A verapamil-sensitive chloroquine-associated H^+ leak from the digestive vacuole in chloroquine-resistant malaria parasites

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Accepted 28 January 2008
Journal of Cell Science 121, 1624-1632 Published by The Company of Biologists 2008
doi:10.1242/jcs.016758

Summary

Chloroquine resistance in the malaria parasite *Plasmodium falciparum* has made malaria increasingly difficult to control. Chloroquine-resistant parasites accumulate less chloroquine than their chloroquine-sensitive counterparts; however, the mechanism underlying this remains unclear. The primary site of accumulation and antimalarial action of chloroquine is the internal acidic digestive vacuole of the parasite, the acidity of which is maintained by inwardly-directed H^+ pumps, working against the (outward) leak of H^+. In this study we have investigated the leak of H^+ from the digestive vacuole of the parasite by monitoring the alkalinisation of the vacuole following inhibition of the H^+-pumping V-type ATPase by concanamycin A. The rates of alkalinisation observed in three chloroquine-resistant strains were two- to fourfold higher than those measured in three chloroquine-sensitive strains. On addition of chloroquine there was a dramatic increase in the rate of alkalinisation in the chloroquine-resistant strains, whereas chloroquine caused the rate of alkalinisation to decrease in the chloroquine-sensitive strains. The chloroquine-associated increase in the rate of alkalinisation seen in chloroquine-resistant parasites was inhibited by the chloroquine-resistance reversal agent verapamil. The data are consistent with the hypothesis that in chloroquine-resistant parasites chloroquine effluxes from the digestive vacuole, in association with H^+, via a verapamil-sensitive pathway.

Key words: Chloroquine, Chloroquine resistance, Lysosome, Malaria

Introduction

The emergence, followed by the spread throughout most malaria-endemic regions, of *Plasmodium falciparum* parasites resistant to the antimalarial drug chloroquine (CQ) has worsened the global malaria situation (Trape, 2001). CQ is thought to exert its toxic effect in the intraerythrocytic malaria parasite's digestive vacuole (DV), an acidic organelle in which haemoglobin endocytosed from the host erythrocyte's cytosol is digested into small peptides and potentially toxic haem monomers (Francis et al., 1997). Uncharged CQ is membrane-permeant and can therefore traverse the multiple membranes required for its entry into the DV from the extracellular milieu (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1984). However, once inside the acidic DV, CQ becomes trapped in its doubly protonated and, hence, less membrane-permeant form (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1984). CQ is thought to exert its antimalarial effect by binding to haem (Bray et al., 1998; Bray et al., 1999), thereby inhibiting its incorporation into inert haemozoin crystals and leading to the buildup of haem and CQ-haem complexes that kill the parasite (Fitch, 2004; Orjih et al., 1994).

CQ resistance is associated with a significant reduction in CQ accumulation by the parasite (Fitch, 1970; Krogstad et al., 1987; Yayon et al., 1984), and has been attributed to mutations in PfCRT (the *P. falciparum* chloroquine resistance transporter) (Fidock et al., 2000; Sidhu et al., 2002), a member of the 'drug/metabolite transporter' superfamily (Martin and Kirk, 2004; Tran and Saier, 2004) that is localised to the DV membrane (Cooper et al., 2002; Fidock et al., 2000). The DV localisation of PfCRT, together with the findings that the majority of the CQ accumulated by CQ-sensitive (CQS) parasites is within the DV (Bray et al., 2006), and that DVs isolated from CQ-resistant (CQR) parasites accumulate less CQ than those from CQS parasites (Saliba et al., 1998a), are consistent with the hypothesis that mutations in PfCRT cause CQ resistance by reducing the intravacuolar CQ concentration. How they do so is the subject of ongoing debate (Sanchez et al., 2007a).

In acidic organelles such as the DV, the resting pH is determined primarily by the rate of H^+ pumping into the organelle and the rate of H^+ leaking from the organelle (Demaurex, 2002; Grabe and Oster, 2001). Under physiological conditions the DV of the malaria parasite is acidified primarily by a V-type H^+-ATPase (Saliba et al., 2003). Inhibition of this pump by concanamycin A leads to DV alkalinisation as H^+ ions exit the organelle via as-yet-uncharacterised leak pathways.

It has been postulated that CQ resistance may arise as a consequence of CQR parasites having a less acidic DV than their CQS counterparts, resulting in decreased 'weak base trapping' of CQ within this compartment (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1985). Recent reports that the pH of the DV (pH_{DV}) of CQR parasites is significantly lower than that of CQS parasites (Bennett et al., 2004; Dzekunov et al., 2000; Ursos et al., 2000), and that expression of the mutant form of PfCRT in human embryonic kidney 293 (HEK293) cells results in a decreased lysosomal pH (Reeves et al., 2006), have given rise to alternate hypotheses for how PfCRT influences CQ accumulation via an effect on pH_{DV}. However these findings are at odds with recent reports that there is no significant difference between the pH_{DV} of CQR- and CQS parasite strains (Hayward et al., 2006; Klonis et al., 2007;

Kuhn et al., 2007). Furthermore, variations in intravesicular pH were unable to account for the acquisition of a CQ resistance phenotype by *Dictyostelium discoideum* that expressed mutant but not wild-type forms of PfCRT on its acidic vesicles (Naude et al., 2005).

