA1; XP_817442.1 and EhENA1 (Enthist1); XM_652424. Black shaded residues are completely conserved, whilst lighter shaded residues are functionally conserved. The start of the proposed ‘ENA’ specific domain is indicated by the asterisk. The start of motif 9 (see Table 5.1) is indicated by the black triangle. The triple-basic motif discussed in the text is shown in a red box.
5.2.2. Spiroindolones perturb $[\text{Na}^+]_i$ and pH$_i$, but not $[\text{Ca}^{2+}]_i$

In initial experiments investigating the relationship between the spiroindolones, PfATP4 and parasite Na$^+$ regulation, two enantiomeric pairs of spiroindolones - NITD246/247 and NITD138/139 (Fig. 5.2A) - were tested for their effect on the resting $[\text{Na}^+]_i$ in saponin-isolated parasites suspended in standard saline. Addition of a 50 nM concentration of NITD246 and 139 resulted in an immediate rapid increase in $[\text{Na}^+]_i$ (Fig. 5.2B). Addition of their enantiomers, NITD247 and NITD138 respectively, had little if any effect at the same concentration (Fig. 5.2B), though both compounds did perturb $[\text{Na}^+]_i$ at much higher concentrations (data not shown).

The most potent of the spiroindolones, NITD246, was investigated for its effect on pH$_i$ and $[\text{Ca}^{2+}]_i$. As shown in Fig. 5.2C, addition of 50 nM NITD246 to isolated parasites suspended in standard saline with a pH of 7.1 caused pH$_i$ to increase significantly, from $7.33 \pm 0.03$ to $7.46 \pm 0.02$ ($n = 6, p = 0.009$), a similar increase to that seen on addition of orthovanadate to isolated parasites (Fig. 4.1). As was seen with the orthovanadate-induced alkalinisation, the NITD246-induced alkalinisation was Na$^+$-dependent; addition of 50 nM NITD246 to parasites suspended in a solution containing choline$^+$ in place of Na$^+$ had no significant effect on pH$_i$ (Fig. 5.2C).

By contrast with its effect on $[\text{Na}^+]_i$ and pH$_i$, NITD246 (50 nM) had no effect on $[\text{Ca}^{2+}]_i$ in isolated parasites suspended in medium containing 1 μM Ca$^{2+}$ (Fig. 5.2D). Resting $[\text{Ca}^{2+}]_i$ under these conditions was $87.7 \pm 9.7$ nM ($n = 10$). By contrast, the SERCA Ca$^{2+}$-ATPase inhibitor cyclopiazonic acid (CPA; 2 μM) caused a transient increase in $[\text{Ca}^{2+}]_i$, as has been observed previously (Alleva and Kirk, 2001).

5.2.3. Dose dependence of the NITD compounds

For all four compounds the effect on $[\text{Na}^+]_i$ was dose dependent (Fig. 5.3A); i.e., higher concentrations of the spiroindolone caused an increase in both the rate and magnitude of the increase in $[\text{Na}^+]_i$. An IC$_{50}$ for $[\text{Na}^+]_i$ disruption was calculated based upon the initial rate of increase of $[\text{Na}^+]_i$ following addition of the spiroindolones.
Figure 5.2. The spiroindolones and their effect on cation regulation in saponin-isolated *P. falciparum* trophozoites. (A) Chemical structure of the enantiomers NITD246/247 and NITD138/139. (B) Traces showing the effects of the four spiroindolones, each at a concentration of 50 nM on [Na$^+$], in SBFI-loaded parasites suspended in standard saline. The spiroindolones were added at the time-point indicated by the closed triangle. (C) Traces showing the effects of the addition of NITD246 (50 nM, at the point indicated by the triangle) on pH$_i$ in BCECF-loaded parasites. The parasites were suspended in either standard saline (dark trace) or Na$^+$-free solution (in which Na$^+$ was replaced with an equimolar concentration of choline$^+$; light trace). (D) Trace showing the effect of the addition of NITD246 (50 nM, at the point indicated by the closed triangle) and CPA (2 μM, at the point indicated by the open triangle) on [Ca$^{2+}$], in Fura-2-loaded parasites suspended in standard saline supplemented with 1 μM Ca$^{2+}$. The traces shown are, in each case, representative of those obtained from at least three independent cell preparations.
Initial influx rates were estimated from the initial slope of an exponential function fitted to the data:

\[
\text{[Na}^+]_i = [\text{Na}^+]_{i0} + \Delta [\text{Na}^+]_i^{\text{max}} \times (1-e^{-at})
\]  

*Equation 5.1*

where \([\text{Na}^+]_{i0}\) is the initial resting \([\text{Na}^+]_i\), \(\Delta [\text{Na}^+]_i^{\text{max}}\) is the maximum increase in \([\text{Na}^+]_i\), \(t\) is the time after the addition of the spiroindolone, and \(a\) is a fitted constant. The initial \(\text{Na}^+\) influx rate (at \(t = 0\)) is \(a \times \Delta [\text{Na}^+]_i^{\text{max}}\).

Dose-response curves were generated by plotting the calculated initial \(\text{Na}^+\) influx rates against the concentration of the spiroindolone (Fig. 5.35). An IC\(_{50}\) for \([\text{Na}^+]_i\) disruption was obtained by fitting a sigmoidal function to the data:

\[
\text{Na}^+ \text{ Influx} = \text{Na}^+ \text{ Influx}^{\text{max}} / (1 + ([\text{NITD}] / \text{IC}_{50})^b)
\]  

*Equation 5.2*

where \(\text{Na}^+ \text{ Influx}^{\text{max}}\) is the maximum rate of \(\text{Na}^+\) influx (i.e., that measured at maximally effective concentration of inhibitors), \([\text{NITD}]\) is the spiroindolone concentration, \(b\) is a fitted constant, and IC\(_{50}\) is the spiroindolone concentration at which \(\text{Na}^+\) influx is 50% of \(\text{Influx}^{\text{max}}\).

The order of potency for the effect of the four compounds on \([\text{Na}^+]_i\), disruption (Table 5.2) was the same as their order of potency for inhibition of parasite proliferation in standard growth assays (i.e., NITD246 > NITD139 > NITD247 > NITD138; Table 5.2). The maximum rates of increase of \([\text{Na}^+]_i\), measured at maximally effective concentrations of NITD246, NITD139 and NITD247 (0.092 ± 0.007 mM/s, 0.098 ± 0.007 mM/s and 0.125 ± 0.013 mM/s, respectively) were not significantly different from one another (\(p > 0.06\); t-test with 8 degrees of freedom), and not significantly different from the rate of increase of \([\text{Na}^+]_i\), seen following the addition of 500 μM orthovanadate (0.13 ± 0.03 mM/s; \(n = 4\); \(p = 0.57\)). For the least potent spiroindolone, NITD138 the rate of increase of \([\text{Na}^+]_i\), did not reach the maximum value at the highest concentration tested, precluding an estimate of a maximum rate of increase of \([\text{Na}^+]_i\); for this compound.
Figure 5.3. Dose-dependence of the effect of the spiroindolones on [Na⁺], SBFI-loaded, saponin-isolated P. falciparum trophozoites. (A) [Na⁺] traces showing the effect of addition (at the start of each trace) of a range of concentrations of NITD246. The traces shown are representative of those obtained from at least three independent cell preparations. (B) Dose-response curves showing the effect of a range of concentrations of each of the four spiroindolones tested (● NITD246; ■ NITD247; ◊ NITD138; ▽ NITD139) on the initial rate of net Na⁺ influx measured following the addition of spiroindolone (as in (A)). Each data point represents the mean Na⁺ influx rate averaged from at least three independent experiments (and is shown ± S.E.M.). For the purpose of the curve-fitting Na⁺ Influx max was set to 0.11 mM/s, the mean of the Na⁺ Influx rates measured using the maximally effective concentrations of the three most potent inhibitors (NITD246, NITD139 and NITD 247). The resulting IC₅₀ values are given in Table 5.2.
Table 5.2. Efficacy with which the four spiroindolones inhibited in vitro parasite proliferation and disrupted $[\text{Na}^+]_i$ regulation in the 3D7 P. falciparum strain. Parasite proliferation was measured using a standard $[^3\text{H}]$hypoxanthine incorporation assay, with the IC$_{50}$ values being the concentration of each spiroindolone required to inhibit proliferation by 50%. The values are the mean (± S.E.M.) of those obtained in the number of independent experiments indicated in parentheses, each performed in triplicate. The IC$_{50}$ values for disruption of $[\text{Na}^+]_i$ regulation are the concentration of each inhibitor required to cause the $[\text{Na}^+]_i$ in SBFI-loaded saponin-isolated trophozoites to increase (from its normal resting value) at half the maximal rate. The IC$_{50}$ values for Na$^+$ disruption were derived from the dose response curves shown in 5.3B.

<table>
<thead>
<tr>
<th>Spiroindolone derivative</th>
<th>IC$_{50}$ for inhibition of parasite proliferation</th>
<th>IC$_{50}$ for disruption of Na$^+$ regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NITD246</td>
<td>0.12 ± 0.02 nM (4)</td>
<td>2.1 ± 0.8 nM</td>
</tr>
<tr>
<td>NITD247</td>
<td>41.6 ± 5.8 nM (4)</td>
<td>407 ± 117 nM</td>
</tr>
<tr>
<td>NITD138</td>
<td>2.3 ± 0.2 µM (4)</td>
<td>819 ± 1.7 µM</td>
</tr>
<tr>
<td>NITD139</td>
<td>4.0 ± 0.9 nM (3)</td>
<td>16.6 ± 5.7 nM</td>
</tr>
</tbody>
</table>

5.2.4. Effect of SERCA/Ca$^{2+}$-ATPase inhibitors on $[\text{Na}^+]_i$

As the P-type ENA Na$^+$-ATPases are very similar to SERCA Ca$^{2+}$-ATPases, the sensitivity of $[\text{Na}^+]_i$ to SERCA inhibitors was investigated. CPA (40 µM) and thapsigargin (2 µM) were tested for their effect on $[\text{Na}^+]_i$ (Fig. 5.4). Whilst thapsigargin had little effect, the addition of CPA caused $[\text{Na}^+]_i$ to increase.

![Figure 5.4. Effect of SERCA Ca$^{2+}$-ATPase inhibitors on $[\text{Na}^+]_i$ in SBFI-loaded, saponin-isolated P. falciparum trophozoites.](image-url)

At the point indicated by the closed triangle (A) cyclopiazonic acid (40 µM) and (B) thapsigargin (2 µM), were added to the cell suspension (as a concentrated stock). The traces shown are representative of those obtained from at least three independent cell preparations.
5.2.5. Effect of antimalarials on $[\text{Na}^+]_i$

The spiroindolones have potent antimalarial activity. To determine whether the effect on $[\text{Na}^+]_i$ was specific, or a general property of multiple drug classes, the effect of other antimalarials on $[\text{Na}^+]_i$ regulation was also investigated. Neither chloroquine nor artemisinin (both at 10 μM) caused a significant disruption of $[\text{Na}^+]_i$ (Fig. 5.5A and B).

![Figure 5.5](image)

**Figure 5.5. Effect of antimalarials on $[\text{Na}^+]_i$ in saponin-isolated, SBFI-loaded *P. falciparum* trophozoites.** $[\text{Na}^+]_i$ traces showing the effect of addition (at the time-point indicated by the closed triangle) of (A) chloroquine (10 μM) and (B) artemisinin (10 μM). For all additions the compounds were added as a concentrated stock. The traces shown are, in each case, representative of those obtained from at least three independent cell preparations.
5.2.6. Effect of NITD246 on the net efflux of Na\(^+\) following an imposed Na\(^+\) load

The Na\(^+\) loading manoeuvre described in Section 3.2.7 was used as a means of testing directly the effect of NITD246, the most potent of the spiroindolones, on the net efflux of Na\(^+\) from the parasite following an imposed Na\(^+\) load. Isolated parasites were incubated in K\(^+\) free medium to induce Na\(^+\) loading. At time-zero 10 mM KCl was added to the medium, with or without NITD246 (1 nM; approximately half the IC\(_{50}\) for disruption of resting [Na\(^+\)]; see Table 5.2). At this concentration the spiroindolone inhibited Na\(^+\) efflux (Fig. 5.6). When higher concentrations of NITD246 were added in conjunction with the 10 mM K\(^+\) the Na\(^+\) loading continued unabated (data not shown).

![Graph showing the effect of NITD246 on the recovery of [Na\(^+\)]\(_i\) from an imposed Na\(^+\) load in saponin-isolated P. falciparum trophozoites.](image)

Figure 5.6. Effect of NITD246 on the recovery of [Na\(^+\)]\(_i\) from an imposed Na\(^+\) load in saponin-isolated P. falciparum trophozoites. SBFI-loaded parasite suspension were subjected to a Na\(^+\) load (by suspension in K\(^-\)-free medium), as illustrated in Fig. 3.6A. The traces commenced with the addition to the suspension of 10 mM KCl either with (light trace) or without (control, dark trace) 1 nM NITD246. The traces shown are representative of those obtained from at least three independent cell preparations.

5.2.7 Effect of spiroindolones on [ATP]\(_i\)

The effect of the spiroindolones on intracellular [ATP] was assessed to determine if the disruption of [Na\(^+\)]\(_i\) regulation was due to depletion of [ATP]\(_i\). In isolated parasites in standard, inhibitor-free saline, [ATP]\(_i\) was constant over the 1 hr timecourse (data not shown). For parasites suspended at time-zero in standard saline (20 mM glucose) there was no effect of any of the spiroindolones (when added at 50 nM) on [ATP]\(_i\) over a 1 hr timecourse (Fig. 5.7).
Figure 5.7. Effect of the spiroindolones on [ATP], in saponin-isolated trophozoites.

Intracellular [ATP] was determined using the firefly luciferase assay (section 2.9) and results were standardised to the resting cytosolic [ATP] under glucose-containing, inhibitor-free conditions (unchanged across the timecourse; data not shown). Inhibitors (● NITD246; ○ NITD247; ▼ NITD138; △ NITD139) were added at time-zero. The data are averaged from three independent experiments (mean ± S.E.M.). For clarity, error bars are shown for the NITD247 series only, and where not shown error bars lie within the symbol. The dashed reference line indicates 100% ATP (control levels).

