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# Role for the *Plasmodium falciparum* Digestive Vacuole in Chloroquine Resistance

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**ABSTRACT.** We have developed a method for the isolation of pure and intact *Plasmodium falciparum* digestive vacuoles capable of ATP-dependent chloroquine (CQ) accumulation *in vitro*. The method is rapid and reliable, and it produces a high yield of vacuoles (20%). CQ accumulation in isolated vacuoles was found to be ATP-, $Mg^{2+}$-, and temperature-dependent. We then investigated the CQ-accumulating capabilities of vacuoles isolated from CQ-resistant (CQR) and CQ-sensitive (CQS) parasites. At external CQ concentrations of 100 and 250 nM, vacuoles isolated from two CQS strains (D10 and RSA3) ($V_{max}$: 380–424 fmol/$10^6$ vacuoles/hr) accumulated significantly more CQ (~3 times) than those isolated from three (FAC8, RSA11, and RSA15) of the four CQ-resistant strains of *P. falciparum* tested ($V_{max}$: 127–156 fmol/$10^6$ vacuoles/hr) ($P \leq 0.05$). We propose that the low level of CQ accumulation observed in vacuoles isolated from most of the CQ-resistant parasites tested contributes to the decreased CQ accumulation seen in these strains and, hence, to CQ resistance. Although it is often suggested that the digestive vacuole of the *P. falciparum* parasite is involved in the mechanism of CQ resistance, to our knowledge this is the first direct confirmation. BIOCHEM PHARMACOL **56**;3: 313–320, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** chloroquine resistance; *Plasmodium falciparum*; isolated digestive vacuoles; drug-transport; *in vitro*

The mechanism of CQ‡ resistance is not clearly understood, and it remains controversial. A decrease in CQ accumulation in CQR parasites is regarded as the basis of CQ resistance [1, 2]. The efflux of CQ is reported to be 40–50 times faster from CQR than CQS parasites, despite comparable initial rates of CQ accumulation [3]. This process is ATP dependent [4]. However, Bray *et al.* [5] have found rapid efflux of CQ in both CQS and CQR strains of *Plasmodium falciparum*, suggesting that efflux rates alone are insufficient to explain CQ resistance. In addition, there is a correlation between the rate of CQ uptake and CQ sensitivity [5]. Using kinetic modeling of CQ uptake in parasitized erythrocytes, Ginsburg and Stein [6] failed to detect drug pumping activity, and concluded that drug efflux is insufficient to explain CQ resistance. They suggested that weakened vacuolar proton pump activity may be responsible for the low level of CQ accumulation in CQR parasites [6, 7].

The accumulation of CQ in acid vesicles of mammalian cells is well described [8, 9]. CQ accumulates within the digestive vacuoles of *P. falciparum* [10]. CQ accumulation in organelles with a low pH has been explained by the weak base properties of the drug. It is estimated that millimolar levels of CQ accumulate in digestive vacuoles at therapeutic concentrations [11], and kinetic modeling suggests that the digestive vacuole membrane is the main barrier controlling CQ accumulation in *P. falciparum* [12]. The low pH in these organelles is maintained by V-ATPase [13]. Bafilomycin $A_1$, a specific inhibitor of V-ATPase in a number of systems [14], decreases CQ accumulation in CQS and CQR parasites; CQR parasites are particularly sensitive [15]. This inhibitor increases CQ resistance in both strains, which supports the idea that CQ resistance is associated with weakened vacuolar proton pumping activity [15].

Other evidence suggesting the involvement of the digestive vacuole in CQ resistance has been the localization of Pgh1, a drug transport protein that is a homologue of P-gp in mammalian multidrug-resistant cancer cell lines, to the digestive vacuole of *P. falciparum* [16]. Pgh1 is overexpressed in certain drug-resistant strains of *P. falciparum* [17, 18]. Expression of wild-type Pgh1 in CHO cells increases CQ accumulation levels in this cell line [19], and mutations in *pfmdr 1*, the gene coding for Pgh1, have been linked to a loss of ability of Pgh1 to transport 4-aminoquinolines [20,

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‡ *Abbreviations:* AchE, acetylcholine esterase; AMP-PCP, 5′-(β, γ-methylene)-triphosphate; CQ, chloroquine; $[CQ]_{ext}$, external chloroquine concentration; CQR, chloroquine-resistant; CQS, chloroquine-sensitive; P-gp, P-glycoprotein; MEF, mefloquine; Pgh1, P-glycoprotein homologue 1; pLDH, parasite lactate dehydrogenase; PRBC, parasitized red blood cells; RBC, red blood cells; TEM, transmission electron microscopy; V-ATPase, vacuolar-adenosine triphosphatase.