An alternative hypothesis is that mutant PfCRT confers CQ resistance by mediating the efflux of CQ from the DV (Bray et al., 2005; Bray et al., 2006; Sanchez et al., 2007b; Warhurst et al., 2002). On the basis of experiments on intact parasitised erythrocytes, Krogstad et al. proposed that CQ resistance involves an energy-dependent CQ efflux mechanism (Krogstad et al., 1987). Work by Sanchez and colleagues, again on intact parasitised erythrocytes, has provided further support for this view. An apparent trans-stimulation of CQ accumulation in CQR parasites is consistent with the presence of a saturable drug efflux system (Sanchez et al., 2003; Sanchez et al., 2004), which has been associated with the CQR form of PfCRT (Sanchez et al., 2005). Recently, Sanchez et al. have reported that a trans-stimulated CQ efflux system is present in CQS parasites as well as CQR parasites, but that it has different kinetic properties that result in less efficient CQ efflux (Sanchez et al., 2007b). Sanchez et al. have argued that the efflux of CQ is an active (i.e. energy-requiring) process (Sanchez et al., 2007a). By contrast, Bray and others have argued for a mechanism involving the passive diffusion of protonated CQ down its concentration gradient and out of the DV (the 'charged-drug leak' hypothesis) (Bray et al., 2005; Bray et al., 2006; Warhurst et al., 2002).

The data underpinning the CQ efflux model for CQ resistance have, thus far, been derived primarily from experiments with intact parasitised erythrocytes. In this study, we have focused specifically on the DV and present evidence that in CQR parasites the presence of CQ gives rise to a substantial outward H^+ leak, consistent with CQ effluxing from the DV of CQR parasites in association with H^+. The CQ-associated leak of H^+ from the DV of CQR parasites is inhibited by verapamil, one of a growing number of compounds that have been shown to increase CQ accumulation by CQR parasites and, thereby, to sensitise them to the drug (Krogstad et al., 1987; Martin et al., 1987; van Schalkwyk and Egan, 2006).

Our results do not distinguish between the active (H^+-coupled) efflux of CQ from the vacuole and the passive efflux of the protonated form of the drug down its concentration gradient. They do, however, provide further evidence for the involvement of enhanced CQ efflux from the DV in the phenomenon of CQ resistance, as well as providing the first insights into the factors governing the leak of H^+ from the DV of the malaria parasite.

Results

The leak of H^+ from the DV is rate-limited by the H^+ permeability of the DV membrane

The pumping of H^+ into the DV of the parasite by the DV membrane H^+-ATPase is counterbalanced by the leakage of H^+ out of the DV via unknown pathways. As is illustrated in Fig. 1, on inhibition of the H^+-ATPase by the addition of concanamycin A (100 nM) there was an immediate alkalinisation of the DV. Fig. 1A,B shows representative traces for CQS D10 and CQR 7G8 parasites in which the DV had been preloaded with dextran-linked fluorescein. Similar results were obtained for 'dye-loaded' CQS 3D7 and CQR K1 parasites (not shown). As summarised both in Fig. 1D and Table 1, the rate of alkalinisation of the DV following inhibition of the H^+-ATPase was two to three times higher in the two dye-loaded CQR strains than in the two dye-loaded CQS strains (P<0.001, unpaired t-tests).