5.2.8. Effect of NITD246 on parasite volume

An increase in [Na⁺], in response to the addition of an inhibitor has the potential to place a significant osmotic burden on the parasite, and to lead, thereby, to cell swelling. The effect of spiroindolone-treatment on the volume of the parasite was therefore investigated, using a Millipore Scepter, with volume estimates made before and 30 min after treatment with 50 nM of the potent NITD246 spiroindolone. On treatment of the parasites with NITD246, the volume increased significantly from 45.3 ± 3.4 fL (n = 4) in untreated cells (after the same 30 min incubation conditions) to 53.3 ± 3.8 fL after 30 min of NITD246-exposure (n = 4; paired t-test, p = 0.003). The average cell size (diameter) also increased significantly from 4.42 ± 0.11 μm (n = 4) to 4.68 ± 0.12 μm (n = 4; paired t-test, p = 0.003).
The volume estimate for the untreated saponin-isolated trophozoites was consistent with that obtained using a second instrument, the Malvern Mastersizer 2000, which indicated an average diameter of 4.44 ± 0.24 μm (n = 3).

5.2.9. Na⁺ regulation in PfATP4 mutant lines

In the original study reporting the antimalarial activity of the spiroindolones (Rottmann et al., 2010), exposure of *P. falciparum* parasites to gradually increasing (sublethal) concentrations of spiroindolones over a period of 3 - 4 months led to the generation of spiroindolone-resistant parasites, all of which had mutations in PfATP4. The role of the PfATP4 mutations in conferring spiroindolone resistance was confirmed by the demonstration that transfection of parasites with genes encoding the mutant PfATP4 proteins found in resistant (spiroindolone-exposed) parasites resulted in reduced spiroindolone sensitivity (Rottmann et al., 2010).

In this study, aspects of Na⁺ regulation were investigated in the NITD609-RDd2-clone#2 parasite line (generated by exposing Dd2 *P. falciparum* parasites to the potent spiroindolone NITD609), and the Dd2attB CAM I398F/P990R parasite line (generated by transfecting the gene encoding a mutant PfATP4 protein from a resistant, NITD609-exposed line into spiroindolone-sensitive Dd2attB parasites (Rottmann et al., 2010)). Representative results are shown in Fig. 5.8 for the NITD609-RDd2-clone#2 parasite line. The outcomes of similar experiments for both mutant lines (and their parent lines) are given in Table 5.3. The spiroindolone resistance of the mutant lines was confirmed in standard parasite growth assays (Fig. 5.8A; Table 5.3); the drug-exposed and transfectant lines showed, respectively, an ~11 fold and ~8 fold increase in IC₅₀ values relative to their parent lines.

In initial experiment, aspects of Na⁺ regulation in the parasites expressing mutant PfATP4 were investigated. The resting [Na⁺]ᵢ was significantly higher in the NITD609-RDd2-clone#2 line at 14.8 ± 1.9 mM (n = 10) compared to its parent line at 7.2 ± 1.1 mM (n = 10; p = 0.003). The resting [Na⁺]ᵢ was also higher in the Dd2attB CAM I398F/P990R line at 11.9 ± 1.8 mM (n = 8) compared to its parent line at 8.4 ± 0.7 mM (n = 11), though this did not quite reach statistical significance (p = 0.06).
Figure 5.8. Comparison of the NITD246-sensitivity and aspects of Na⁺ regulation in NITD609-RDd2 clone#2 parasites (generated by exposing Dd2 P. falciparum parasites to the potent spiroindolone NITD609) and the Dd2 parent line. (A) Dose-response curves for the effects of NITD246 on the proliferation of the two parasite lines, as measured using a standard [³²P]-hypoxanthine incorporation assay. The values shown represent the means (± S.E.M.) from at least three independent experiments, each performed in triplicate. (B) [Na⁺] traces showing the recovery of SBFI-loaded saponin-isolated trophozoites from an additional Na⁺ load, imposed by the removal then restoration of extracellular K⁺ (as illustrated in Fig. 3A). The black trace shows the recovery for the Dd2 parent and the grey trace shows the recovery for the NITD609-RDd2 clone#2 parasites. The traces are representative of those obtained from at least three independent cell preparations. (C) Dose-response curves for the effects of supra-physiological extracellular Na⁺ concentrations on the proliferation of the two parasite lines, as measured using a standard [³²P]-hypoxanthine incorporation assay. ‘Excess [Na⁺]’ denotes the increase in [Na⁺] above that normally present in the parasite culture medium (~133 mM). The values shown represent the means (± S.E.M.) from at least three independent experiments, each performed in triplicate. (D) Dose-response curves for the effect of a range of concentrations of NITD246 on the initial rate of the net Na⁺ influx measured following the addition of the compound to each of the two strains (as in Fig. 5.3A). Each data point represents the mean Na⁺ influx rate averaged from at least three independent experiments (and is shown ± S.E.M.). For all four panels the relevant parameters are summarised in Table 5.3.
<table>
<thead>
<tr>
<th>Parasite line</th>
<th>$IC_{50}$ for inhibition of parasite proliferation by NITD246 (nM)</th>
<th>$IC_{50}$ for inhibition of parasite proliferation by excess $[Na^+]_0$ (nM)</th>
<th>$IC_{50}$ for disruption of $Na^+$ regulation by NITD246 (nM)</th>
<th>Half-time for recovery of $[Na^+]_i$ following $Na^+$ loading (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dd2 parent</td>
<td>0.08 ± 0.01 (5)</td>
<td>57.5 ± 4.8 (5)</td>
<td>1.62 ± 0.31</td>
<td>153 ± 22 (5)</td>
</tr>
<tr>
<td>NITD609-R$Dd2$ clone#2</td>
<td>0.89 ± 0.12 (7)*</td>
<td>31.3 ± 2.4 (7)*</td>
<td>10.37 ± 8.39</td>
<td>345 ± 10 (4)*</td>
</tr>
<tr>
<td>Dd2$attB$ parent</td>
<td>0.08 ± 0.01 (5)</td>
<td>48.8 ± 7.9 (5)</td>
<td>1.66 ± 0.54</td>
<td>158 ± 7 (4)</td>
</tr>
<tr>
<td>Dd2$attB$ CAM I398F/P990R</td>
<td>0.61 ± 0.14 (5)*</td>
<td>32.2 ± 5.2 (5)</td>
<td>3.87 ± 1.72</td>
<td>335 ± 55 (4)*</td>
</tr>
</tbody>
</table>

Table 5.3. Characterisation of PfATP4 mutant parasites (and their parent lines) in terms of their sensitivity to spiroindolones, and their $[Na^+]_i$ regulation properties. The parameters were estimated as described in the legend for Fig. 5.8 and in Sections 5.2.3 and 3.2.7. All of the values shown represent mean ($\pm$ S.E.M.) of the number of independent experiments shown in parentheses. For $Na^+$ disruption, the cited value is the fitted parameter from at least three independent experiments per [NITD]. Where indicated by an asterisk, comparison between the parent line and PfATP4 mutant is significant where $p < 0.05$.

The finding of higher resting $[Na^+]_i$ in parasite lines expressing mutant PfATP4 is consistent with the mutations giving rise to some impairment in the $Na^+$ efflux mechanism. To test this directly the parasites were pre-loaded with $Na^+$ (by removing extracellular K$, as per Section 3.2.7) and the rate of $Na^+$ extrusion measured. For both of the parasite strains expressing mutant PfATP4 there was a significant increase in the half-time for $Na^+$ efflux, relative to the parental lines (Fig. 5.8B; Table 5.3).

The possible implications of an impaired $Na^+$ efflux mechanism for the degree of ‘$Na^+$ tolerance’ of the parasites was investigated by growing both mutant and parent strains in the presence of increasing extracellular $Na^+$ concentrations. Increasing $[Na^+]_0$ above its normal value (of ~133 mM) inhibited parasite proliferation in a concentration-dependent manner, with the lines expressing mutant PfATP4 having lower $IC_{50}$ values than their corresponding parent lines (Fig. 5.8C; Table 5.3; though once again the observed decrease only reached statistical significance in the NITD609-RDd2-clone#2 line).
As well as showing impaired Na$^+$ efflux and tolerance the two parasite lines expressing mutant PfATP4 also showed a decrease in the [Na$^+$]-disrupting effects of the spiroindolone NITD246, relative to their parent lines (Figure 5.8D, Table 5.3). However, although the dose response curves were shifted, the difference in the IC$_{50}$ values was not statistically significant ($p = 0.31$ and $p = 0.24$ for the NITD609-RDd2-clone#2 and Dd2$^{antb}$ CAM I398F/P990R lines respectively) reflecting the large S.E.M. for each of the fitted IC$_{50}$ values.

5.3. Discussion

The work presented in this chapter identifies PfATP4 as a candidate for the parasite’s plasma membrane Na$^+$-ATPase. PfATP4 is on the plasma membrane of the intraerythrocytic parasite (Dyer et al., 1996; Rottmann et al., 2010) and although it was originally annotated as a Ca$^{2+}$-ATPase (Krishna et al., 2001) it shows significant sequence similarities to the Na$^+$-extruding ENA-type ATPases of lower plants, fungi and some protozoa. It is likely that the recent review of protozoal ENA-type Na$^+$-ATPases (Rodriguez-Navarro and Benito, 2010) did not include PfATP4 as a candidate ATPase because despite no functional data demonstrating Ca$^{2+}$ translocation, multiple literature reports state that PfATP4 is a Ca$^{2+}$-ATPase (Trottein et al., 1995; Dyer et al., 1996; Krishna et al., 2001; Martin et al., 2005; Martin et al., 2009a).

5.3.1. Bioinformatic analysis of PfATP4 implicates it as being an ENA-type Na$^+$-ATPase

PfATP4 possesses all nine key motifs of P-type ATPases (Table 5.1), and the amino acid sequences closely match the consensus motifs for ENA-ATPases of other single cell eukaryotes determined in a comprehensive bioinformatic analysis of eukaryotic P-type ATPases (Thever and Saier, 2009). The MIEALHRR motif (sequence from ScENA) has been proposed to be important in Na$^+$ translocation by ENA-type ATPases (Rodriguez-Navarro and Benito, 2010). Based upon the secondary structure of SERCA Ca$^{2+}$-ATPases it has been predicted that this motif lies near, but not close enough to motifs 5 - 7 to play a role in the catalytic cycle (Rodriguez-Navarro and Benito, 2010). When the N. crassa ENA (NcENA1) $^{742}$HRR residues
were mutated (to SYD residues present in the corresponding positions in rabbit SERCA Ca\(^{2+}\)-ATPase) the Na\(^{+}\) transport capacity was lost (Rodriguez-Navarro and Benito, 2010). This indicates that this conserved area of basic residues is somehow important in Na\(^{+}\) translocation. Whereas PfATP4 possessed the \(854\text{KRK}\) triple-basic motif, the only other \(P. falciparum\) protein currently annotated as a Ca\(^{2+}\)-ATPase, the SERCA-like PfATP6, contained \(902\text{KDL}\) (in the same position of an alignment), in which the last two residues are occupied by acidic and hydrophobic residues. PfATP6 is therefore unlikely to be a Na\(^{+}\)-ATPase.

5.3.2. The effect of the spiroindolones on parasite [Na\(^{+}\)]\(_{o}\) regulation

The four different spiroindolones tested for their effect on ion regulation in the parasite varied widely in their relative potencies, with \(IC_{50}\) values for disruption of the resting [Na\(^{+}\)]\(_{i}\), ranging from 2.1 nM to 819 \(\mu\)M. The finding that the order of potency for the effect of the spiroindolones on [Na\(^{+}\)]\(_{i}\) was the same as that for inhibition of parasite proliferation (i.e., NITD246 > NITD139 > NITD247 > NITD138) is consistent with the inhibition of parasite growth being a consequence of the disruption of ion regulation.

The increase in the resting [Na\(^{+}\)]\(_{i}\), caused by the spiroindolones (or orthovanadate; Fig. 3.7E) could, in theory, be attributed to either an increased influx or decreased efflux of Na\(^{+}\). An increased influx might reflect a general membrane disruption, allowing Na\(^{+}\) and other ions to move down their electrochemical gradients. However, this is unlikely because: (i) the addition of the spiroindolone NITD246 caused pH\(_{i}\) to increase from its initial resting value of 7.3, away from the extracellular pH of 7.1 (i.e., the spiroindolone caused the transmembrane pH gradient to increase) and, furthermore, this increase was seen only in the presence of extracellular Na\(^{+}\); (ii) the same concentration of NITD246 had no effect on [Ca\(^{2+}\)]\(_{i}\), under conditions in which there was a steep inward Ca\(^{2+}\) electrochemical gradient (with an approximately 10-fold inward [Ca\(^{2+}\)] gradient and a large inwardly negative membrane potential; (Allen and Kirk, 2004b)). These data do not rule out the induction or activation of a Na\(^{+}\)-specific leak. However, this provides no ready explanation for why the spiroindolones should cause pH\(_{i}\) to increase in the same manner as the P-type ATPase inhibitor orthovanadate (Fig. 4.1).
Furthermore, the finding that the rate of increase of $[\text{Na}^+]_i$, seen on addition of maximally-effective concentrations of the three most active spiroindolones was similar in each case, and similar to that seen on addition of orthovanadate (Section 3.2.8) is consistent with the alternative hypothesis that the spiroindolones, like orthovanadate, exert their effect on $[\text{Na}^+]_i$ by inhibiting the extrusion of $\text{Na}^+$ via a plasma membrane $\text{Na}^+\text{-ATPase}$, thereby revealing, in each case, the endogenous influx of $\text{Na}^+$ into the parasite.

The finding that common antimalarials had no effect on $[\text{Na}^+]_i$ (Fig. 5.5), and the fact that the parasites were able to maintain constant ATP levels for up to an hour after spiroindolone exposure (Fig. 5.7), is also evidence against the spiroindolones having a non-specific effect on cell health.

Given that the ENA-type $\text{Na}^+\text{-ATPase}$ (class IID) is structurally similar to $\text{Ca}^{2+}\text{-ATPases}$ (class IIA, B), it is noteworthy that the SERCA $\text{Ca}^{2+}\text{-ATPase}$ inhibitor CPA caused a disruption of $\text{Na}^+$ regulation (Fig. 5.4). This might be accounted for by the CPA binding pocket being conserved in PfATP4. The effect of CPA on ENA-type $\text{Na}^+\text{-ATPases}$ from other organisms has not been reported. Thapsigargin and CPA bind to nearby, yet distinct sites near the membrane/cytosol interface of SERCA $\text{Ca}^{2+}\text{-ATPases}$; thus the finding that thapsigargin had little effect on $[\text{Na}^+]_i$, does not preclude conservation of the CPA-binding site (Lape et al., 2010). The pharmacological profile of high-CPA sensitivity and weak-thapsigargin sensitivity has been observed previously for PfATP6 (Arnou et al., 2011) consistent with this being a property of the type IIA/B/D P-type ATPases in $P. falciparum$. However, the possibility that the CPA-induced increase in $[\text{Na}^+]_i$ is secondary to the CPA-induced increase in $[\text{Ca}^{2+}]_i$, cannot be excluded and was not investigated here.