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21]. However, CQ resistance can occur without mutations to the *pfmdr 1* gene [22], and results from a genetic cross between CQR and CQS parasites showed no correlation between the CQ-resistance phenotype and *pfmdr 1* [23]. CQ resistance was argued to be controlled by one gene, or a group of linked genes, later shown to reside within a 400-kb region of chromosome 7, a different chromosome to the location of *pfmdr 1* [24].

Studies of the mechanism of CQ accumulation in the parasite food vacuole have been complicated by an inability to exclude the role of other membranes (red blood cell membrane, parasite plasma membrane, and parasitophorous vacuole membrane) in concentrating CQ in PRBC. Cell-free models of CQ accumulation have been examined previously [25]. Although these provide interesting information, purified preparations of specific organelles, particularly the digestive vacuole, are necessary for a conclusive study of the mechanism(s) of CQ accumulation and resistance [16, 25, 26].

Digestive vacuoles were first isolated after loading with ferric oxide granules, liberation from the parasites by homogenization, and purification by centrifugation on discontinuous sucrose gradients [27]. Vacuoles isolated in this way were used for partial characterization of V-ATPase activity [27]. Digestive vacuoles isolated by combined differential centrifugation and density gradient separation appear to be pure and intact, and able to degrade haemoglobin at pH 5 to 5.5 [28]. No published data exist on the uptake of CQ in vacuoles purified by either of the two methods.

In this study we have used pure, intact, and functional digestive vacuoles isolated from *P. falciparum* to determine whether this organelle has a role in CQ resistance.

## MATERIALS AND METHODS

### P. falciparum *Parasite Culture*

*P. falciparum* parasites were cultured as described by Trager and Jensen [29], with minor modifications. The CQS-cloned strain D10 and CQR strains FAC8 and W2mef were donated by A. Cowman, Walter and Eliza Hall Institute of Medical Research. The culture-adapted South African isolates RSA3 (CQS), RSA11 (CQR), and RSA15 (CQR) were obtained from J. Freese, Medical Research Council of South Africa, Malaria Research Programme. Human serum/blood was donated by the Western Province Blood Transfusion Service and Haematology Department. Parasitemia was kept between 5 and 10% by the addition of $O^+$ RBC. Parasites were maintained at 37° in desiccator cabinets in an atmosphere of 93% $N_2$, 4% $CO_2$, and 3% $O_2$. Parasites were synchronized by treatment with D-sorbitol [30].

### *Vacuole Isolation*

A 5-mL sample of PRBC, synchronized in the late trophozoite stage with a 10% parasitemia, was washed twice in 50 mL PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$/$KH_2PO_4$). The pellet was resuspended in 50 mL PBS containing 0.5 μg/mL of saponin and immediately centrifuged (Heraeus Sepatech, Omnifuge 2.0 RS) at 1500 *g* for 10 min. Isolated trophozoites were collected and washed twice with 1 mL of ice-cold PBS. Trophozites were then resuspended in 10 vol. of ice-cold water, pH 4.5, and immediately triturated four times through a 27-G 1.2-cm needle. The crude vacuole preparation was centrifuged at 13,000 rpm in a desk-top microfuge (model mentioned above) for 2 min. The pellet was resuspended in 1 mL of uptake buffer, pH 7.4, containing 2 mM $MgSO_4$, 100 mM KCl, 10 mM NaCl, 25 mM HEPES, 25 mM $NaHCO_3$, and 5 mM sodium phosphate, to which was added 10 μL of 5 mg/mL of DNase 1, and incubated at 37° for 5 min. Then the suspension was microfuged for 2 min at 13,000 rpm. The resulting pellet was resuspended in 100 μL of ice-cold uptake buffer followed by the addition of 1.3 mL of ice-cold 42% Percoll™ containing 0.25 M sucrose and 1.5 mM $MgSO_4$, pH 7.4. The suspension was again triturated 2 times through a 27-G 1.2-cm needle and microfuged at 13,000 rpm at 4° for 10 min. The preparations regularly contained purified vacuoles identified in a dark band in the bottom 50 μL of the gradient. The purified vacuoles were collected, resuspended in 1 mL of uptake buffer, and microfuged at 13,000 rpm for 2 min to wash off the Percoll™.