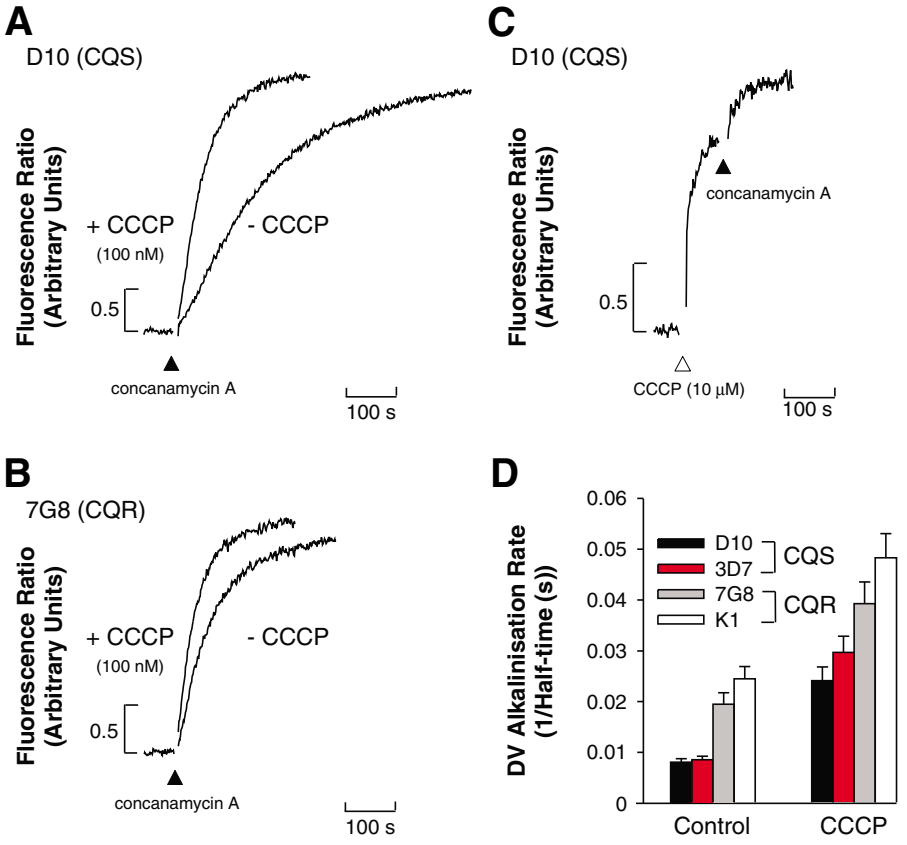

Fig. 1. Alkalinisation of the DV following inhibition of the V-type H^+-ATPase by concanamycin A. (A,B) Representative fluorometer traces for mature trophozoite-stage D10 (CQS; A) and 7G8 (CQR; B) parasites. The parasites were isolated using saponin-permeabilisation of the erythrocyte and parasitophorous vacuole membranes, and suspended in minimal saline solution at pH 7.1. The H^+ ionophore CCCP (100 nM) or solvent (DMSO) was added to parasites 1 minute prior to the addition of concanamycin A (100 nM). In A and B the baseline fluorescence ratio (before the addition of concanamycin A) is that for the DMSO control trace. Concanamycin A addition is indicated by black arrowheads. Traces are representative of those obtained in seven independent experiments for 7G8 parasites and 12 independent experiments for D10 parasites. (C) Representative fluorometer trace for saponin-isolated mature-trophozoite-stage D10 parasites to which 10 μM CCCP was added (indicated by white arrowhead) 1 minute prior to the addition of 100 nM concanamycin A (black arrowhead). Similar data were obtained with other strains (not shown). (D) Averaged data for the rate of DV alkalinisation (expressed as the inverse of the alkalinisation half-time) in the CQS strains D10 and 3D7 and in the CQR strains 7G8 and K1, to which had been added (1 minute before the addition of 100 nM concanamycin A) either CCCP in DMSO (giving a final CCCP concentration of 100 nM) or DMSO alone. Data are the average from at least five independent experiments (+ s.e.m.) for each condition and strain.

Table 1. Half-times for the concanamycin-A-induced alkalinisation of the DV for the strains used in this study

Strain	DV alkalinisation half-time (seconds)			
	–CQ, –verapamil (*n*)	–CQ, +verapamil (*n*)	+CQ, –verapamil (*n*)	+CQ, +verapamil (*n*)
D10	129±7 (8)	149±13 (8)	239±18 (8)	195±16 (5)
3D7	123±10 (13)	142±12 (12)	215±16 (13)	206±18 (9)
3D7-PM2-GFP	167±10 (9)	188±16 (8)	269±37 (9)	294±40 (8)
7G8	57±4 (10)	62±7 (10)	17±1 (10)	65±7 (6)
K1	45±3 (9)	39±3 (7)	12±1 (9)	48±3 (4)
Dd2-PM2-GFP	69±8 (3)	69±8 (3)	19±2 (3)	52±5 (3)

Values are the mean (± s.e.m.), *n*=number of experiments; +verapamil, 50 μM verapamil; +CQ, 10 μM chloroquine. Concanamycin A was used at 100 nM. For each strain there was a significant difference between the digestive vacuole (DV) alkalinisation half-time in the absence of CQ and the half-time in the presence of 10 μM CQ ($P<0.03$; paired *t*-tests). For the 7G8, K1 and Dd2-PM2-GFP strains (all CQR), there was a significant difference between the half-time for DV alkalinisation in the presence of 10 μM CQ alone and the half-time in the presence of 10 μM CQ together with 50 μM verapamil ($P<0.02$; paired or unpaired *t*-tests as appropriate). There was no significant difference in the case of the CQS strains ($P>0.1$; unpaired *t*-tests). In the absence of CQ there was no significant difference between the half-times for DV alkalinisation in the presence and absence of verapamil in any of the strains ($P\geqslant0.2$; paired or unpaired *t*-tests as appropriate).