5.3.3. The effect of NITD246 on $pH_i$ is consistent with $H^+$-coupling of the $\text{Na}^+\text{-ATPase}$

As was discussed in relation to the effect of orthovanadate on $pH_i$ (Fig. 4.1), the $\text{Na}^+$-dependent increase in $pH_i$ seen on addition of NITD246 might be accounted for if the extrusion of $\text{Na}^+$ via the $\text{Na}^+\text{-ATPase}$ is coupled to the influx of $H^+$. As described in Chapter 4, the influx of $H^+$ constitutes a substantial ‘acid load’ on the parasite; inhibition of the $\text{Na}^+\text{-ATPase}$ and the resultant removal of this acid load might
therefore be expected to result in a cytosolic alkalinisation, as was observed (Fig. 5.2C). Under Na\(^+\) free conditions [Na\(^+\)], is close to zero (Fig. 3.5A), the Na\(^+\)-ATPase is therefore inactive, and there is therefore no associated acid load.

5.3.4. PfATP4 as an ENA-type ATPase and the target of the spiroindolones

The hypothesis that PfATP4 serves as the parasite’s primary Na\(^+\) extrusion mechanism, and that it is the target of the spiroindolones would provide an explanation for why parasite lines expressing mutant PfATP4 show altered Na\(^+\) regulation (a significant increase in the resting [Na\(^+\)], a decrease in the rate of efflux of Na\(^+\) following Na\(^+\) loading, and an increased sensitivity to the growth-inhibitory effects of excess extracellular Na\(^+\); Fig. 5.8, Table 5.2), as well as showing reduced sensitivity to both the [Na\(^+\)]-disrupting effects and growth-inhibitory effects of the spiroindolones. The finding that the transgenic (Dd2\(^{int}\) CAM I398F/P990R) parasite line shows both a lower level of spiroindolone resistance and a greater sensitivity to the [Na\(^+\)]-disrupting effects of the spiroindolones than the drug-selected mutant (NITD609-RDd2-clone#2) parasite line is consistent with this hypothesis. The finding of (slightly) impaired Na\(^+\) regulation in parasites expressing the mutant PfATP4 is consistent with the spiroindolone-resistance-conferring mutations having a small but significant fitness cost to the parasite.

5.3.5. How might inhibition of Na\(^+\) extrusion inhibit parasite growth?

The hypothesis that the spiroindolones exert their antiproliferative effect by disrupting Na\(^+\) homeostasis in the intraerythrocytic parasite raises the question of why an increase in [Na\(^+\)], should inhibit parasite growth. Possibilities include: (i) high levels of cytosolic Na\(^+\) may be ‘toxic’, (ii) disruption of the Na\(^+\) electrochemical gradient across the parasite plasma membrane may inhibit parasite growth, or (iii) the osmotic imbalance arising from the increased [Na\(^+\)], and the resulting cell swelling might be lethal to the parasite.

In plants, Na\(^+\) toxicity (and hence the need to maintain [Na\(^+\)], at low levels) has been linked to the ability of Na\(^+\) to interfere with the regulation of various metabolic pathways (including glycolysis) by K\(^+\) (Munns and Tester, 2008). As seen in Fig. 5.8, parasites were not able to proliferate at supra-physiological [Na\(^+\)], though there
was no attempt made to distinguish between the osmotic consequences of raising \([\text{Na}^+]_o\) and Na⁺-specific effects. Brand et al. have shown that parasites grow normally in media in which the extracellular \([\text{Na}^+]\) is decreased (by replacement with NMDG⁺) by two-thirds, from 120 to 40 mM; it is only as the extracellular \([\text{Na}^+]\) is decreased below 40 mM that parasite growth decreases (Brand et al., 2003). If the inhibition of parasite growth by the spiroindolones is a consequence of Na⁺ toxicity, then, in future experiments, decreasing \([\text{Na}^+]_o\) should result in decreased potency (i.e., increased IC\(_{50}\)) for the spiroindolones.

The parasite is known to utilise the inward Na⁺ electrochemical gradient to energise the uptake of at least one important nutrient, inorganic phosphate (Saliba et al., 2006). Whether there are other nutrients for which uptake into the parasite is energised by the Na⁺ gradient is not known. The possibility that the inhibition of parasite proliferation is due to the reduction or elimination of the Na⁺ gradient, resulting in a reduced driving force for the uptake of key nutrients, could be tested by measuring the sensitivity of parasite proliferation to the spiroindolones in parasites grown in media supplemented with an excess of the various nutrients present in the growth medium. The increase in nutrient concentrations would result in increased uptake of the nutrients into the parasite. If it is found that this counters the inhibitory effect of the spiroindolones on parasite growth then further experiments could be carried out in which the concentrations of different classes of nutrient (and, ultimately, individual nutrients) are varied, to identify those nutrients for which uptake is compromised by the spiroindolones.

Finally, as outlined in Section 5.2.8, inhibition of the Na⁺-ATPase by the spiroindolones caused the parasites to swell significantly, consistent with a breakdown of the normal osmotic balance in the parasite. The capacity of the parasite to regulate its volume in response to volume perturbation is unknown and it is unclear whether the parasite swelling is occurring despite the activation of volume-regulatory mechanism, or what might happen to the volume on a longer timescale (> 30 min). It is possible that it is the cell swelling, perhaps leading ultimately to membrane lysis, that is the key lethal event. To investigate this possibility further, the efficacy of the spiroindolones could be tested in parasite proliferation assays in which parasites are grown in media having a range of osmolalities (from 250 mOsM
to 400 mOsM, with the extracellular [Na⁺] maintained constant throughout). An osmolality-dependence of the efficacy (i.e., IC₅₀ values) for the spiroindolones would be consistent with the spiroindolones exerting their antiproliferative effect via a disruption of osmoregulation by the parasite.

5.4. Conclusion

PfATP4, a P-type ATPase on the parasite plasma membrane, shows significant sequence similarities to the Na⁺-extruding ENA-type Na⁺-ATPases of lower eukaryotes. A recent study showed that mutations in PfATP4 confer resistance to a newly described class of antimalarials, the spiroindolones (Rottmann et al., 2010). The results presented in this chapter are consistent with the spiroindolones causing a profound disruption in parasite Na⁺ homeostasis. In parasites with mutant PfATP4 there is both an impairment of Na⁺ regulation and a decrease in the spiroindolone sensitivity of Na⁺ regulation. These results are consistent with PfATP4 being a Na⁺-ATPase and the target of the spiroindolones.
Chapter 6:

Investigating the function of PfNHE, a putative Na$^+$/H$^+$-exchanger
6.1. Declaration

The work presented in this chapter is all my own work. There are three instances (Fig. 6.1, 6.5 and 6.6) where the data presented is based on preliminary results that were included in my own Honours thesis (Spillman, 2007). However, the data shown here were generated as part of this current study.

The work described in section 6.3.2 has been published in the following paper:


6.2. Introduction

Although a putative NHE has been annotated in the *P. falciparum* genome (Pf13_0019), its physiological function remains unclear. PfNHE is of particular significance to this study for two reasons: firstly it is a putative Na⁺ transporter that could be involved in [Na⁺]ᵢ regulation; secondly it has been linked with quinine resistance (reviewed in Okombo *et al.*, 2011).

In an early study of the regulation of pHᵢ in the parasite it was reported that the maintenance of resting pHᵢ, and recovery from an intracellular acidification, was dependent on the presence of extracellular Na⁺ and inhibited by compounds known to block NHEs in other cell types (Bosia *et al.*, 1993). It was concluded that the parasite, like many animal cells, uses an NHE to extrude H⁺ and thereby control its pHᵢ. Contradictory results were obtained in a subsequent study (Saliba and Kirk, 1999) in which it was shown that neither the maintenance of a steady resting pHᵢ, nor the recovery from an intracellular acidification was Na⁺ dependent, but that both processes were inhibited by V-type H⁺-ATPase inhibitors. The data in this second study were consistent with a V-type H⁺-pump, rather than an NHE, playing the primary role in the extrusion of H⁺ from the parasite. The effects of V-type H⁺-ATPase inhibitors on parasite pH regulation were confirmed in a subsequent study.
that also demonstrated that a subunit of the V-type H⁺-ATPase was localised to the parasite plasma membrane and the digestive vacuole membranes (Hayashi et al., 2000). However, in a recent study an NHE was again implicated in pHᵢ regulation, and Bennett et al. presented data showing a Na⁺-dependent pHᵢ recovery of parasites subjected to an intracellular acidification using the H⁺ ionophore nigericin (Bennett et al., 2007). In considering their results the authors made no mention of a possible role for a plasma membrane V-type H⁺-ATPase, and the pHᵢ recovery observed following an intracellular acidification imposed using nigericin was attributed to a parasite NHE. The physiological role of PfNHE is thus controversial and although it has been proposed that the role of PfNHE may be in regulating [Na⁺]ᵢ, rather than pHᵢ (Ginsburg, 2002; Spillman et al., 2008), the work presented in Chapters 3 – 5 of this thesis is consistent with Na⁺ extrusion from the parasite being via an ENA-type Na⁺-ATPase rather than via an NHE.

This chapter describes efforts to determine the role of the putative NHE in *P. falciparum*. This study used two main approaches: (i) bioinformatic analyses of the PfNHE sequence, and (ii) measurements of [Na⁺]ᵢ, and pHᵢ using fluorescent ion indicators to probe the relationship between Na⁺ and H⁺ transport (in both wild type and NHE knock-down strains) across the plasma membrane. This chapter reports that although PfNHE is predicted bioinformatically to transport Na⁺, and to be functionally similar to NHEs found in plants, it is unlikely that it plays a significant role in either pHᵢ or [Na⁺]ᵢ regulation in the mature asexual blood-stage of the parasite under the conditions tested here.
6.3. Results

6.3.1 PfNHE bioinformatics

6.3.1.1. Confirmation that PfNHE belongs to the ‘NhaP/SOS1’ phylogenetic clade of NHEs

The PfNHE amino acid sequence was used as the query sequence in a pBLAST search against the non-redundant protein databank (excluding *Plasmodium* spp) and the results (summarised in Table 6.1) were consistent with previous classification of PfNHE as a member of the NhaP/SOS1 family of transporters (Fig. 1.7 and Section 1.6.3.5; Brett *et al.*, 2005).

The sequence with the most significant alignment was TgNHE1 from the closely related apicomplexan *T. gondii*, which has four putative NHEs annotated in its genome. Of the top 22 hits shown, four are protozoan, 15 are from plants (including seven halophytes), two are from bacteria, and one is from a sea urchin. Five of the proteins had previously been assigned to the NhaP/SOS1 family (Brett *et al.*, 2005).

6.3.1.2 Peculiarities of the C- and N-termini of PfNHE

The PfNHE sequence is long (1920 amino acids) with a C-terminus of ~1300 amino acids (see the annotation of the predicted transmembrane domains in Fig. 6.1), which compares to C-terminal lengths of < 500 amino acids in human NHEs (Brett *et al.*, 2005). TgNHE1 is even longer (2097 amino acids), with a C-terminal length of ~1800 amino acids (Arrizabalaga *et al.*, 2004). An investigation of NhaP/SOS1 family C-terminal lengths was performed using raw data retrieved from Supplementary Table 1 in Brett *et al.* (2005). The mean eukaryotic NhaP/SOS1 family C-terminal length was 1225 ± 398 amino acids (n = 8), which was significantly longer than the mean C-terminal length of 546 ± 75 amino acids (n = 32; p = 0.008) for prokaryotic NhaP/SOS1 family members. Plants also have a second, distinct clade of NHEs (NHX). NHX proteins localise to the plant vacuole (Section 1.6.3.2), and members of this clade have a mean C-terminal
<table>
<thead>
<tr>
<th>Organism</th>
<th>Annotation Detail and GenBank Accession Number</th>
<th>E-value</th>
<th>Clade</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em> (protozoan)</td>
<td>sodium/hydrogen exchanger (XP_001349762.1)</td>
<td>0.0</td>
<td>NhaP/SOS1</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em> (protozoan)</td>
<td>sodium/hydrogen exchanger 1 (AAR858890.1)</td>
<td>3e-160</td>
<td>-</td>
</tr>
<tr>
<td><em>Thelponentia annulata</em> (protozoan)</td>
<td>sodium/hydrogen exchanger XP_952081.1</td>
<td>5e-125</td>
<td>-</td>
</tr>
<tr>
<td><em>Cryptosporidium muris</em> (protozoan)</td>
<td>sodium/hydrogen exchanger family protein XP_002140147.1</td>
<td>2e-64</td>
<td>NhaP/SOS1</td>
</tr>
<tr>
<td><em>Babesia bovis</em> (protozoan)</td>
<td>sodium/hydrogen exchanger 1 XP_001610187.1</td>
<td>2e-61</td>
<td>-</td>
</tr>
<tr>
<td><em>Zyophylum xanthoxylum</em> (halophyte)</td>
<td>plasma membrane Na+/H+ antiporter ACZ57357.1</td>
<td>3e-41</td>
<td>-</td>
</tr>
<tr>
<td><em>Strongylcentrus purpuratus</em> (purple sea urchin)</td>
<td>similar to sperm-specific sodium proton exchange XP_001184091.1</td>
<td>3e-41</td>
<td>-</td>
</tr>
<tr>
<td><em>Chenopodium quinoa</em> (halophyte; grain)</td>
<td>salt overly sensitive 1B e</td>
<td>6e-40</td>
<td>-</td>
</tr>
<tr>
<td><em>Lolium perenne</em> (ryegrass)</td>
<td>SOS1 AAY42598.1</td>
<td>6e-40</td>
<td>-</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> (thale cress)</td>
<td>SOS1 (Salt Overly Sensitive 1); sodium: hydrogen antiporter NP_178307.2</td>
<td>1e-39</td>
<td>NhaP/SOS1</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> (wheat)</td>
<td>plasma membrane Na+/H+ antiporter ACB47885.1</td>
<td>7e-39</td>
<td>-</td>
</tr>
<tr>
<td><em>Suaeda japonica</em> (halophyte; salt marsh plant)</td>
<td>putative Na+/H+ antiporter BAE95196.1</td>
<td>2e-38</td>
<td>-</td>
</tr>
<tr>
<td><em>Limonium gmelinii</em> (sea lavender; salt marsh plant)</td>
<td>Na+/H+ antiporter protein AC05808.1</td>
<td>3e-38</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacteriodes coprocola</em> (bacteria)</td>
<td>hypothetical protein BACCOP_00379 ZP_03008536.1</td>
<td>6e-35</td>
<td>-</td>
</tr>
<tr>
<td><em>Thellungiella halophila</em> (salt cress)</td>
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<td>-</td>
</tr>
<tr>
<td><em>Chloroherpeton thalassium</em> (marine bacteria)</td>
<td>cyclic nucleotide-binding protein YP_001996721.1</td>
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<td>-</td>
</tr>
<tr>
<td><em>Populus trichoca</em> (poplar plant)</td>
<td>sodium proton exchanger XP_002315837.1</td>
<td>8e-36</td>
<td>-</td>
</tr>
<tr>
<td><em>Phragmites australis</em> (reed)</td>
<td>Na+/H+ antiporter BAF41924.1</td>
<td>2e-36</td>
<td>-</td>
</tr>
<tr>
<td><em>Cymodocea nodosa</em> (sea grass)</td>
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<td>9e-35</td>
<td>NhaP/SOS1</td>
</tr>
<tr>
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<td>salt overly sensitive 1 ACY03274.1</td>
<td>2e-34</td>
<td>-</td>
</tr>
<tr>
<td><em>Physcomitrella patens</em> (moss)</td>
<td>SOS1B putative Na+/H+ antiporter CBG92827.1</td>
<td>2e-33</td>
<td>NhaP/SOS1</td>
</tr>
<tr>
<td><em>Brachypodium sylvaticum</em> (grass)</td>
<td>sodium/proton antiporter AC087666.1</td>
<td>4e-32</td>
<td>-</td>
</tr>
<tr>
<td><em>Mesembryanthemum crystallinum</em> (halophyte)</td>
<td>salt-overly-sensitive 1 ABN04585.1</td>
<td>5e-32</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.1. pBLAST results using PfNHE as the query sequence against the non-redundant protein databank. The top sequences retrieved, and their available annotation from the NCBI databank [http://www.ncbi.nlm.nih.gov/] are listed in order of E-value (expectation score). The E-value is a statistic that describes the significance of alignment between two sequences. The smaller the E-value, the more likely it is that the sequences are related (Zvelebil and Baum, 2008). It is generally accepted that an E-value < 1x10^{-50} provides "high confidence" that there is homology between matches, and an E-value between 0.01 and 1x 10^{-50} also suggest homology (Xiong, 2006). Values >0.01 are considered not significant. Where the protein was included in the NHE phylogenetic analyses of Brett et al. (2005) the clade to which the protein was allocated in this study is indicated.
length of 542 ± 8 amino acids (n = 32). This is significantly less (p = 0.001) than the C-terminal length of eukaryotic NhaP/SOS1 family members.