### *Electron Microscopy of* P. falciparum *Isolated Vacuoles*

Samples were fixed for 2 hr in 2.5% glutaraldehyde, pH 8.4, washed once in PBS, and post-fixed in 2% osmium for 1 hr. They were then washed once in PBS and once in water followed by stepwise dehydration in 50, 70, 80, 90, and 100% ethanol and acetone. Ultra-thin sections were automatically (Reithert Ultracut S) obtained from Spurrs resin-mounted samples using a glass knife and stained with Reynolds lead citrate and 2% uranyl acetate. Samples were viewed on a Jeoll 200 CX transmission electron microscope.

### *AchE Activity*

AchE activity was measured by a modified method of Vander Jagt *et al.* [31]. Isolated vacuoles or erythrocyte membranes were incubated in freshly prepared 0.1 M sodium phosphate (Merck)/0.5 mM 2,2′-dinitro-5,5′-dithiobenzoic acid (Merck)/0.6 mM *S*-acetylthiocholine iodide (Merck), pH 7.5, in a 96-well microtitre plate at room temperature for 20 min. Absorbance values at 405 nm were obtained using a 7520 Microplate Reader (Cambridge Technology, Inc.).

### *pLDH Activity*

The Malstat™ (Flow, Inc.) reagent was used for the measurement of pLDH [32]. Measurements were carried out in microtitre 96-well plates. Malstat™ (100 μL) and 25 μL of 0.24 mM phenazine ethosulphate (PES)/1.96 mM nitro

blue tetrazolium were added to each well. Then 20 μL of PBS containing between 3–5 × $10^5$ isolated trophozoites or 0.5–5 × $10^6$ isolated vacuoles was added to the well. Twenty microliters of PBS was added to the control wells. Absorbance values at 620 nm were obtained using a 7520 Microplate Reader (Cambridge Technology, Inc.).

### *Vacuole Number Determination*

A spectrophotometric method was developed for vacuole quantitation. Vacuoles isolated from D10 and FAC8 parasite cultures, tightly synchronized in the late trophozoite stage and suspended in PBS, were scanned between 250 and 800 nm (Shimadzu UV 160 1 PC dual beam spectrophotometer) and found to have a maximum optical density at 662 nm. A standard curve was set up by obtaining optical densities (662 nm) of serially diluted vacuole suspensions. Vacuole counts of suspensions were obtained using a haemocytometer (Spencer Bright–Line® improved Neubauer 1/10 mm deep). Subsequent vacuole concentrations estimated using the standard curve correlated well with the values using the haemocytometer.

### CQ *Accumulation in Parasitized RBC*

Parasitized erythrocytes, synchronized in the trophozoite stage, with a parasitemia between 1 and 5% and the haematocrit set at 1% in culture medium, were exposed to [$^3$H]CQ (18 Ci/mmol; Amersham) in 1.5-mL Eppendorf vials. The vials were incubated at 37° in a heating block for 1 hr, unless otherwise indicated. At the end of the incubation period, the PRBC were centrifuged through 100 μL of dibutyl phthalate (Sigma) for 30 sec in a bench top centrifuge (Beckman microfuge B). The Eppendorf tip containing the PRBC was cut off and placed in a scintillation vial. Then 5 mL of liquid scintillation fluid and 1 mL of 3.5% (m/v) sodium hypochlorite, to bleach the red colour of the haemoglobin, were added to the vial and shaken (Labcon) overnight. The radioactivity in the vials was counted in a Packard Tri-Carb 4640 liquid scintillation spectrometer.