To confirm that the difference in the rates of DV alkalinisation between CQS and CQR strains was not caused by dye-induced variations in pH_{DV} (Hayward et al., 2006), the experiment was repeated with a lower fluorescein concentration (~10 μM rather than ~110 μM). At this dye concentration, pH_{DV} estimates in CQS and CQR parasites are not significantly different from one another, and are proposed to be close to the true physiological values (Hayward et al., 2006). DV alkalinisation was again faster in both CQR strains than in both CQS strains under these conditions (not shown). The half-time values obtained with the lower fluorescein concentration were comparable with those obtained with the higher concentration, consistent with fluorescein not exerting a significant buffering effect in the DV. A shorter half-time for DV alkalinisation in CQR parasites compared with CQS parasites was also observed when fluorescein-dextran was replaced with dextran-linked forms of either 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) or DM-NERF (not shown).

Klonis et al. have recently reported the generation of CQS and CQR transfectant *P. falciparum* strains expressing pH-sensitive chimeras of green fluorescent protein (GFP) with the DV haemoglobinase plasmepsin II (PM2) (Klonis et al., 2007). In mature trophozoite-stage parasites, most of the PM2-GFP is in the DV compartment, thus allowing changes in pH_{DV} to be monitored without the need to load the DV with a pH-sensitive dye (Klonis et al., 2007). The results obtained here with two of these transfectant strains – CQS 3D7 transfectants (3D7-PM2-GFP) and CQR Dd2 transfectants (Dd2-PM2-GFP) – were similar to those obtained with the dye-loaded parasites; on addition of concanamycin A, the rate of DV alkalinisation in the CQR Dd2-PM2-GFP strain was 2.4-fold higher than that in the CQS 3D7-PM2-GFP strain (Table 1; $P<0.001$, unpaired *t*-test). It should be noted that the rate of DV alkalinisation in the CQS 3D7-PM2-GFP parasites was slightly lower than that in dye-loaded CQS parasites (3D7 and D10) (Table 1; $P<0.01$, unpaired *t*-tests). Similarly, DV alkalinisation in CQR Dd2-PM2-GFP parasites was slightly slower than in dye-loaded CQR K1 and 7G8 parasites (Table 1). This difference was statistically significant when comparing Dd2-PM2-GFP with K1 ($P=0.004$, unpaired *t*-test) but not with 7G8 ($P=0.2$, unpaired *t*-test).

The leak of H^+ from the DV could be rate-limited either by the inherent permeability of the DV membrane to H^+, or by its permeability to ions such as K^+ or Cl^- that counterbalance the charge movement associated with H^+ efflux (Lukacs et al., 1991). To discriminate between these possibilities, we investigated the effect of the conductive H^+ ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the rate of alkalinisation of the DV seen on inhibition of the V-type H^+-ATPase by concanamycin A. If the alkalinisation is rate-limited by the permeability of the DV membrane to counterions rather than by its H^+ permeability, further increasing the H^+ permeability with a H^+ ionophore should not affect the rate of alkalinisation (Lukacs et al., 1991).

As shown in the representative traces (Fig. 1A,B) and the averaged data (Fig. 1D), the addition of CCCP (100 nM, added 1 minute prior to the addition of concanamycin A) caused the rate of DV alkalinisation that is seen following inhibition of the H^+-ATPase to increase significantly in all strains ($P<0.001$, paired *t*-tests), including the PM2-GFP transfectants (not shown). This is consistent with the leak of H^+ from the DV of the parasite being limited by the inherent H^+ permeability of the DV membrane rather than by its permeability to counterions. In the majority of the experiments 100 nM CCCP (in the absence of concanamycin A) had little effect on the resting pH_{DV} in any of the strains, presumably because the parasites were able to counter the enhanced H^+ leak with an increased rate of H^+ pumping. In some experiments, the addition of 100 nM CCCP did cause a slight increase in the fluorescence ratio (<10% of the maximum increase in fluorescence ratio seen following the addition of concanamycin A), and at higher concentrations the ionophore consistently caused a significant increase in resting pH_{DV}. Fig. 1C illustrates the effect of 10 μM CCCP on pH_{DV} in dye-loaded D10 parasites; the addition of the ionophore resulted in a marked alkalinisation, presumably because at this CCCP concentration the enhanced H^+ leak was too great to be countered effectively by the H^+ pumps of the parasite. The subsequent addition of concanamycin A caused a further small alkalinisation.

The finding that for all the strains tested the final fluorescence ratio reached (following the concanamycin-A-induced alkalinisation) was similar in the presence and absence of CCCP (see Fig. 1A,B) suggests that the concanamycin-A-sensitive H^+-ATPase is the sole H^+ pump operating in the DV, and that the previously characterised DV H^+-PPase (Saliba et al., 2003) did not contribute significantly to the acidification of the DV under the conditions used here.