Goswami et al. (2010) recently solved the crystal structure of a bacterial member of the NhaP family and they demonstrated that, contrary to what had been thought previously, an N-terminal transmembrane domain is not a signal peptide, but is actually involved in ion translocation. In previous studies (Ferdig et al., 2004), an N-terminal transmembrane domain of PfNHE (distinct from the main bundle of twelve transmembrane domains which form the ion translocation pore) was annotated as a signal peptide; however the findings of Goswami et al. (2010) call this into question. The number of putative transmembrane domains in PfNHE was confirmed using the MacVector program (Fig. 6.1). The hydophobicity plot is consistent with the presence of one N-terminal transmembrane domain distinct from the 12 main transmembrane domains.

![Hydophobicity plot for PfNHE](image)

**Figure 6.1. Hydophobicity plot for PfNHE.** PfNHE is predicted to contain 12 transmembrane domains (highlighted in yellow) and a conserved N-terminal transmembrane domain (highlighted in red). The predicted secondary structure is consistent with the number of transmembrane domains found in other members of the NhaP/SOS1 family of NHEs (Goswami et al., 2010). The prediction diagram was generated using MacVector software.

### 6.3.1.3. PfNHE lacks the consensus sequence for binding to amiloride and its derivatives

The NHE inhibitor amiloride and its derivatives, including ethylisopropylamiloride (EIPA), bind to NHE proteins at a conserved region near transmembrane domains 3 and 4 (Kagami and Suzuki, 2005). Darley et al. have previously shown that human
NHE isoforms, yeast NHE and plant vacuolar NHX are highly conserved at the amiloride binding site\textsuperscript{164}VFFLPLLPI (sequence from HsNHE1; see Section 1.5.3.1 Darley \textit{et al.}, 2000). Mutations of the HsNHE1 sequence of either one or two amino acids (L167F and F168Y) led to a five-fold increase in the IC\textsubscript{50} for amiloride inhibition (Counillon\textit{et al.}, 1993), highlighting the importance of this conserved sequence. Kagami and Suzuki have previously shown that the sequence of the NhaP/SOS1 clade member, \textit{A. thaliana} SOS1 (AtSOS1; Figure 1.7 and Section 1.6.3.2) lacks the amiloride binding consensus region (Kagami and Suzuki, 2005), and physiological studies are consistent with AtSOS1 being amiloride-insensitive (Qiu\textit{et al.}, 2003; Guo\textit{et al.}, 2009).

The potential amiloride binding site of PfNHE was investigated by comparing the relevant amino acid sequence to that found in several other organisms (Fig. 6.2). The alignment of human NHE1 and 2 (HsNHE1,2), the yeast \textit{S. cerevisiae} NHX1 (ScNHX1), \textit{A. thaliana} NHX1 (AtNHX1) and \textit{Oryza sativa} NHX1 (OsNHX1) showed strong homology over the crucial 10 amino acid region (Fig. 6.2; Group A). By contrast, sequences from members of the NhaP/SOS1 clade, including PfNHE (as well as other proteins of interest from Table 6.1) show little homology at the amiloride binding region (Fig. 6.2; Group B). These sequences include those of PfNHE, \textit{P. yoelii} NHE (PyNHE1), \textit{T. gondii} NHE (TgNHE1), \textit{B. coprocola} NHE (BeNHE1) and SOS1 proteins from \textit{C. parvum} (CpSOS1), \textit{C. quinoa} (CqSOS1), \textit{A. thaliana} (AtSOS1), \textit{C. nodosa} (CnSOS1), and \textit{P. patens} (PpSOS1). As PfNHE lacks the consensus amiloride binding sequence, it is predicted that it will be insensitive to amiloride-class inhibitors.

\subsection*{Identification of conserved residues involved in Na\textsuperscript{+} transport in PfNHE}

Multiple studies have identified important conserved cation-binding residues in NhaP family members (Hamada \textit{et al.}, 2001; Goswami \textit{et al.}, 2010; Resch \textit{et al.}, 2011). In a recent study Resch \textit{et al.} identified several polar or charged residues and, in addition, identified residues important for NHE Na\textsuperscript{+}/K\textsuperscript{+} selectivity (Resch \textit{et al.}, 2011). Sequence homology was compared between several NhaP transporters, with different (known) cation selectivities, including \textit{Vibrio cholera} and \textit{Synechocystis} \textit{spp:} Vc/Syn-NhaP1 (transports Na\textsuperscript{+} or K\textsuperscript{+}), Vc/Syn-NhaP2 (K\textsuperscript{+} preferential, but can
still transport Na\(^+\)), Vc/Syn -NhaP3 (only K\(^+\)). Fig. 6.3 shows multiple, partial alignments of these aforementioned sequences, with those of plant and protozoal members of the SOS/NhaP1 family from Table 6.1 and Fig. 6.2. Highly conserved residues in PfNHE include: T379, D380, E404, N408, D409, R603 and T646. No residues specific to NhaP3-type (K\(^+\)-transporting) NHEs were identified in the PfNHE sequence. This is consistent with PfNHE transporting Na\(^+\) preferentially, and not K\(^+\).

Figure 6.2. Comparison of the amino acid sequence in the amiloride binding region in known amiloride-sensitive NHEs (Group A), and multiple SOS-like NHE amino acid sequences (Group B). Sequences were aligned with Clustal W and a region containing the amiloride-binding site was examined. Residue conservation in this region is indicated by dark shading. Sequences in Group A are highly conserved at the amiloride binding sequence, and the amiloride-sensitivity of HsNHE1 and 2, ScNHX1 and AtNHX1 has been demonstrated experimentally (Counillon et al., 1993; Darley et al., 2000; Kagami and Suzuki, 2005). By contrast, sequences in Group B are poorly conserved at the amiloride binding site. AtSOS1 has previously been shown to lack the amiloride binding sequence, and physiological studies are consistent with AtSOS1 being amiloride-insensitive (Qu et al., 2003; Kagami and Suzuki, 2005; Guo et al., 2009). Sequences were retrieved from GenBank with accession numbers as per Table 1, additionally: HsNHE1 (NP_003038.2), HsNHE2 (NP_003039.2), ScNHX1 (NP_010744.1), AtNHX1 (NP_198067.1), OsNHX1(NP_001060571.1), PyNHE1(EAA22449.1), CpSOS1 (CAD98616.1).
Alignment 1:
PpSOS1 SFSAMDFHQIKRGFMQLMLAAPPGVISTIFLGVLVVK
CqSOS1 SFSMEIHQIKRCQAMILLAGPGVLITFCQLGAKL
AtSOS1 SFSMEVHQIKRCQLGMVLLAVPGVLSTACQLSLVVK
CpSOS1 SATLNFHSVLRPNSALLAGPGAIIVMVLAFAIK
TaSOS1 TQTIDWYSFCNNLAGISVGIVQVAILGLHY
TgNHE1 TQSINWHFQRFGMGLLLAVGVAVVQVGLGVFMVY
PfNHE TQIANYAFKHFLYGGIEAAGVGVAGVQVGLGVFMVY
VcNhaP1 GLGKIKLPNLKDQKWEITVLAAGLTFSTFFGFTLYG
VcNhaP2 CMTRVAVAFVAPWPSVSLATLGVATTTLTLGALLM
VcNhaP3 SLTINFEKIGSVSNTVWSITVGALESVGATSAHHK
Syn-NhaP1 AWINLQWRLKINWFPILFALTGLVGCVGAIAFPLS
Syn-NhaP2 AINTDISRLSTIKPLTVLAAPGVGVSAITAVISLKL
Syn-NhaP3 GLNLGRELGVQGSRLNVTIGTLTLVGGLAHHK

Figure 6.3. Multiple partial alignments (1-3) of plant, protozoal and reference NhaP family members with conserved polar and charged residues shaded. Plant SOS1 members are distinguished in red, protozoal members are in purple, and the reference NhaPs are in black. Residues discriminating between NHEs of varying cation selectivity (Na⁺ or K⁺) are highlighted in yellow in alignments 1 and 2. Accession numbers are as per Table 6.1 and Figure 6.2 legend, with the following additional sequences included: Ve-NhaP1 (NP_230043.1), Ve-NhaP2 (NP_232330.1), Ve-NhaP3 (NP_230338.1), Syn-NhaP1 (NP_441245.1), Syn-NhaP2 (NP_441812.1), Syn-NhaP3 (NP_442407.1).
6.3.2. Investigating the role of PfNHE in pHi regulation

The bioinformatic analyses presented in Section 6.3.1 are consistent with PfNHE sharing characteristics with NhaP/SOS1 transporters. However, the physiological role of PfNHE has not been demonstrated, and there are conflicting results regarding the contribution of PfNHE to pHi regulation in asexual blood-stage *P. falciparum* parasites (Bosia *et al.*, 1993; Saliba and Kirk, 1999; Bennett *et al.*, 2007). These conflicting studies used somewhat differing methodologies. There were differences in the methods used to isolate the parasite from its host erythrocyte (in order to gain access to the extracellular surface of the parasite). There were also differences in the techniques used to impose an acid load on the parasite: either an ammonium pre-pulse (used in Saliba and Kirk, 1999) or an ionophore-mediated acidification (used in Bosia *et al.*, 1993; Bennett *et al.*, 2007). The effect of these variables on pHi regulation and NHE activity was investigated.

### 6.3.2.1. Parasites isolated using 0.05 % (w/v) saponin are viable

In their study, Bennett *et al.* (2007) isolated parasites from their host erythrocytes by exposing parasitised erythrocytes to saponin (of unspecified sapogenin content) for 3 min, then removing the detergent by repeated washing (Bennett *et al.*, 2007). They reported that under these conditions parasites treated with 0.03 % (w/v) saponin retain a low permeability to H⁺ whereas parasites treated with 0.04 % or 0.05 % (w/v) saponin show a significant H⁺ leak and are unable to maintain a pHi independent of pH₀ (Bennett *et al.*, 2007). In this study, as in previous work in this laboratory, parasites were isolated by exposure to 0.05 % (w/v) saponin (of which ≥ 10 % was the active agent sapogenin) for less than 20 s before washing the cells. There is ample evidence that parasites isolated using this protocol are capable of maintaining large transmembrane gradients for H⁺ (Saliba and Kirk, 1999) and Ca^{2+} (Alleva and Kirk, 2001), as well as generating and maintaining a large membrane potential (Allen and Kirk, 2004b).

To address this discrepancy, the membrane permeability of parasites isolated using either 0.03 % or 0.05 % (w/v) saponin was investigated. In both cases > 98 % of the parasites excluded trypan blue (data not shown). Fig. 6.4 shows that in both cases,
and as has been demonstrated previously for parasites isolated using 0.05 % (w/v) saponin (Lehane et al., 2004), a reduction of pH₀ from 7.1 to 6.5 (i.e., a decrease of 0.6 pH units) resulted in a much smaller decrease in pHᵢ, from 7.3 to ~7.2 (i.e., a decrease of 0.1 pH units), with pHᵢ being maintained well above pH₀. This is consistent with the isolated parasites actively regulating pHᵢ. On addition of the H⁺ ionophore CCCP there was an immediate acidification, with pHᵢ decreasing to pH₀ (i.e., pH 6.5). The data indicate that for parasites isolated from their host cells by very brief exposure to saponin there was no significant difference in the H⁺ permeability of parasites treated with 0.03 % and 0.05 % (w/v) saponin; in both cases the parasite plasma membrane retained the ability to maintain a significant transmembrane pH gradient.