### CQ *Accumulation in Isolated Digestive Vacuoles*

CQ accumulation experiments were carried out in 1.5-mL Eppendorf vials. Between 4 and 8 × $10^6$ isolated vacuoles were suspended in 1 mL of uptake buffer in the presence or absence of 2 mM ATP (Boehringer Mannheim). Experiments were initiated by the addition of [$^3$H]CQ (18 Ci/mmol; Amersham). When CQ concentrations greater than or equal to 100 nM were required, unlabelled CQ was included. The vials were placed in a heating block (Techne Dri-block DB-3) at 37° for the required period of time. Accumulation experiments were terminated by centrifugation at 13,000 rpm (Beckman centrifuge B) for 2 min and then removal of the supernatant. The vacuoles were washed twice in 1 mL of ice-cold uptake buffer. The Eppendorf tips were then cut off (Bel-Art micro tube cutter) and transferred to scintillation vials containing 5 mL of scintillation fluid (Packard). Samples were shaken overnight, and radioactivity was counted in a Packard Tri-Carb 4640 liquid scintillation spectrometer.

### In Vitro P. falciparum *Cytotoxicity Assay*

Parasite viability in the presence of drug was calculated using the method described by Makler *et al.* [33]. The Malstat™ (Flow Inc.) reagent was used for the measurement of pLDH activity as an indicator of parasite viability.

### *Statistics*

Statistical significance ($P$ values) was established using Student's two-tailed $t$-test.

## RESULTS

CQ concentration–response relationships were determined for the six strains used in this study. D10 and RSA3 were CQS, with $IC_{50}$ concentrations of 40 ± 17 (N = 4) and 20 ± 3 nM (N = 3), respectively. FAC8, RSA11, RSA15, and W2mef were CQR, with $IC_{50}$ values of 160 ± 23 (N = 3), 340 ± 87 (N = 4), 336 ± 52 (N = 3), and 311 ± 86 nM (N = 4), respectively. These differences in CQ sensitivity, between 4- and 17-fold, confirm that the strains that were studied are suitable for the investigation of CQ resistance.

CQ accumulation in parasitized erythrocytes was evaluated over a range of external CQ concentrations. The CQS strain D10 accumulated more CQ than the CQR strains FAC8 and W2mef at all the concentrations tested (Fig. 1). This is consistent with published data [1], and it supports the idea that CQ resistance in *P. falciparum* is the result of an inability of CQR parasites to accumulate CQ to toxic levels.

Parasites synchronized in the late trophozoite stage were released from erythrocytes by saponin lysis. Then the trophozoites were lysed by a combination of hypotonic lysis at a low pH and trituration through a fine needle. This crude vacuole preparation was resuspended in isotonic buffer and exposed to DNase 1. The vacuoles were purified subsequently using Percoll™ density gradient centrifugation. Vacuoles routinely migrated to the bottom 50 μL of the gradient, visible as a dark band.

TEM of isolated vacuoles, from two separate isolations, revealed no contamination by other organelles or membranes (Fig. 2). The vacuoles were noted to have an intact vacuolar membrane surrounding hemozoin crystals, and they were homogeneous with a diameter of 1.00 ± 0.22 μm (N = 6). Vacuole preparations were assayed for pLDH and AchE as markers for trophozoite and erythrocyte contamination, respectively, and for ouabain-sensitive ATPase activity as a plasma membrane marker. Vacuoles from two separate isolations exhibited less than 0.34% ($P < 0.007$)