A CQ-associated H^+ leak from the DV in CQR but not CQS parasites

If CQ effluxes from the DV as either the mono- or diprotonated species, and/or is transported from the vacuole in symport with H^+, then this will constitute a H^+ leak from the DV. We therefore

investigated the effect of CQ on the rate of the concanamycin-A-induced alkalinisation in the CQS strains D10, 3D7 and 3D7-PM2-GFP and in the CQR strains 7G8, K1 and Dd2-PM2-GFP. In all of the CQR strains the addition of CQ (10 μM) 1 minute prior to the addition of concanamycin A increased the rate of DV alkalinisation following H^+ pump inhibition, reducing the half-times by 3.5±0.2-fold (mean ± s.e.m.) for 7G8 (P<0.001, paired t-test), 3.9±0.3-fold for K1 (P<0.001, paired t-test), and 3.6±0.2-fold for Dd2-PM2-GFP (P=0.02, paired t-test). The variations in fold-differences between the strains were not significant (P>0.3, unpaired t-tests). Fig. 2A,B (see also Fig. 4B) show representative traces, and the mean half-times for DV alkalinisation (with and without the addition of CQ) obtained in paired experiments are summarised in Table 1. A similar CQ-induced increase in the rate of DV alkalinisation was seen for both 7G8 and K1 parasites when the fluorescein concentration in the loading solution was reduced approximately tenfold (to ~10 μM; data not shown).

In all the CQR strains the addition of CQ was seen to cause a small increase in fluorescence ratio (or fluorescence intensity in the case of Dd2-PM2-GFP) (Fig. 2A,B and Fig. 4B). In in-vitro experiments the addition of CQ (10 μM) to simple aqueous solutions of fluorescein-dextran (with pH values between 5.6 and 6.2) resulted in a small increase in fluorescence ratio, raising the possibility that the increase in fluorescence ratio seen on addition of CQ to dye-loaded cells was due to, at least in part, a direct effect of CQ on fluorescein fluorescence. However, the fact that a similar increase was seen for the parasites in which the fluorophore was GFP (rather than fluorescein) is consistent with CQ having caused a genuine increase in the resting pH_{DV} in the CQR parasites. This is most likely to arise from the H^+ pump not being able to counter completely the additional (CQ-associated) H^+ leak. The finding that the addition of 400 nM CCCP to K1 parasites increased the H^+ leak (measured following the addition of concanamycin A) to the same extent as did 10 μM CQ (giving a half-time of 12±1 seconds, n=3; data not shown) and caused the fluorescence ratio (prior to the addition of concanamycin A) to increase to an extent that was comparable with that seen using 10 μM CQ (not shown) is consistent with this interpretation.