Figure 6.4. H⁺ permeability of parasites isolated using varying [saponin]. Parasites isolated using either 0.05 % (dark trace) or 0.03 % (light trace) (w/v) saponin were suspended initially in standard saline at pH 7.1 then, at the point indicated by the black triangle, transferred to standard saline at pH 6.5. At the point indicated by the white triangle, 10μM of the H⁺-ionophore CCCP was added to the suspension, causing pHᵢ to decrease to pH₀. The top pH₀ panel and horizontal dashed lines indicate the extracellular pH values of 7.1 and 6.5.
6.3.2.2. Recovery from an ammonium pre-pulse is Na\(^+\)-independent, but recovery from an ionophore-mediated acidification is Na\(^+\)-dependent

Parasites were subjected to an intracellular acid load either via an ammonium pre-pulse or by treating cells suspended in an acidic medium with 0.8 \(\mu\)M nigericin (the same concentration used by Bennett et al., 2007). The initial resting pH\(_i\) of cells in Na\(^+\)-containing standard saline was 7.29 ± 0.02 \((n = 8)\); see for example Fig. 6.5A). In cells subjected to an NH\(_4\)\(^+\)-prepulse manoeuvre (Fig. 6.5A), addition of 40 mM NH\(_4\)Cl led to a transient alkalinisation which was followed, on removal of the NH\(_4\)Cl, by a cytosolic acidification, with pH\(_i\) decreasing to 7.01 ± 0.03 \((n = 3)\). Over the following minutes pH\(_i\) recovered to 7.32 ± 0.04 \((n = 3)\), consistent with H\(^+\) being extruded by the parasites. The pH\(_i\) recovery of cells acidified in this manner was not Na\(^+\)-dependent, and was effectively inhibited by the specific V-type H\(^+\)-pump inhibitor concanamycin A. The data are consistent with the recovery of pH\(_i\) from an NH\(_4\)\(^+\) pre-pulse induced acidification being due to the extrusion of H\(^+\) by the plasma membrane V-type H\(^+\)-ATPase.

For parasites acidified using ionophore, the manoeuvre entailed permeabilising the parasite plasma membrane to H\(^+\) with nigericin, then exposing the parasites to an acidic medium (pH 6.2), thereby reducing pH\(_i\). Bovine serum albumin was then added to the solution in order to ‘scavenge’ the nigericin from the membrane (a manoeuvre intended to ‘lock’ pH\(_i\) at the lower pH) after which the cells were returned to media at pH 7.1 (in the presence or absence of Na\(^+\)) and the pH\(_i\) recovery was monitored. As shown in Fig. 6.5B, in Na\(^+\)-containing medium pH\(_i\) recovered to 7.09 ± 0.01 \((n = 5)\) within a few minutes. However, for parasites in a Na\(^+\)-free (NMDG\(^+\)-containing) medium the recovery was slower and incomplete. The pH\(_i\) recovery following a nigericin-induced acidification was unaffected by concanamycin A, or the NHE inhibitor EIPA. The Na\(^+\)-dependence of the pH\(_i\) recovery following a nigericin-induced acidification contrasts with the Na\(^+\)-independence of the pH\(_i\) recovery following a NH\(_4\)\(^+\)-prepulse induced acidification (Fig. 6.5A).
Figure 6.5. pH<sub>i</sub> recovery from an intracellular acidification via an ammonium pre-pulse (A) or an ionophore mediated acidification (B). (A) Saponin-isolated parasites were acidified by the addition (black triangle) then removal (white triangle) of 40 mM NH<sub>4</sub>Cl (i.e., an NH<sub>4</sub><sup>+</sup> pre-pulse) then resuspended in either: standard (Na<sup>+</sup>-containing) saline (pH 7.1; dark trace); a Na<sup>+</sup>-free medium (in which NaCl is isosmotically replaced with NMDG-Cl; light trace); or the Na<sup>+</sup>-saline to which had been added the V-type H<sup>+</sup>-pump inhibitor concanamycin A (75 nM; dark grey trace). The pH<sub>i</sub> recovery was independent of Na<sup>+</sup> but inhibited by concanamycin A. The extracellular NH<sub>4</sub>Cl concentration over the course of the experiment is shown above the trace. (B) Saponin-isolated parasites were acidified by suspension for 2 min in an acidic Na<sup>+</sup>-free solution (pH 6.2) containing 0.8 μM nigericin. The parasites were then centrifuged and resuspended for 2 min in a solution of the same composition but containing 5 mg/mL BSA (Fraction V) in place of nigericin. The trace begins when the parasites were resuspended at an extracellular pH of 7.1 in either Na<sup>+</sup>-saline (± 75 nM concanamycin A, ± 200 μM EIPA) or Na<sup>+</sup>-free (NMDG<sup>+</sup>-containing) solution. For cells acidified using this protocol there was a Na<sup>+</sup>-dependent pH<sub>i</sub> recovery, unaffected by concanamycin A or EIPA. All traces are representative of those obtained in at least three independent experiments.
6.3.2.3. The rate of recovery from an ionophore mediated acidification shows the selectivity $K^+ > Rb^+ > Na^+$

In the context of pH$_i$ regulation studies in other cell types, exposure of cells to nigericin can give rise to artefacts; specifically, the presence of residual concentrations of nigericin in the cell membrane can give results mimicking K$^+/H^+$ and Na$^+/H^+$ exchange (Richmond and Vaughan-Jones, 1997; Bevensee et al., 1999). One possibility is that the Na$^+$-dependence of the pH$_i$ recovery seen in isolated parasites following a nigericin-induced acidification is due to the transport of H$_+$ (in exchange for Na$_+$) through residual nigericin, rather than via an NHE. To examine this possibility, the effect of different extracellular cations on the pH$_i$ recovery of isolated parasites following a nigericin-induced acidification was investigated.

Fig. 6.6 shows that for parasites suspended in a solution containing Rb$^+$ the pH$_i$ recovery was faster than that for cells suspended in the presence of Na$^+$. In parasites suspended in K$^+$ the recovery was faster still, with the pH$_i$ reaching a value of $\sim$7.1 within a few seconds. The selectivity series evident in Fig. 6.6 (i.e., $K^+ > Rb^+ > Na^+$) is the same as that exhibited by nigericin (Pressman, 1976), consistent with the hypothesis that the pH$_i$ recovery seen under these conditions is due to H$^+/cation exchange via residual ionophore.

6.3.2.4. Recovery from an ionophore mediated acidification is dependent on [BSA]

The effect of different concentrations of the nigericin-scavenging agent BSA on the rate of the subsequent pH$_i$ recovery was investigated. As the concentration of BSA to which the parasites were exposed prior to initiating the pH$_i$ recovery increased from 1.25 to 15 mg/mL the rate of the recovery (for parasites in a Na$^+$-containing solution) decreased (Fig. 6.7). This finding is consistent with higher concentrations of BSA removing greater amounts of nigericin from the parasite membrane, resulting in there being less residual ionophore in the membrane and hence a slower pH$_i$ recovery.
Figure 6.6. Cation selectivity series for the recovery of $\text{pH}_i$ following an ionophore induced acidification. Saponin isolated trophozoites were acidified using 0.8 $\mu$M nigericin (as described in the legend to Fig. 6.5) and $\text{pH}_i$ recovery recorded in solutions containing different extracellular cations ($\text{Na}^+$, $\text{Rb}^+$ and $\text{K}^+$; all at pH 7.1). The traces are representative of those obtained from at least three separate cell preparations.

Figure 6.7. Effect of different concentrations of BSA on the $\text{pH}_i$ recovery from an ionophore mediated acidification. Saponin isolated trophozoites were acidified using 0.8 $\mu$M nigericin (as described in the legend to Fig. 6.5) and $\text{pH}_i$ recovery recorded in Na$^+$-containing solution. The [BSA] was varied during the 2 min prior to resuspension in the pH 7.1 medium. As [BSA] was increased the $\text{pH}_i$ recovery seen following the increase in the [pH]o slowed. The traces are representative of those obtained from at least three separate cell preparations.
As shown previously (Lehane et al., 2004) and noted in Section 6.3.2.1, isolated parasites exposed to extracellular media having a range of pH values regulate their pH within a narrow range. This is illustrated by the broken line in Fig. 6.8 (data from Lehane et al., 2004). Contrastingly, for parasites subjected to an ionophore mediated acidification, then permitted to undergo pHj recovery in media with varying pHo values, the final pHj reached was, in each case, equal to pHo (Fig. 6.8). This indicates that parasites subjected to this manoeuvre have lost the capacity to regulate its pHj independently of pHo, which would not be true had the parasites recovered their pH via an endogenous pHj regulatory mechanism.

Figure 6.8. Relationship between final pHj value achieved and pHo for the pHj recovery from an ionophore mediated acidification. The black triangles show the relationship between the pHj and pHo in cells acidified using 0.8 μM nigericin (as described in the legend to Fig. 6.5) then allowed to recover their pHj in Na+-containing media at a range of different pH values. The solid line, indicating the relationship pHj = pHo, provides a good fit to the data; i.e., in each case the final pHj reached by the cells subjected to nigericin/BSA pretreatment was close to pHo. The open circles and broken line are taken directly from a previous study (Lehane et al., 2004) and show the relationship between pHj and pHo in isolated parasites not subjected to the nigericin/BSA pre treatment. The data points are collated from four independent experiments.
6.3.3. Characterising cation regulation in an NHE knock-down strain

The results of Section 6.3.2 are consistent with the Na\textsuperscript{+}-dependent recovery from an ionophore-induced acid load being via residual nigericin and not PfNHE. To investigate a possible role for PfNHE in cation regulation, \([\text{Na}^+]_i\) and \(p\text{H}_i\) regulation were characterised in a PfNHE knock-down strain generated by Nkrumah et al. (2009). In this study, the AXA4 strain (referred to henceforth in Chapter 6 as PfNHE-kd), generated from the GC03 parent line, was characterised.

6.3.3.1. PfNHE-kd does not have increased sensitivity to excess \([\text{Na}^+]_o\)

Nkrumah et al. (2009) investigated sensitivity of the PfNHE-kd line to quinine. Although a decrease in quinine IC\textsubscript{50} was observed in the PfNHE-kd line, relative to the parent line (GC03), the difference was not statistically significant (Nkrumah et al., 2009). In this study, the IC\textsubscript{50} values determined for quinine using a standard \(^{3}\text{H}\)-hypoxanthine growth assay were 92 ± 19 nM (n = 4) in the parent strain and 87 ± 18 nM (n = 4) for the PfNHE-kd strain (Fig. 6.9A). These values were consistent with the trend reported previously (Nkrumah et al., 2009), but once again the difference did not reach statistical significance (p = 0.85).

The ability of the PfNHE-kd line to proliferate in the presence of supra-physiological \([\text{Na}^+]_o\) was compared to that of the parent strain (as per Section 5.2.9). The IC\textsubscript{50} values for excess Na\textsuperscript{+} (above the \(\sim 133\) mM normally present in parasite culture medium) were between 38 and 75 mM Na\textsuperscript{+} for both strains (i.e., growth was \(\sim 100\%\) at 38 mM excess Na\textsuperscript{+}, but \(\sim 0\%\) at 75 mM excess Na\textsuperscript{+}; intermediate concentrations were not tested). This result is consistent with the PfNHE-kd line having similar susceptibility to the growth-inhibitory effects of supra-physiological \([\text{Na}^+]_o\) as the parental GC03 strain.
Figure 6.9. Comparison of dose-response curves for (A) the QN sensitivity and (B) the sensitivity to excess [Na\(^+\)]\(_{o}\) of the PfNHE-kd line and the parent line (GC03). Parasite proliferation was measured using a standard \(^{3}\)Hhypoxanthine incorporation assay (Section 2.10). The values shown are the mean (± S.E.M.) from four experiments, each performed in triplicate. In panel (B) ‘Excess [Na\(^+\)]\(_{o}\)’ denotes the increase in [Na\(^+\)]\(_{o}\) above that normally present in the parasite culture medium (~133 mM).
6.3.3.2. \([\text{Na}^+]_i\) regulation is not altered in the PfNHE-kd line

Aspects of \([\text{Na}^+]_i\) regulation were investigated in the PfNHE-kd and GC03 strains using the \(\text{Na}^+\)-sensitive dye SBFI. The resting \([\text{Na}^+]_i\) was not significantly different between the two strains at 13.5 ± 1.1 mM (n = 10) in PfNHE-kd compared to 13.6 ± 1.6 mM (n = 9; p = 0.95) in the GC03 parent. There was also no significant difference in the rate of \(\text{Na}^+\) efflux following a \(\text{Na}^+\) load, imposed as per Section 3.2.7. The \(t_{1/2}\) to complete recovery in the PfNHE-kd line was 169 ± 39 s (n = 4) compared to the parent line 148 ± 11 s (n = 6; p = 0.55). These observations are consistent with knock-down of PfNHE resulting in no impairment of the parasite’s \(\text{Na}^+\) extrusion mechanism.

6.3.3.3. \(p\text{Hi}_i\) regulation is not altered in the PfNHE-kd line

Aspects of \(p\text{Hi}_i\) regulation were investigated in the PfNHE-kd and GC03 strains using the \(p\text{Hi}_i\)-sensitive dye BCECF. As was found by Nkrumah et al. (2009), in this study the resting \(p\text{Hi}_i\) was not significantly different between the two strains: 7.34 ± 0.06 pH units (n = 4) in PfNHE-kd parasites compared to 7.37 ± 0.06 pH units (n = 3; p = 0.75) in the GC03 parent. The parasite strains were subjected to an acidification using the \(\text{NH}_4^+\) pre-pulse technique (described in Section 6.3.2.2). If PfNHE activity contributes to the \(p\text{Hi}_i\) recovery (\(\text{H}^+\) efflux) from a \(\text{NH}_4^+\) pre-pulse-mediated acidification then slower recovery would be observed under \(\text{Na}^+\)-free conditions compared to recovery in a \(\text{Na}^+\)-containing solution. As shown in Fig. 6.10, both parasite strains recovered in a \(\text{Na}^+\)-independent manner (using both choline\(^+\) and NMDG\(^+\) as \(\text{Na}^+\)-replacements), and recovery was concanamycin A-sensitive. No differences were observed in the recovery between the GC03 and PfNHE-kd strains. These observations are consistent with the knock-down of PfNHE resulting in no impairment of the mechanisms by which the parasite regulates its \(p\text{Hi}_i\).
Figure 6.10. Comparison of pH$_i$ recovery from an intracellular acidification imposed via an ammonium pre-pulse in the PfNHE-kd and GC03 parent strains. Saponin-isolated parasites were acidified by the addition (black triangle) then removal (white triangle) of 40 mM NH$_4$Cl (i.e., an NH$_4^+$ pre-pulse) then resuspended in either standard (Na$^+$-containing) saline (pH 7.1), Na$^+$-free medium (in which Na$^+$ was replaced with NMDG$^+$ (or choline$^+$; data not shown for clarity)) or the Na$^+$-containing saline to which had been added the V-type H$^+$-pump inhibitor concanamycin A (75 nM). For both strains the pH$_i$ recovery was independent of Na$^+$ but inhibited by concanamycin A. For clarity the full data set for the GC03 parent is not shown. The extracellular NH$_4$Cl concentration over the course of the experiment is shown above the trace. The traces are representative of those obtained from at least three separate cell preparations.
6.4. Discussion

6.4.1. PfNHE has characteristics of the Na\(^{+}\)-extruding NhaP1/SOS1 family of NHEs

Brett et al. (2005) separated the NHEs within the CPA1 family into four distinct clades (Fig. 1.7), and NHEs phylogenetically related within each clade were found to show similar physiological properties in terms of cation selectivity, inhibitor sensitivity and localisation (Brett et al., 2005). PfNHE (named PlfSOS1 in the Brett et al. study) clustered in the plant dominated ‘NhaP/ SOS1’ clade, including NHEs from \(A.\) thaliana, \(O.\) sativa, \(P.\) patens, \(C.\) nodosa and another protozoan, \(C.\) parvum (Brett et al., 2005). The sessile nature of plants, and the increasing problem of salinity in agricultural zones means salinity tolerance and transporters involved in [Na\(^{+}\)]\(_{i}\) regulation have been extensively studied in plants (see detailed recent review in Flowers and Colmer, 2008; Munns and Tester, 2008; Plett and Moller, 2009; Kronzucker and Britto, 2010). Several members of the ‘SOS1’ clade have been characterised (Shi et al., 2000; Garciadeblas et al., 2007; Martinez-Atienza et al., 2007; Fraile-Escanciano et al., 2010), and are implicated in amiloride-insensitive, electroneutral Na\(^{+}\) regulation, localised to the plant plasma membrane (Qiu et al., 2003). This phylogenetic analysis, and the results of the pBLAST search in Table 6.2, are consistent with the hypothesis that the role of PfNHE is in regulating [Na\(^{+}\)]\(_{i}\), rather than pH\(_{i}\). It is important to note that PfNHE is not in either of the two clades which contain all nine human NHE isoforms (Fig 1.7 and Brett et al., 2005).