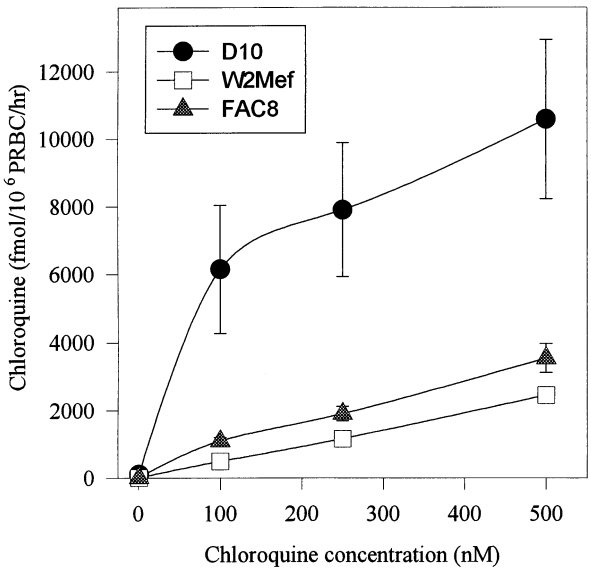


**FIG. 1. CQ accumulation in erythrocytes parasitized with the *P. falciparum* strains D10, FAC8, and W2mef. CQ accumulation in unparasitized erythrocytes was subtracted. The range of $[CQ]_{ext}$ was 1 to 500 nM. CQ accumulation is expressed as fmol/$10^6$ parasitized erythrocytes/hr. Error bars represent SDs from the means of three separate experiments (each experiment performed in duplicate).**

pLDH activity compared with isolated trophozoites. AchE activity in isolated vacuoles was low. AchE activity from three separate vacuole isolations was 0.66 ± 0.24 nmol/hr/$10^6$ vacuoles. AchE activity from isolated RBC membranes was 11,640 nmol/hr/mg of protein. From this it follows that vacuole preparations of $10^6$ vacuoles (~1 μg of protein) contained less than 60 ng of RBC membrane protein. Ouabain-sensitive ATPase activity was not detectable in isolated vacuole preparations.

CQ accumulation in isolated vacuoles was ATP dependent, 2 mM ATP giving maximum accumulation. Incubation in the presence of 2 mM ATP resulted in a 3.33 ± 0.98-fold (N = 6) increase in CQ accumulation. The ATP-dependent CQ accumulation was abolished by removal of $Mg^{2+}$, maintenance of vacuoles at 4°, or replacement of ATP with 2 mM 5′-AMP-PCP, an unhydrolysable analogue of ATP (Fig. 3).

**FIG. 2. Transmission electron micrograph of digestive vacuoles isolated from the D10 strain of *P. falciparum* (magnification 40,000×).**

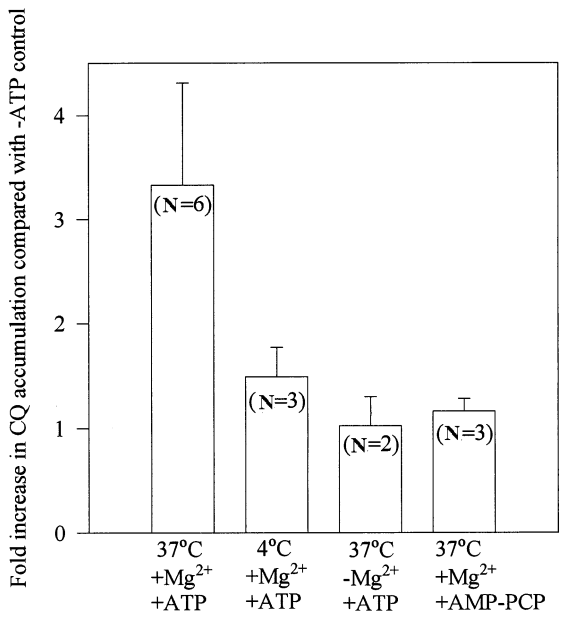


**FIG. 3. CQ accumulation in digestive vacuoles isolated from the D10 strain of *P. falciparum* in the presence and absence of ATP, AMP-PCP, and $Mg^{2+}$, maintained at 37° or 4°. $[CQ]_{ext}$ = 1 nM. The number of experiments (each experiment performed in duplicate) from separate vacuole isolations is indicated within the bars. Error bars represent SD except for the column having an N-value of 2; here the value is given as mean ± range.**