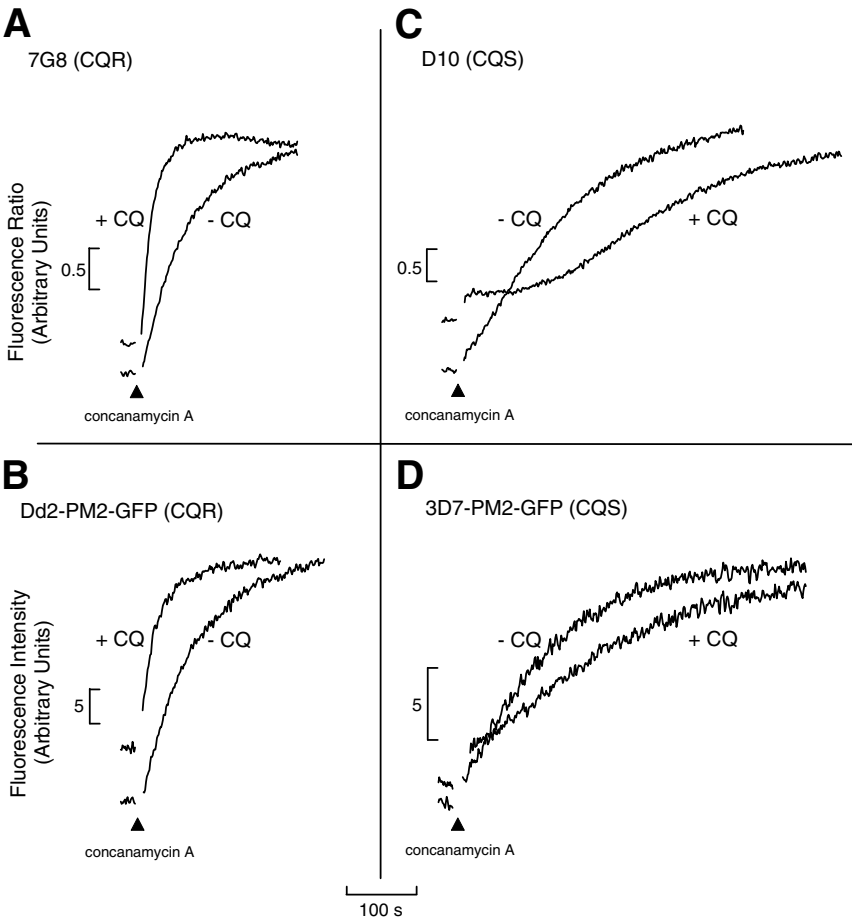

Fig. 2. (A-D) Fluorometer traces showing the effect of CQ on the alkalinisation rate of the DV in (A) CQR 7G8, (B) CQR Dd2-PM2-GFP, (C) CQS D10 and (D) CQS 3D7-PM2-GFP parasites following the addition of concanamycin A. CQ (10 μM) was added to saponin-isolated mature trophozoites 1 minute before the addition of concanamycin A (100 nM; black arrowheads). The data are from single experiments, representative of those obtained in at least three separate experiments for each strain.

In contrast to the situation with the CQR strains, in all CQS strains the addition of 10 μM CQ slowed DV alkalinisation. The addition of CQ introduced a significant lag phase into the pH_{DV} traces, giving them a sigmoidal appearance, and the half-time for alkalinisation increased by >1.6-fold (P<0.03, paired t-tests; Fig. 2C,D and Fig. 4A; Table 1). As can be seen in Fig. 2C,D and Fig. 4A (and as was seen for the CQR strains), the addition of 10 μM CQ caused a small increase in the resting fluorescence ratio or intensity, consistent with the intravacuolar accumulation of CQ (which is much higher in the CQS strains than in the CQR strains) causing a small increase in pH_{DV}. Klonis et al. reported that 10 μM CQ completely alkalinises the DV in 3D7-PM2-GFP (Klonis et al., 2007); however in their study the drug was applied to infected erythrocytes rather than isolated parasites, and for a much longer time (1.5 hours) than was used here.

The CQ-associated increase in the rate of alkalinisation seen in the CQR parasites was investigated in more detail in the CQR 7G8 strain. The rate of DV alkalinisation (presented as the inverse of the half-time in Fig. 3A and as the initial rate of alkalinisation in Fig. 3B) increased with increasing CQ concentration, with a significant effect observed with CQ concentrations as low as 2.5 μM (P<0.02, paired t-test). The immediate CQ-induced rise in fluorescence ratio (discussed above) was not observed at CQ concentrations below 5 μM. The CQ-induced decrease in the rate of alkalinisation seen in the CQS parasites was investigated further in the D10 strain. The rate of DV alkalinisation decreased with increasing CQ concentration and the half-time was 2.4-fold greater in the presence of 20 μM CQ than in the absence of CQ (Fig. 3).

To test whether the CQ-induced decrease in the alkalinisation rate in CQS parasites might be due to CQ actually inhibiting the leak of H^+ from the DV, the experiment was repeated in cells that had been treated with the H^+ ionophore CCCP (100 nM) in order to provide an alternative (much faster) H^+ leak. As shown in Fig. 3, the effect of CQ on the rate of DV alkalinisation in CCCP-treated

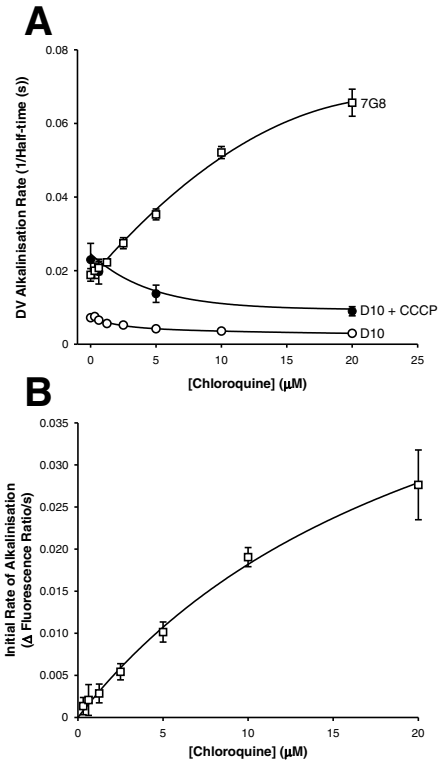

Fig. 3. (A) Effect of increasing CQ concentrations on the rate of DV alkalinisation (expressed as the inverse of the half-time) following H^+ pump inhibition in CQS D10 (circles) and CQR 7G8 (squares) parasites. For D10 parasites, the experiment was performed both in the absence (○) and presence (●) of the H^+ ionophore CCCP (100 nM). CQ and CCCP were added to saponin-isolated mature trophozoites 1 minute prior to the addition of the H^+ pump inhibitor concanamycin A (100 nM). Data are averaged from three separate experiments for D10 and from four separate experiments for 7G8, and are shown as mean ± s.e.m. Where not shown, error bars fall within the symbol. (B) CQ-concentration-dependence of the initial rate of alkalinisation of the DV in CQR 7G8 parasites (estimated from the initial slope of the fluorescence traces) following inhibition of the H^+ pump by concanamycin A (100 nM). CQ was added to saponin-isolated mature trophozoites 1 minute before the addition of concanamycin A. The fluorescence ratio was normalised between experiments by dividing by the maximum fluorescence ratio. The initial rate of alkalinisation in the absence of CQ was subtracted from the initial rates in the presence of CQ. The data (± s.e.m.) are averaged from four independent experiments; the line was drawn using a rectangular hyperbola, fitted to the data [$y=ax/(b+x)$, where y is the initial rate of alkalinisation, x is the CQ concentration, a=0.06 second^{-1} and b=23 μM].