The C-terminal region of PfNHE (~1300 amino acids) is substantially longer than the C-terminal domain of human NHE1 (315 amino acids) (Fliegel, 2005) and longer than NHEs belonging to the ‘plasma membrane’ or ‘intracellular clades’ with an average C-terminal length of 199 and 365 amino acids respectively (Brett et al., 2005). Comprehensive studies of \(S\)ynechocystis and \(Aphanothece\) NHEs (both \(NhaP\) clade NHEs) indicated that the C-terminal region is essential for transport activity and ion selectivity (Hamada et al., 2001; Waditee et al., 2001; Waditee et al., 2006), and the C-terminal region is also believed to be involved in regulation of exchange activity through binding of signalling molecules and phosphorylation (Slepkov et al., 2007). Therefore, the length of the C-terminus in PfNHE and other SOS family members may reflect complex regulation or an additional role specific to this clade.
Consistent with this, recently in *Arabidopsis* AtSOS1, an auto-inhibitory domain was identified in the C-terminal domain. Phosphorylation of this auto-inhibitory domain by the kinase AtSOS2 results in activation of the NHE (Quintero *et al.*, 2011). It has also been suggested that in AtSOS1 the long C-terminal domain is essential to stabilise the protein under conditions of salt stress through direct interactions with Na\(^+\) (Zhu, 2002). Fifteen potential phosphorylation sites have been identified *in silico* in the PfNHE C-terminal (Treeck *et al.*, 2011).

NhaP/SOS1 transporters have a distinctive N-terminal region, containing a single transmembrane domain, distinct from the 12 main helices that form the ion translocation pore (Fig. 6.1 and Goswami *et al.*, 2010). Goswami *et al.* demonstrated that this N-terminal transmembrane helix was involved in ion translocation, as no NHE activity was observed in N-terminal truncation mutants despite normal folding, dimerisation and orientation (Goswami *et al.*, 2010). It was hypothesised that the involvement of the N-terminal transmembrane helix in ion translocation was unlikely to be by direct interaction with the cations, as the crystal structure showed that the helix is not in contact with the main transport bundle. Instead they proposed that the N-terminal helix is involved in allosteric regulation of the transporter (Goswami *et al.*, 2010). The function of the predicted, conserved N-terminal transmembrane domain in PfNHE is a further avenue for research, and a potential novel region for antimalarials targeting PfNHE as human NHEs do not have this extra helix.

PfNHE was found to lack the consensus sequence for amiloride binding (Fig. 6.2). Therefore, the lack of effect of EIPA on [Na\(^+\)]\(\text{i}\) observed in Chapter 3 (Fig. 3.7) does not disprove the hypothesis that PfNHE may be involved in Na\(^+\) efflux from the parasite. Conversely, the report from Bennett *et al.* (2007) of an effect of EIPA on pH\(\text{i}\) regulation in intracellular parasites (unable to be reproduced here) is not necessarily indicative of PfNHE involvement. In human NHE isoforms, binding of amiloride and other benzoylguanidine derivatives (including HOE694, cariporide and EMD85131) involves residues in transmembrane domain 4 (Counillon and Pouyssegur, 2000; Slepkov *et al.*, 2007). This region is conserved in vacuolar plant and yeast NHX proteins which show amiloride-sensitivity (Gaxiola *et al.*, 1999; Darley *et al.*, 2000). In Fig. 6.2, the human NHE sequences were more tightly conserved at the amiloride binding site than the plant NHX sequences. Consistent
with this observation, the IC$_{50}$ for amiloride against HsNHE1 is 3 μM (Counillon et al., 1993), whereas 120 μM amiloride is required for 100% inhibition of AtNHX1 (Darley et al., 2000).

The Arabidopsis AtSOS1 was predicted on the basis of sequence analysis to lack an amiloride binding site (Zhu, 2000; Kagami and Suzuki, 2005), and a study of amiloride treated seedlings demonstrated that although amiloride caused a disruption of the transmembrane H$^+$ gradient this was not due to amiloride interactions with AtSOS1 (Guo et al., 2009). This disruption of the H$^+$ gradient by EIPA has also been observed in P. falciparum (Bosia et al., 1993). In this study it was shown that both amiloride and EIPA inhibited parasite growth, with IC$_{50}$ values of 12 μM and 120 μM respectively (Bosia et al., 1993). In a later study, EIPA was shown to inhibit parasite growth with a more potent IC$_{50}$ of < 10 μM (Brand et al., 2003), which the authors suggested was due to inhibition of a parasite-induced erythrocyte-membrane cation channel, together with host and parasite NHEs. TgNHE1, like PfNHE, does not have a strong consensus sequence for amiloride binding (Fig. 6.2), however some amiloride-sensitivity of H$^+$ efflux from Toxoplasma has been reported (Arrizabalaga et al., 2004). Further characterisation of both TgNHE1 and PfNHE is necessary to confirm the predicted amiloride-insensitivity proposed in this study.

Amiloride inhibits multiple Na$^+$ transporters, including Na$^+$ channels, NHEs and Na$^+$/Ca$^{2+}$ exchangers (Kleyman and Cragoe, 1988), as well as a variety of other proteins such as the adenosine A1 receptor in brain tissues (Garritsen et al., 1992), the anticancer chemotherapy target urokinase-type plasminogen activator (Matthews et al., 2011), various tyrosine/serine kinases and phosphatases (Kim and Lee, 2005) and RNA polymerases (Gazina et al., 2011). Clearly with so many targets, it is difficult to use amiloride and its derivatives as specific tools to examine NHE function.

6.4.2. Conserved residues of Na$^+$-transporting NHEs are present in PfNHE

PfNHE has several highly conserved polar and charged residues that were previously identified in NhaP family members from V. cholera and Synechocystis spp (Hamada et al., 2001; Goswami et al., 2010; Resch et al., 2011). With the exception of
CpSOS1 and TaSOS1, a lysine is present in position 328 (using the enumeration of residues in PfNHE; Alignment 1, Figure 6.3) in all NHE sequences included in the alignment apart from Vc/Syn NhaP2/3 (which transport K⁺). This indicates that this residue may play a role in Na⁺ selectivity. In alignment 2, the hydrophilic S377 and S405 and the polar N408 are replaced with neutral valine, glycine and isoleucine in Syn/Vc NhaP3, consistent with these residues being involved in Na⁺-selectivity (Resch et al., 2011). Notably at these positions PfNHE (and other members of the SOS1 clade) contain the SxTD/ESxxND aspartate motifs consistent with Na⁺-selectivity.

The hydroxyl group provided by the side chain of either serine or threonine in position 345 was absent in the closely related Plasmodium, Toxoplasma and Theileria NHE sequences, in which it was replaced with a glutamine. This also occurred in position 639 (Alignment 3), in which all sequences contained a serine or threonine apart from those of Plasmodium, Toxoplasma and Theileria which had an isoleucine. It is unclear what the precise role of these residues is, and whether the absence of a serine/threonine in these closely related parasites is significant.

The amino acid region shown in Alignment 2 (Fig. 6.3) contains several charged lysine and glutamate residues, along with two conserved aspartate residues (D380 and D409) that are essential for transporter activity (Hellmer et al., 2003). The first aspartate residue is essential for Na⁺ antiport (Resch et al., 2011) and, consistent with this, it is replaced with glycine in K⁺-transporting Vc/Syn NhaP3. Mutation of the first aspartate to glutamate or tyrosine abolished Na⁺/H⁺ antiport activity in Syn-NhaP1 (Hamada et al., 2001). The second aspartate is completely conserved in the CPA superfamily of transporters. In CPA2 members, including NhaA there is a DD motif at this position, whereas in CPA1 members, including the NhaP/SOS1 members in this study, the motif is ND (Goswami et al., 2010). The additional charge in the CPA2 family has been proposed to allow the exchange of two H⁺, whereas CPA1 members only exchange one H⁺ (Goswami et al., 2010). Thus, the presence of the ⁴⁰⁸ND motif in PfNHE is consistent with a prediction of electroneutral antiport in P. falciparum.
Finally, consistent with NhaP/SOS1 NHEs being part of the CPA1 superfamily, in alignment 3 (Fig. 6.3), all NHEs (apart from SynNhaP2) investigated in this study contain the RG motif, which is conserved in all CPA1 families, and is IG in CPA2 transporters (Goswami et al., 2010).

In summary, on the basis of sequence comparisons with transporters of known function, PfNHE is predicted to be an amiloride-insensitive, plasma membrane Na⁺/H⁺ antiporter, catalysing the electroneutral exchange of Na⁺ for H⁺.

6.4.3. NHEs of the closely related apicomplexan parasite *T. gondii*

PfNHE was found to share the highest sequence similarity with NHEs from other protozoa; *T. gondii, T. annulata, C. muris* and *B. bovis* (Table 6.1). Transport proteins of the latter three organisms have not been studied in any detail. The closest homologue to PfNHE was *Toxoplasma* TgNHE1, which is localised to the *Toxoplasma* plasma membrane (Arrizabalaga et al., 2004). TgNHE1 was identified in a random insertion mutagenesis screen for parasites that failed to exit the parasitophorous vacuole when exposed to the Ca²⁺ ionophore A23187 (Arrizabalaga et al., 2004). A TgNHE1 knock-out line was shown to have increased [Ca²⁺], and the authors hypothesised that this may be due to a decrease in Ca²⁺/H⁺-ATPase activity, resulting from a reduction in the pH-gradient in the NHE knock-out parasites (Arrizabalaga et al., 2004). Alternately, they proposed that if the role of TgNHE1 was in Na⁺ efflux, than an increased [Na⁺], in the TgNHE1 knock-outs may cause Ca²⁺ release from intracellular stores (Arrizabalaga et al., 2004) as a Na⁺-stimulated Ca²⁺-release from acidocalcisomes has been previously demonstrated in trypanosomatids (Vercesi and Docampo, 1996; Vercesi et al., 2000). There were no measurements of [Na⁺] in the study by Arrizabalaga et al. (2004).

Only one NHE has been annotated in the *P. falciparum* genome, whereas four putative NHEs have been annotated in the *T. gondii* genome (Gajria et al., 2008). TgNHE2 is localised to an invasion organelle, the rhoptries (Karasov et al., 2005). It has been hypothesised that TgNHE2 is involved in acidification of the organelle, as it has been estimated to have a pH of 3.5 - 5.5 when young, which alkalinises to pH 5-7 as the rhoptry matures (Shaw et al., 1998). TgNHE3 localises to the parasite’s plant-
like vacuole, and TgNHE3 knock-out strains were more sensitive to both hyperosmotic shock and increased levels of extracellular Na⁺ (Francia et al., 2011). The TgNHE3 knock-out parasites also had significantly higher [Ca²⁺]ᵢ and reduced host invasion efficiency through abnormal secretion of the micronemes (an organelle involved in invasion; Francia et al., 2011). TgNHE4 remains uncharacterised. It is unclear why these two closely related apicomplexan parasites would have a varying number of NHEs, and it remains to be investigated if PfNHE has multiple roles in Plasmodium physiology that are fulfilled by distinct NHEs in Toxoplasma.

6.4.4. PfNHE is not involved in pHᵢ regulation in P. falciparum

Although the bioinformatic analyses provided in this study are consistent with the role of PfNHE being in Na⁺ efflux, there have been several studies implicating PfNHE in H⁺ efflux (Bosia et al., 1993; Bennett et al., 2007), despite the report that the V-type H⁺-ATPase plays the primary role in pHᵢ regulation in P. falciparum (Saliba and Kirk, 1999). The estimates reported in this chapter for pHᵢ of 7.29 - 7.37 (Section 6.3.2.2 and Section 6.3.3.3) are consistent with previously reported values of pHᵢ ~7.3 (Wunsch et al., 1998; Saliba and Kirk, 1999; Hayashi et al., 2000; Kuhn et al., 2007).

If subjected to an experimentally imposed acidification the parasite extrudes H⁺, thereby undergoing a pHᵢ recovery. When the parasites were acidified via an ammonium pre-pulse, pHᵢ recovery was Na⁺-independent, and was inhibited by the V-type H⁺-ATPase inhibitor concanamycin A (Fig. 6.5A). However, when parasites were acidified via an ionphore induced acidification, the pHᵢ recovery was Na⁺-dependent and was not sensitive to concanamycin A or EIPA (Fig. 6.5B). The lack of EIPA sensitivity observed in this study contrasts with the report from Bennett et al. that the recovery of pHᵢ from a nigericin-induced acidification is inhibited by EIPA, with a computed \( Kᵢ \) of 0.9 µM (Bennett et al., 2007). They did not present the data relating to this finding.