CQ accumulation in vacuoles isolated from strains of *P. falciparum* with various degrees of CQ resistance was evaluated. Three $[CQ]_{ext}$ concentrations (1, 100, and 250 nM) were chosen for this purpose. Three data points have been used in previous experiments to investigate the effect of increasing medium pH on $[CQ]_{int}$ of PRBC, over a range of $[CQ]_{ext}$ (1–500 nM) [34]. The vacuoles were isolated immediately prior to the uptake experiments. Although vacuoles isolated from either CQR or CQS parasites accumulated similar amounts of CQ at a $[CQ]_{ext}$ of 1 nM, vacuoles isolated from the CQS strains D10 and RSA3 accumulated more CQ at 100 and 250 nM than vacuoles isolated from the CQR FAC8, RSA11, and RSA15 (Fig. 4). Vacuoles isolated from the MEF-pressured, CQR W2mef strain accumulated CQ at similar levels to D10 and RSA3 at each of the concentrations tested (Fig. 4). Lineweaver–Burk plots of Fig. 4 (not shown) yielded CQ $V_{max}$ values for D10, RSA3, and W2mef that were significantly different ($P \leq 0.05$) from those of FAC8, RSA11, and RSA15 (Table 1).

## DISCUSSION

A method is described here for the isolation of vacuoles capable of ATP-dependent accumulation of CQ. The method is an improvement of that of Goldberg *et al.* [28], which yields pure vacuoles unable to accumulate CQ in an

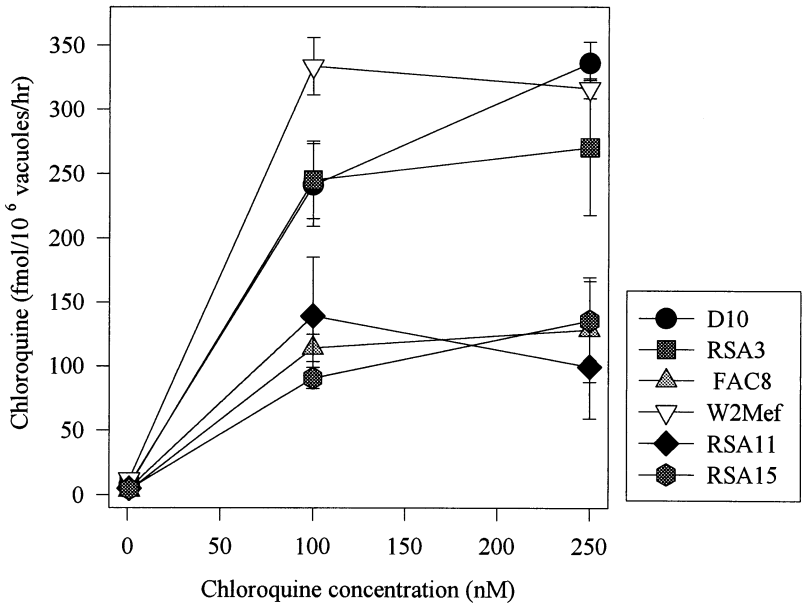


**FIG. 4. CQ accumulation in vacuoles isolated from D10, FAC8, W2mef, RSA3, RSA11, and RSA15, expressed as fmol/$10^6$ vacuoles/hr, at a $[CQ]_{ext}$ of 1, 100, and 250 nM in the presence of ATP after a 1-hr exposure. CQ accumulation in vacuoles not exposed to ATP was subtracted. Data points represent means of 2–4 experiments; error bars indicate SEM for D10 (N = 4), W2mef (N = 4), FAC8 (N = 3), RSA11 (N = 3), and RSA15 (N = 3) and range for RSA3 (N = 2). Vacuoles used in each experiment were from separate isolations.**

energy-dependent manner. We use saponin to lyse the erythrocytes, and have found that brief exposure of PRBC to saponin is sufficient to release intact trophozoites. Lengthy exposure of the trophozoites to saponin was avoided to minimize membrane damage. No detergents were included in subsequent isolation steps.

Trophozoites were suspended in water at low pH to release the vacuoles with a minimum of shearing forces, while maintaining a low vacuolar pH. Parasite lysis in distilled water has been used previously to obtain the internal constituents of the parasite [35]. As observed by phase contrast microscopy, vacuole lysis during this step is minimal (not shown).