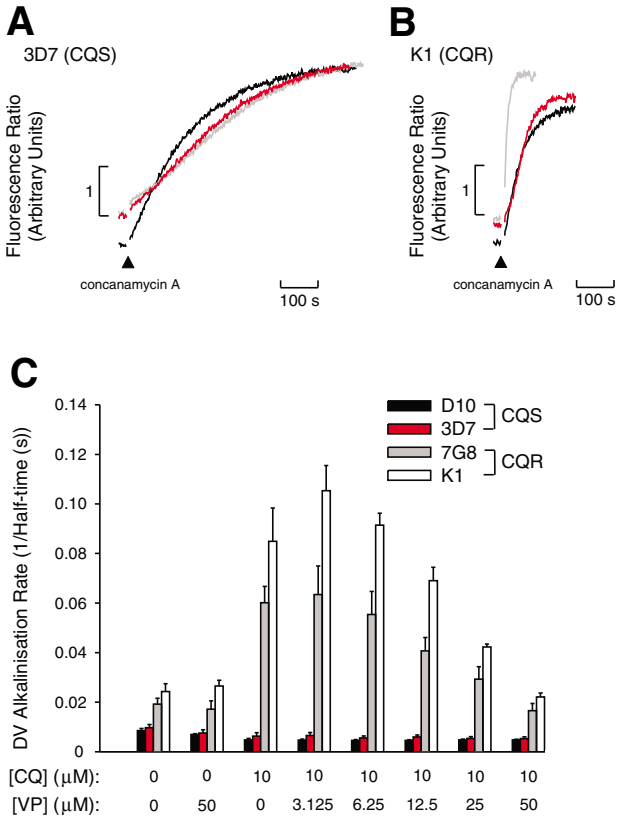

Fig. 4. Effect of verapamil on CQ-induced changes in the concanamycin-A-induced alkalinisation of the DV in saponin-isolated mature trophozoite-stage CQS and CQR parasites. (A,B) Fluorometer traces showing DV alkalinisation in the absence of CQ and verapamil (black), in the presence of 10 μM CQ (light grey), and in the presence of 10 μM CQ and 50 μM verapamil (red) in the (A) CQS 3D7 strain and the (B) CQR K1 strain. Data are representative of nine independent experiments for 3D7 and four independent experiments for K1. (C) Averaged data obtained from at least three independent experiments (+ s.e.m.) for the CQS 3D7 and D10 strains and the CQR 7G8 and K1 strains. In all cases, CQ and verapamil were added to the parasites 1 minute before the addition of concanamycin A (100 nM).

cells was similar to that seen in cells in the absence of CCCP. CQ caused a marked slowing of the alkalinisation; 20 μM CQ caused a 2.4-fold increase in the half-time (P=0.003, paired t-test), just as was found in the absence of CCCP. The CQ-induced slowing of the alkalinisation of the DVs of CQS parasites following H^+ pump inhibition is therefore not because of an effect on the H^+ permeability of the DV membrane.

The CQ-associated H^+ leak in CQR parasites is inhibited by the CQ-resistance-reverser verapamil

In the absence of CQ, the CQ-resistance-reverser verapamil (50 μM) had no significant effect on the half-times for DV alkalinisation in any of the CQR strains ($P \geq 0.2$, paired or unpaired t-tests as appropriate; Fig. 4C; Table 1), but caused a slight (although not statistically significant) increase in the half-times for the CQS strains ($P \geq 0.2$, unpaired t-tests; Fig. 4C; Table 1). In all six strains the addition of verapamil caused a small increase in the fluorescence ratio (or fluorescence intensity in the case of the PM2-GFP transfectants), ranging from 3-14% of the total increase observed after concanamycin-A-induced DV alkalinisation, consistent with there being a small verapamil-induced rise in pH_{DV}.

As shown for the CQR 7G8 and K1 strains in Fig. 4C, verapamil reversed the CQ-associated increase in the rate of alkalinisation of the DV following H^+ pump inhibition. For both of the CQR strains, a verapamil concentration of 25 μM was required for significant inhibition (P<0.03, paired t-tests), and 50 μM for complete inhibition, of the increase in the rate of alkalinisation induced by 10 μM CQ. Verapamil (tested at a single concentration of 50 μM) also inhibited the H^+ leak induced by 10 μM CQ in CQR Dd2-PM2-GFP parasites (Table 1). The verapamil concentrations used in these experiments are higher than those required to reverse CQ resistance in parasite proliferation assays with the same strains (Table 2).