In their study, Bennett et al. assumed that the Na⁺-dependent pHᵢ recovery was mediated by a plasma membrane NHE (Bennett et al., 2007). However the results presented in Section 6.3.2 are consistent with an alternative explanation of the data:
that, as has been reported previously (Saliba and Kirk, 1999), the recovery from an ammonium-induced acidification is mediated by the endogenous V-type H\(^+\) pump, whereas the recovery seen following a nigericin-induced acidification represents H\(^+\)/cation exchange via residual nigericin remaining in the parasite plasma membrane. Several lines of evidence support this hypothesis: (i) the rates of recovery from an ionophore-induced acidification were in the same order as the selectivity series for nigericin (K\(^+\) > Rb\(^+\) > Na\(^+\); Fig 6.6), (ii) the recovery was faster at lower concentrations of the nigericin-scavenging agent, BSA (Fig. 6.7), and (iii) in the parasites acidified using nigericin pH\(_i\) recovered to pH\(_o\), and not the normal resting pH\(_i\), of the parasite (Fig. 6.8). Some NHEs characterised to date do have selectivity for K\(^+\) over Na\(^+\), such as Vc/Syn NhaP3 (Resch et al., 2011), human HsNHE7 (Numata and Orlowski, 2001) and LxNHE2 from tomatoes (Venema et al., 2003); point (i) alone therefore does not rule out PfNHE involvement (if, despite the bioinformatics predictions, PfNHE is K\(^+\) selective). However, the results of the other experiments were also consistent with nigericin contamination of the parasite plasma membrane.

Nigericin (and other ionophores) can bind to plastic (i.e., tubing) in perfusion systems, thus contaminating experimental solutions, and residual nigericin in cell membranes can give results which mimic K\(^+\)/H\(^+\) and Na\(^+\)/H\(^+\) exchangers (Richmond and Vaughan-Jones, 1997; Bevensee et al., 1999). Residual nigericin in the parasite plasma membrane would also account for the observation by Bennett et al. (2007) that in cells treated with nigericin, followed by the ionophore scavenger BSA in an acidic medium, there was no pH recovery until pH\(_o\) was increased; if the nigericin were effectively removed by BSA then the endogenous pH\(_i\) regulatory mechanism(s) might be expected to have mediated at least some degree of recovery in the acidic medium. When nigericin was present, the concanamycin A-sensitive H\(^+\) pump was short-circuited by the flux of H\(^+\) via residual nigericin, and the observed increase in pH\(_i\) seen on transferring the cells from an acidic medium to medium at a physiological pH simply reflected the (concanamycin A-insensitive) ionophore-mediated equalisation of the intra- and extracellular pH.

This raises obvious questions regarding the reported correlation between the rate of pH\(_i\) recovery following a nigericin-induced acidification and the sensitivity of the
parasite to quinine (Bennett et al., 2007). Following the publication of the results contained in Section 6.3.2 (Spillman et al., 2008), another research group investigating quinine resistance and pH\textsubscript{i} regulation in PfNHE knock-down strains (used in Section 6.3.3, and see discussion in Section 6.4.5) failed to obtain reproducible results using the nigericin acidification technique, stating “variability from experiment to experiment” that “could be due to differing residual amounts of nigericin in the parasite plasma membrane” (Nkrumah et al., 2009).

### 6.4.5. [Na\textsuperscript{+}]\textsubscript{i} and pH\textsubscript{i} regulation in \textit{P. falciparum} is not altered in the PfNHE-kd

To address the question of the physiological function of PfNHE, aspects of ion regulation were investigated in a PfNHE knock-down strain, previously generated by Nkrumah et al. (2009). Nkrumah et al. generated multiple PfNHE knock-down strains using the 1BB5 and 3BA6 strains (both chloroquine resistant, with low levels of quinine resistance) and the GC03 strain (chloroquine and quinine sensitive). Two knock-down strains were generated from each parent and the level of transcript reduction was shown to vary between 40 - 70 % for the six strains, resulting in a 50 - 80 % reduction in protein expression (Nkrumah et al., 2009). All four knock-down parasite strains from the 1BB5 and 3BA6 parent strains had a significantly lower IC\textsubscript{50} for quinine compared to the parent strains, however the two knock-down strains derived from the GC03 parent did not display a significant shift in quinine IC\textsubscript{50} (Nkrumah et al., 2009). Due to parasite import permit regulations changing during the process of acquiring these parasite strains from Dr David Fidock at Columbia University, only the GC03 parent and its mutant (AXA4, referred to here as PfNHE-kd) were available for investigation during this study.

As shown in the results presented in Sections 6.3.3.1-2, there were no differences in multiple aspects of [Na\textsuperscript{+}]\textsubscript{i} regulation investigated between the PfNHE-kd and the parent strain (including the ability to proliferate in excess [Na\textsuperscript{+}]\textsubscript{o}, maintenance of resting [Na\textsuperscript{+}]\textsubscript{i}, and the ability to recover from an imposed Na\textsuperscript{+} load). Similarly, there were no differences in the aspects of pH\textsubscript{i} regulation investigated between the PfNHE-kd and the parent strain (Section 6.3.3.3; including maintenance of resting pH\textsubscript{i} and the ability to recover from an NH\textsubscript{4}\textsuperscript{+} pre-pulse mediated acidification). The results for resting pH\textsubscript{i} under normal physiological conditions reported in this study (7.37 in the
Parent strain compared to 7.34 in PfNHE-kd parasites) are consistent with the pH\texttext{\textsubscript{i}} measurements of Nkrumah \textit{et al.} (2009). Nkrumah \textit{et al.} found the GC03 parent strain had a resting pH\texttext{\textsubscript{i}} of 7.27, and the knock-down strains were not significantly different, with pH\texttext{\textsubscript{i}} falling within the range of 7.27 - 7.34 (Nkrumah \textit{et al.}, 2009).

One possibility to explain the lack of phenotype observed between the GC03 and PfNHE-kd strains is that the degree of protein reduction in the knock-down strain is not sufficient to disrupt ion regulation. Perhaps the remaining 20 – 50 % PfNHE protein is capable of maintaining normal cation homeostasis. For example, if PfNHE is involved in pH\texttext{\textsubscript{i}} regulation it is plausible that with ~50 % the normal number of transporters the cell can still maintain a normal resting pH\texttext{\textsubscript{i}}. However, even if this were the case it might be expected that if PfNHE played a major role in the extrusion of H\textsuperscript{+} from the parasite the rate of recovery from an acid load would be diminished in the PfNHE-kd strain. The fact that this was not observed (Fig. 6.10) again argues against a role for PfNHE in parasite pH regulation.

In \textit{Toxoplasma} TgNHE\textsubscript{1} knock-out parasites, although resting pH\texttext{\textsubscript{i}} was unaffected (and [\textit{Na}\textsuperscript{+}], was not measured), the resting [Ca\textsuperscript{2+}], was found to be higher in the NHE knock-out strain compared to the parent strain (Arrizabalaga \textit{et al.}, 2004). It may be relevant to measure [Ca\textsuperscript{2+}], in the GC03 and PfNHE-kd strains using the Fura-2 Ca\textsuperscript{2+}-sensitive dye (as per Section 2.7).

Another possibility to be considered is that PfNHE is not on the plasma membrane, but on an intracellular organellar membrane, for example the digestive vacuole membrane. The localisation of PfNHE is presently under investigation (this work is not reported here).
6.5. Conclusion

The physiological role of the putative Na⁺/H⁺-exchanger PfNHE was investigated, because previous studies on its contribution to regulation of [Na⁺]ᵢ and pHᵢ have been inconclusive (and remain the source of significant controversy). The studies reported here have failed to clarify the role of PfNHE in the trophozoite stage of the parasite. Despite bioinformatic predictions of PfNHE playing a role in electroneutral, amiloride-insensitive plasma membrane Na⁺ efflux, no significant role for such an NHE in either [Na⁺]ᵢ or pHᵢ regulation in the P. falciparum trophozoite was identified.
Chapter 7:

General Discussion
7.1. General Discussion

The maintenance of a low \([Na^+]_i\) is essential in almost all cell types. Animal, plant, fungal and protozoal cells use different mechanisms to efflux Na\(^+\), and thereby regulate their \([Na^+]_i\). The intraerythrocytic malaria parasite maintains a low \([Na^+]_i\), shown in this thesis to be \(\sim 11\) mM. Prior to this project, the transporter/s that the parasite uses to maintain this low \([Na^+]_i\) were unknown.

The work reported in this study identified a Na\(^+\)-ATPase as being the key mechanism responsible for Na\(^+\) efflux (Chapter 3). Further experiments led to the proposal that it is H\(^+\)-coupled, imposing a significant (Na\(^+\)-dependent) acid load on the parasite, and perhaps \(\Delta\Psi\)-sensitive (Chapter 4). A molecular candidate for the Na\(^+\)-ATPase activity, PfATP4, was identified and Na\(^+\) efflux (and hence parasite Na\(^+\) regulation) was found to be disrupted by the potent, new antimalarial drugs, the spiroindolones (Chapter 5). The function of another putative Na\(^+\) transporter, PfNHE was investigated, however no significant role for this protein in either Na\(^+\) or pH regulation in the trophozoite stage was identified (Chapter 6).

This thesis provides crucial information towards our understanding of global ion fluxes of the malaria parasite. Understanding the basic physiology of the parasite can only aid in the fight against this deadly disease.

7.2. The importance of the parasite Na\(^+\)-ATPase

7.2.1. The Na\(^+\)-ATPase contributes to \([Na^+]_i\) and pH\(_i\) homeostasis and is a promising drug target

\([Na^+]_i\) regulation in *P. falciparum* trophozoites was found to be sensitive to orthovanadate, CPA and the potent, new antimalarial drug class, the spiroindolones. It was insensitive to ouabain and several NHE inhibitors. The data are consistent with the presence, on the parasite plasma membrane, of a P-type Na\(^+\)-ATPase, acting to extrude Na\(^+\), and thereby maintain a low \([Na^+]_i\). When Na\(^+\)-ATPase activity was inhibited, there was a significant flux of Na\(^+\) into the parasite. Assuming that orthovanadate or the spiroindolones do not inherently increase the Na\(^+\) leak into the
parasite (for instance by allowing a Na\(^{+}\) transporter to act as a non-specific cation channel, such as the effect of the inhibitor palytoxin on the Na\(^{+}/K^{+}\)-ATPase (Takeuchi et al., 2008)), this indicates that under resting conditions the Na\(^{+}\)-ATPase has to counter a large Na\(^{+}\) influx. It is therefore likely that the parasite’s putative plasma membrane Na\(^{+}\)-ATPase plays an essential housekeeping role.

The malaria parasite, like other cells, maintains a steady resting pH\(_{i}\) through a balance of H\(^{+}\) import and H\(^{+}\) extrusion mechanisms. A V-type H\(^{+}\)-ATPase and a lactate:H\(^{+}\) symporter both operate to extrude H\(^{+}\) from the parasite (Kanaani and Ginsburg, 1991; Saliba and Kirk, 1999; Elliott et al., 2001). To date, only one acid-loading transporter has been characterised in P. falciparum, a putative Cl\(^{-}/OH^{-}\) (or HCO\(_{3}^{-}\)) transporter (Henry et al., 2010). In this study, the activity of the Na\(^{+}\)-ATPase was shown to be coupled to a significant acid load that was countered by the activity of the V-type H\(^{+}\)-ATPase. Consistent with this, inhibition of the Na\(^{+}\)-ATPase (with either orthovanadate or a spiroindolone) caused a cytosolic alkalisation of ~0.1 pH units. It is likely that activity of the Na\(^{+}\)-ATPase contributes to setting the resting pH\(_{i}\) value of the parasite. Inhibition of the Cl\(^{-}/OH^{-}\) acid loader causes a cytosolic acidification of ~0.07 pH units (Henry et al., 2010), whereas inhibition of the V-type H\(^{+}\)-ATPase causes a dramatic acidification of ~0.4 pH units (Fig. 4.5A and Saliba and Kirk, 1999). It is likely that the activity of the Na\(^{+}\)-ATPase, Cl\(^{-}/OH^{-}\) acid loader, V-type H\(^{+}\)-ATPase and other less-well characterised H\(^{+}\)-transport pathways all contribute towards maintaining a stable resting pH\(_{i}\) of ~7.3. A summary of Na\(^{+}\) and pH regulation in the parasite is shown in Fig. 7.1.
Fig. 7.1. Summary of pH and Na⁺ homeostasis in the intraerythrocytic P. falciparum parasite. (A) Around 12-16 hrs after erythrocyte invasion by the parasite, NPPs are induced in the plasma membrane of the erythrocyte. The NPPs mediate the flux of a range of solutes including inorganic cations, such as Na⁺. (B) By the time the parasite is a mature trophozoite, the influx of Na⁺ via the NPPs, combined with a decrease in the activity of the endogenous erythrocyte Na⁺/K⁺-ATPase (Staines et al., 2001) results in a dramatic increase in the [Na⁺] in the infected erythrocyte cytosol (the Na⁺ concentration approaches that in the blood plasma, ~130 mM). Na⁺ (and H⁺) are thought to move freely across the parasitophorous vacuole (PV) membrane (Desai et al., 1993); the ions concentration within the PV are therefore expected to be similar to those in the erythrocyte cytosol. (C) The parasite itself maintains a low cytosolic [Na⁺] (~11 mM). There is therefore a large inwardly directed [Na⁺] gradient across the parasite plasma membrane and this serves to energise the uptake of the essential nutrient inorganic phosphate (Saliba et al., 2006). Na⁺ also enters the parasite through other uncharacterised pathways. (D) The low [Na⁺], of ~11 mM in the parasite is maintained by a Na⁺-ATPase, postulated (here) to be H⁺-coupled. (E) H⁺ ions that enter the parasite through the P-type Na⁺-ATPase and other H⁺-coupled transporters are extruded by the plasma membrane V-type H⁺-ATPase (Saliba and Kirk, 1999; Hayashi et al., 2000). The intracellular pH is maintained at ~7.3 through the concerted action of the V-type H⁺-ATPase, lactate/H⁺ symporter (F) (Elliott et al., 2001) and the acid loading Cl⁻ uptake mechanism (G) (Henry et al., 2010). H⁺ ions are also pumped into the digestive vacuole (H) by the V-type H⁺-ATPase and the V-type H⁺-PPase (Saliba et al., 2003). The contribution of the putative NHE, PfNHE, to cation regulation is unknown, as is its subcellular localisation.
In light of the central role of the Na\(^{+}\)-ATPase in malaria parasite physiology, maintaining a low [Na\(^{+}\)]\(_{i}\) (and influencing pH\(_{i}\)), the protein involved is of obvious interest as an antimalarial drug target. The spiroindolones cause a profound disruption in Na\(^{+}\) homeostasis, and this disruption was attenuated in drug-resistant parasites bearing mutations in PfATP4. These data are consistent with the hypothesis that PfATP4 is the Na\(^{+}\)-ATPase, as represented schematically in Fig. 7.2.