The number of triturations through a 27-G needle was critical for vacuole integrity. Vacuoles triturated ten times in distilled water through the 27-G needle were fragmented (by TEM; micrographs not shown), and unsuitable for drug transport studies. However, four triturations resulted in no visible damage (Fig. 2).

Parasites can adhere to sticky DNA during their isolation [36]. Previously, saponin with streptomycin was used to prevent vacuolar adherence to other cellular components [28]. In this study, the vacuoles adhered to DNA released during parasite lysis, making purification difficult. DNase 1 was included in the isolation process to eliminate the DNA liberated during hypotonic lysis. This resulted in a significantly improved yield and purity of the isolated vacuoles. Purification of the vacuoles was achieved by density gradient centrifugation in 42% Percoll™. In the absence of DNase 1, resuspension of the vacuoles in 42% Percoll™ solution was difficult, and often they would not migrate to the bottom of the centrifuge tube.

Vacuole purity was confirmed by the virtual absence of AchE, pLDH, and ouabain-sensitive ATPase activity. TEM revealed no contaminating membranes or organelles in preparations of isolated vacuoles. These observations were confirmed in all the vacuole preparations tested. The integrity of the vacuolar membrane and the homogeneity of the vacuoles were confirmed using TEM.

CQ accumulation in the purified vacuole preparations was enhanced markedly by ATP. The inability of AMP-PCP, a nonhydrolysable analogue of ATP, to stimulate CQ accumulation indicates that ATP does not act as an allosteric activator, and that hydrolysis is necessary. The requirement for ATP hydrolysis may be due either to the generation of a low vacuolar pH by V-ATPase, followed by CQ accumulation due to its weak base properties, or to the

**TABLE 1. $V_{max}$ values of CQ accumulation in vacuoles isolated from CQR and CQS strains of *P. falciparum***

| Strain | $IC_{50}$ (nM) | $V_{max}$ (fmol/$10^6$ vacuoles/hr) |
|---|---|---|
| D10 | 40 ± 17 (4) | 424.31 ± 126.96 (4) |
| RSA3 | 20 ± 3 (3) | 380.30 ± 132.0 (2) |
| FAC8 | 160 ± 23 (3) | 155.87 ± 60.94 (3) |
| W2mef | 311 ± 86 (4) | 406.41 ± 27.24 (4) |
| RSA11 | 340 ± 87 (4) | 126.85 ± 85.67 (3) |
| RSA15 | 336 ± 52 (3) | 129.50 ± 30.53 (3) |

Values are means ± SD except for the $V_{max}$ value of RSA3, which is given as mean ± range. Numbers in parentheses represent the number of experiments performed. $V_{max}$ for D10, RSA3, and W2mef was significantly different from that of FAC8, RSA11, and RSA15: $P \leq 0.02$, 0.05, and 0.002, respectively.

direct transport of CQ by a transporter present on the vacuolar membrane.

Herwaldt *et al.* [25] previously investigated CQ uptake in heterogeneous membrane preparations from CQR and CQS PRBC, but they were unable to demonstrate whether the two strains have different CQ-accumulating capabilities. In this study, vacuoles isolated from different strains demonstrated different CQ-accumulation profiles. The two CQS strains tested (D10 and RSA3) had CQ $V_{max}$ values significantly larger than three (FAC8, RSA11, and RSA15) of the four CQR strains. These results suggest that the vacuole plays a direct role in determining CQ resistance. If one assumes that one vacuole is isolated *per* parasite, the level of CQ accumulation in isolated vacuoles is about 20-fold less than that in the same number of PRBC (Fig. 1). Several possibilities, or combinations thereof, exist for this observation. First, as we have not been able to measure the internal pH of our isolated vacuoles, we are unable to establish whether decreased CQ accumulation is due to an increased pH in the isolated vacuoles compared with that found in the intact parasite. Second, there may be CQ-binding compartments in the parasite other than the vacuole. LDH has been identified recently as a cytosolic CQ-binding protein [37]. Third, haem has also been shown to bind CQ [38]. In PRBC, haem is continuously generated in the vacuole due to a regular delivery of haemoglobin. We have been unable to detect haemoglobin in our isolated vacuoles. If haem is a significant contributor to CQ accumulation in the vacuole, then the low levels of CQ accumulated by isolated vacuoles may be due to the absence of haem. Finally, a CQ importer has recently been identified and postulated to reside on the parasite plasma membrane [39]. Such an importer could generate high cytosolic levels of CQ, accounting for the higher levels of accumulation observed in PBRC.

The mechanism(s) responsible for the differential handling of CQ by vacuoles isolated from the various strains of *P. falciparum* is unclear. An elevated intravacuolar pH in CQR parasites due to weakened V-ATPase should result in lower levels of CQ accumulation in the vacuole. However, there is, as yet, no molecular evidence that links V-ATPase to CQ resistance [40, 41]. Localization of Pgh1 on the vacuole [16], and the fact that expression of Pgh1 in yeast and CHO cells is linked with accumulation of 4-aminoquinolines [19, 21], strengthen the case for a role for Pgh1 in the transport of these drugs across the parasite vacuolar membrane, directly or indirectly, by controlling $Cl^-$ flux and, hence, vacuolar pH. Any changes in Pgh1 expression, or expression of mutated Pgh1 in CQR strains, may affect CQ and other 4-aminoquinoline accumulation in the parasite digestive vacuole. The cell-free system that we have developed will allow careful examination of these ideas.

It is not clear why we found no difference in CQ accumulation at 1 nM $[CQ]_{ext}$ between all the strains tested. If the difference in CQ accumulation by sensitive and resistant parasites at higher CQ concentrations is due to a difference in vacuolar CQ binding sites, then one would expect to find less difference in accumulation at lower CQ concentrations. At 1 nM CQ, erythrocytes parasitized with CQR strains accumulate significantly less CQ than sensitive strains, although 1 nM CQ has no appreciable effect on the viability of either CQR or CQS parasites. This suggests that additional mechanisms exist to control the accumulation of CQ in PRBC. This point is strengthened by the fact that verapamil, which has been shown to increase CQ accumulation in resistant parasites and reverse CQR [3, 42], has no effect on CQ accumulation in vacuoles isolated from CQR parasites at any $[CQ]_{ext}$ (1–250 nM) tested (data not shown). Reversal of CQ resistance thus appears to take place at a site(s) other than the vacuole, sites that are perhaps also involved in CQR. However, since the site of CQ action is the digestive vacuole, the regulation of CQ accumulation by the vacuole would be crucial in determining CQ susceptibility.

W2mef (CQR) vacuoles accumulated CQ at levels similar to those of the sensitive strains at all three $[CQ]_{ext}$ tested. W2mef, the only drug-pressured strain used in this study, is the MEF-pressured daughter line of W2, a CQR but MEF-sensitive clone derived from the Sierra Leone I/CDC isolate and Indochina III/CDC [43]. W2mef is 4–6 times more MEF-resistant than W2, and slightly more sensitive to CQ, although still within the CQ-resistance threshold [44]. Perhaps the elevated levels of CQ accumulation in vacuoles isolated from W2mef (compared with vacuoles isolated from other unpressured CQR strains) is due to the larger vacuoles reported in this strain [45]. Alternatively, it could be due to increased activity or expression of either V-ATPase or Pgh1, resulting from MEF pressure.

Erythrocytes parasitized with W2mef accumulate CQ at levels similar to those of erythrocytes parasitized with other unpressured CQR strains (Fig. 2). It may be that CQ is removed from the W2mef parasite before it accumulates in the vacuole. This does not necessarily apply to other CQR strains as the lower accumulation capacity of their vacuoles may be sufficient to explain CQ resistance.

In conclusion, we have developed a method that yields pure and intact *P. falciparum* vacuoles that accumulate CQ in an ATP-dependent manner. The parasite digestive vacuole appears to play a crucial role in CQ resistance. This has important implications in understanding the mechanism of CQ resistance, and in devising ways of overcoming it.

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