Fig. 7.2. Schematic representation showing the proposed role of PfATP4 in Na\(^{+}\) homeostasis in the intraerythrocytic *P. falciparum* parasite. (A) PfATP4 is postulated to function as an ENA-type Na\(^{+}\)-ATPase, actively extruding Na\(^{+}\) from the intraerythrocytic parasite, countering the inward leak of Na\(^{+}\) (via unknown pathways) and maintaining a [Na\(^{+}\)]\(_{i}\) (~11 mM) more than 10-fold lower than the extracellular [Na\(^{+}\)] \((125 \text{ mM in the experiments conducted here)}\). The PfATP4-mediated efflux of Na\(^{+}\) is accompanied by an influx of H\(^{+}\) ions and this constitutes a significant ‘acid-load’ on the parasite. (B) PfATP4 is inhibited by the spiroindolones, by orthovanadate, and by CPA. Inhibition of PfATP4 results in an increase in [Na\(^{+}\)]\(_{i}\), as Na\(^{+}\) moves into the cell, down its electrochemical gradient, via the Na\(^{+}\) leak pathways. At the same time there is an increase in pH\(_{i}\), caused by the elimination of the PfATP4-mediated acid load. The alkalinisation seen following inhibition of PfATP4 is not seen for parasites suspended in Na\(^{+}\)-free medium as, under these conditions [Na\(^{+}\)]\(_{i}\) is close to zero (Fig. 3.54), PfATP4 is non-functional and there is therefore no PfATP4-mediated acid-load.
The development of the spiroindolones at the Novartis Institute for Tropical Diseases was the Medicine for Malaria Venture’s (MMV’s) ‘Project of the Year’ in 2009 and, as noted by MMV’s Chief Scientific Officer, Timothy Wells, in a comment in Science last year, the compounds have moved very rapidly from being ‘hits’ to being ‘preclinical drug candidates’ (Wells, 2010). Phase 1 clinical trials for one of the spiroindolones, NITD609, are already underway in Brisbane. Identifying the target and the mechanism of action of a particular drug is often fraught with difficulty; the fact that we are still not sure how artemisinin kills parasites (Krishna et al., 2010) is testament to this. The results presented in this project, are consistent with PfATP4 being the molecular target of the spiroindolones, and with the mechanism of action of the spiroindolones involving disruption of parasite ion regulation.

Although spiroindolone-resistant parasites can be generated in a laboratory setting (Rottmann et al., 2010), it is unclear whether this will be clinically relevant as the pharmacokinetics of the drug in vivo are unknown. It should also be emphasised that in those parasites showing maximum resistance to the spiroindolones, the IC$_{50}$ values are still in the low nM range, concentrations that may be readily achievable clinically. Actually knowing the molecular target of resistance is useful as it can enable rational drug design through an understanding of structure/activity relationships. The work described here therefore has the potential to contribute to the further development and refinement of this new class of antimalarials.

7.2.2. Further experiments to confirm that PfATP4 is the Na$^+$-ATPase

The physiological function of PfATP4, and its inhibition by the spiroindolones, could be confirmed directly by expression of the protein in a heterologous system. The two systems that have proven to be most successful for the functional expression of Plasmodium-encoded membrane transport proteins are X. laevis oocytes and the yeast S. cerevisiae.

PfATP4 has previously been expressed in X. laevis oocytes (Krishna et al., 2001), though the characterisation was limited to the demonstration of an increased Ca$^{2+}$-dependent ATPase activity in oocytes expressing the protein. If PfATP4 is the parasite Na$^+$-ATPase and the target of the spiroindolones, then expression of PfATP4
in *Xenopus* oocytes may give rise to a spiroindolone-sensitive increase in Na⁺-ATPase activity. Previous studies have demonstrated that codon harmonisation leads to improved expression of membrane proteins in the oocyte system (Martin *et al.*, 2009b) and this approach may help in the functional expression of PfATP4. The membrane fraction of *Xenopus* oocytes expressing PfATP4 would be purified and assayed for Na⁺-dependent ATPase activity. In subsequent experiments, the mutations shown by Rottmann *et al.* to confer spiroindolone-tolerance on the parasites (Rottmann *et al.*, 2010) could be introduced to the codon-harmonised, *Xenopus*-expressed sequence to assess the effect of these mutations on the spiroindolone-sensitivity of the ATPase activity.

PfATP4 could also be expressed in the mutant Na⁺-sensitive B31 strain of *S. cerevisiae*. Previous studies have demonstrated that expression of an ENA-type Na⁺-ATPase in B31 mutant yeast confers resistance to a high extracellular [Na⁺] in complementation assays (Lunde *et al.*, 2007). The PfATP4-expressing B31 yeast could be assayed for Na⁺-dependent ATPase activity or used in complementation assays in which the yeast are grown under high (300 mM) [Na⁺] conditions (as in (Lunde *et al.*, 2007)) to establish whether PfATP4 confers an increased Na⁺ tolerance on the mutant B31 strain. If PfATP4 is the parasite Na⁺-ATPase and the target of the spiroindolones, then expression of PfATP4 in *S. cerevisiae* strain B31 may give rise to a spiroindolone-sensitive increase in Na⁺-ATPase activity, and confer a spiroindolone-sensitive increase in [Na⁺] tolerance. As with the oocyte system, initial experiments could be carried out with the wild-type form of the protein, and subsequent experiments could investigate the ability of the spiroindolone-tolerance-inducing mutations in PfATP4 to confer resistance to the spiroindolones in the yeast assays. There has been one reported case of an ENA-type Na⁺-ATPase (from *Trypanosoma* TcENA) failing to express in the B31 yeast system (Iizumi *et al.*, 2006); it remains to be seen whether expression of PfATP4 in this system is any more straightforward.

The development of heterologous expression systems, such as the two outlined in this section, could pave the way for the development of high throughput assays that could be used for testing and refining antimalarial compounds of this class.
7.2.3. ENA-class \( \text{Na}^+ \)-ATPases in other pathogens

Finally, as ENA-class \( \text{Na}^+ \)-ATPases are found in many pathogenic fungi and parasites (Rodriguez-Navarro and Benito, 2010) the possibility exists that the spiroindolones could be a more general anti-parasitic/anti-fungal drug. The growth inhibitory effects, and the ion-regulation disrupting effects of the spiroindolones should be tested in other protozoa/fungi. The identification of the spiroindolones as a broad anti-parasitic/ anti-fungal drug would be a significant breakthrough in our current age of multi-drug resistant pathogens.

7.3. The physiological role of PfNHE

7.3.1. PfNHE does not contribute to \([\text{Na}^+]_i\) or \(\text{pH}_i\) homeostasis in the trophozoite stage

mRNA encoding PfNHE is expressed at all stages throughout the blood stage of the parasite life cycle, possibly increasing towards schizont/late schizont stages (Bozdech et al., 2003; Le Roch et al., 2003). It is therefore an ideal candidate as a \(\text{Na}^+\) efflux transporter. Despite this, no evidence for involvement of an NHE in \([\text{Na}^+]_i\) or \(\text{pH}_i\) regulation was identified in the work carried out for this thesis. It is possible that any \(\text{Na}^+\) efflux via PfNHE is masked by the dominant activity of the proposed ENA-type \(\text{Na}^+\)-ATPase.

7.3.2. The link between quinine resistance and PfNHE

Without understanding the physiological role of PfNHE it is very difficult to understand how mutations in PfNHE might result in resistance to the antimalarial drug quinine. Additionally, the antimalarial mechanism of action of quinine is not entirely understood, although it is predicted to act in the same manner as chloroquine, by accumulating in the DV and inhibiting the crystallization of the toxic haem to inert haemozoin (Fitch, 2004). Length variations in several repeats in the ms4760 microsatellite region of PfNHE (DNNND and DDNNNDNHNDD) have been significantly associated with quinine resistance in some (but not all) studies (reviewed by Okombo et al., 2011).
The ms4760 microsatellite region is found in the C-termini of PfNHE, a domain involved in regulation of exchanger activity. In a recent study, Alexander and colleagues demonstrated that the C-terminus of human NHE3 associates with anionic membrane domains (Alexander et al., 2011), and the phospholipid membrane composition, in particular the (polyanionic) phosphoinositide content, has been implicated in regulation of HsNHE3 activity (Aharonovitz et al., 2000; Lee-Kwon et al., 2003). When basic residues in the C-terminus of HsNHE3 were mutated (replaced with neutral alanine) the electrostatic interactions between the C-terminus and the plasma membrane inner leaflet were disrupted, which led to a loss of NHE activity (Alexander et al., 2011). The microsatellite repeat variations observed in PfNHE ms4760 involve aspartate (charged, acidic), asparagine (hydrophilic) and histidine (charged, basic) residues. It might be postulated that changes in the number of repeats of DNNND and DDNNNDNHNDD sequences change the net charge of the C-termini domain, and that this disrupts PfNHE-mediated ion exchange by disrupting the PfNHE C-termini interactions with the parasite plasma membrane. This hypothesis could explain the link between microsatellite variation and Na\(^+\)/H\(^+\)-exchanger activity. At this stage (with our current lack of understanding of both how PfNHE and quinine function) it is not possible to extend this concept to how PfNHE activity is related to quinine susceptibility.

7.3.3. Possibility of a dual mechanism of Na\(^+\) regulation

In yeast, and lower plants including the moss *P. patens*, an ENA-type Na\(^+\)-ATPase and an NHE are proposed to play complementary roles in Na\(^+\) regulation (Banuelos et al., 1995; Hahnenberger et al., 1996; Fraile-Escanciano et al., 2010; Arino et al., 2011). It has been proposed that the ENA-type Na\(^+\)-ATPase acts to extrude Na\(^+\) under alkaline conditions (when the H\(^+\) gradient is unsuitable for a Na\(^+\)-extruding NHE), and that an electroneutral NHE is more important under acidic conditions (Rodriguez-Navarro and Benito, 2010). Having two Na\(^+\) efflux mechanisms ensures that fungi and bryophytes can always maintain a low [Na\(^+\)]\(_i\), under varying pH environments (Rodriguez-Navarro and Benito, 2010).
The theoretical ability of PfNHE to extrude Na\textsuperscript{+} at varying pH\textsubscript{0} can be calculated using the Gibbs Free energy equation for the movement of solutes across a membrane:

\[
\Delta G_{G, j} = N \times (RT \times \ln([S]_i/[S]_0) + zF\Delta\Psi)
\]

where \(\Delta G\) is the Gibbs free energy (-ve \(\Delta G\) releases energy and +ve \(\Delta G\) requires energy), \(N\) is the number of moles of solute transported, \(R\) is the universal gas constant (8.314 JK\textsuperscript{-1}mol\textsuperscript{-1}), \(T\) is the temperature (in K), \([S]_0\) is the external [solute] of interest (M), \([S]_i\) is the internal [solute] of interest (M), \(z\) is the valency of the ion and \(F\) is the Faraday constant (96487 JV\textsuperscript{-1}mol\textsuperscript{-1}).

In saponin-isolated trophozoites under resting conditions (when \([Na^+]_i = 11\) mM, \([Na^+]_o = 125\) mM, pH\textsubscript{0} = 7.1, pH\textsubscript{i} = 7.3 and \(\Delta\Psi = -95\) mV) the energy needed to extrude one mole of Na\textsuperscript{+} (+13.5 kJ) is greater than the energy released from the movement of one mole of H\textsuperscript{+} into the parasite (-10.4 kJ). In other words, the energy in the prevailing H\textsuperscript{+} gradient is insufficient to generate the observed transmembrane Na\textsuperscript{+} gradient, if the parasite were reliant on an (ATP-independent) electroneutral Na\textsuperscript{+}/H\textsuperscript{+} exchange process. Under more acidic extracellular conditions the inward H\textsuperscript{+} gradient is increased and it would, in principle, be possible for an electroneutral NHE to maintain \([Na^+]_i\) at the required (low) physiological level. Further studies carried out under such conditions may yet reveal some role for PfNHE in parasite Na\textsuperscript{+} regulation.

### 7.3.4. Further experiments to elucidate the physiological role of PfNHE

Determining the sub-cellular localisation of PfNHE will be critical if its physiological role is to be understood. This is presently under investigation using two, complementary immunolocalisation techniques. Parasites expressing HA-tagged and GFP-fusion forms of the PfNHE protein are being generated by 3' replacement into the endogenous PfNHE locus (i.e., under the control of the native promoter). The GFP-tag is suitable for live-cell imaging whereas visualisation of the HA-tag requires the cells to be fixed.
If PfNHE is localised to a membrane other than the plasma membrane, alternative experimental approaches (to those utilised in this study) will be needed to characterise its role in ion transport. For example, if PfNHE is localised to the DV, pH$_{DV}$ can be measured in parasites in which the vacuole is loaded with the pH-sensitive probe fluorescein conjugated to macromolecular dextran (Hayward et al., 2006; Lehane et al., 2008). The Na$^+$-dependence of the H$^+$ leak across the DV membrane could be measured to investigate possible PfNHE activity.

Additionally, to investigate the link between PfNHE microsatellite repeat regions and quinine resistance, parasites expressing differing numbers of the DNNND and DDNNNDNHND repeat sequences are being generated. Introducing these changes in one wild type (3D7) strain (in the absence of other changes throughout the genome), will help to clarify whether it is mutations specifically in PfNHE that cause quinine resistance. Constructs with altered microsatellite regions have been synthesised commercially, and transfection of the parasite with these constructs is currently underway.
7.4. Concluding Remarks

The maintenance of a low [Na$^+$], is likely to play a crucial role in the intraerythrocytic malaria parasite. This thesis identifies the key transporter involved in Na$^+$ efflux in the trophozoite stage as a P-type, ENA-class Na$^+$-ATPase (postulated to be PfATP4). It describes in detail the biochemical characteristics of the transporter and, additionally, provides evidence that the proposed Na$^+$-ATPase is the target of the potent, new antimalarial class, the spiroindolones. At the same time the studies reported here have revealed the presence in the parasite of a substantial inward Na$^+$ leak which is normally countered by the parasite's Na$^+$ efflux mechanism. The presence of this leak is likely to be a key factor in the parasite’s vulnerability to agents (such as the spiroindolones) that inhibit Na$^+$ efflux; the mechanisms involved (i.e., the pathways by which Na$^+$ enters the cell) remain to be elucidated.

What role, if any, the parasite’s putative Na$^+$/H$^+$ exchanger, PfNHE, plays in ion regulation in the parasite, and how it might influence the parasite’s sensitivity to the antimalarial action of quinine, remain open questions, and are the subject of ongoing work.
Chapter 8:

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