In contrast to the verapamil sensitivity of the effect of CQ on DV alkalinisation in the CQR strains, verapamil (50 μM) had no significant effect on the half-times for DV alkalinisation in the presence of 10 μM CQ in the CQS D10, 3D7 and 3D7-PM2-GFP strains (P>0.1, unpaired t-tests; Fig. 4; Table 1). Representative

Table 2. Chloroquine sensitivities of the strains used in this study

Strain	CQ IC_{50} (nM)		
	–Verapamil	+Verapamil (1.56 μM)	+Verapamil (6.25 μM)
D10	10±2	14±4	13±2
3D7	9±1	13±3	9±1
3D7-PM2-GFP	5±2	4±1	4±2
7G8	78±5	40±11	21±3
K1	149±7	46±8	22±0
Dd2-PM2-GFP	54±3	13±1	11±1

CQ IC_{50}, concentration (in nM) at which 50% of parasite proliferation is inhibited. Values are the mean (± range÷2) from two independent experiments for 3D7, 3D7-PM2-GFP, K1 and Dd2-PM2-GFP, and the mean (± s.e.m.) from three independent experiments for D10 and 7G8. All experiments were performed in duplicate.

traces showing the effect of 10 μM CQ on the rate of DV alkalinisation in the presence and absence of 50 μM verapamil for the CQS 3D7 strain and the CQR K1 strain are shown in Fig. 4A and B, respectively.

Discussion

The H^+ leak from the DV

On inhibition of the DV H^+-ATPase there was an immediate alkalinisation of the DV, consistent with there being a rapid efflux of H^+ from this organelle. In order for this to occur it is necessary that the charge movement associated with the efflux of H^+ is counterbalanced by the movement of other ions either into or out of the DV (Demaurex, 2002). The finding in this study that addition of the conductive H^+ ionophore CCCP increased the rate at which the DV alkalinised following inhibition of the H^+ pump (in both CQS and CQR parasites) is consistent with the leak of H^+ from the DV being rate-limited by the inherent H^+ permeability of the membrane, rather than by its permeability to counterions. The same conclusion has been drawn with regard to the leak of H^+ from acidic organelles in a range of different cell types (Demaurex et al., 1998; Llopis et al., 1998; Lukacs et al., 1991; Schapiro and Grinstein, 2000; Wu et al., 2001).

Neither the identity nor characteristics of the pathway(s) underlying the leak of H^+ (or counterions) from the DV of the malaria parasite have been investigated. In other cell types, acidic organelles have H^+ channels (Demaurex, 2002; Schapiro and Grinstein, 2000), as well as transporters that use the outward transmembrane H^+ gradient to energise the influx or efflux of solutes such as sugars, amino acids, nucleosides and organic and inorganic anions (Martinoia et al., 2000; Schuldiner et al., 1995). The parasite's DV might also be endowed with H^+-coupled transporters, although this remains to be demonstrated.

The rates of DV alkalinisation following inhibition of the H^+ pump were significantly higher in the two dye-loaded CQR parasite strains than in the two dye-loaded CQS parasite strains tested. DV alkalinisation was also faster in CQR Dd2-PM2-GFP parasites than in CQS 3D7-PM2-GFP parasites, although the rates for these transfectant strains were somewhat lower than those of dye-loaded CQR and CQS parasites, respectively. The reason for this difference between the dye-loaded and transfectant parasites is not known, although one possibility is that PM2-GFP exerts a significant buffering effect in the DV.

The finding that the DVs of CQR parasites alkalinised faster than those of CQS parasites upon H^+ pump inhibition with concanamycin A is consistent with the recent report that DV alkalinisation in the CQR strain Dd2 is faster than that in the CQS strain HB3 following inhibition of the H^+ pump by ATP depletion (Gligorijevic et al., 2006). The reason for this difference remains to be elucidated. It is possible that mutations in PfCRT, which may be a H^+-coupled transporter (Kirk and Saliba, 2001; Martin and Kirk, 2004), account for the greater H^+ leak from the DV in CQR parasites compared with CQS parasites. This could arise if the mutations uncouple the co-transport of H^+ and substrate, such that PfCRT becomes a H^+ channel. It has been shown that a single mutation (arginine 282 to glutamate) in the rabbit proton-peptide co-transporter PepT1 uncouples the movement of peptides and H^+ (Meredith, 2004). Alternatively, the mutations could result in a greater leak of H^+ by affecting other factors, such as the H^+/substrate stoichiometry or the binding affinity of either the substrate or H^+.

The CQ-associated H^+ leak from the DV in CQR parasites

The major finding in this study is that, in the presence of CQ, the rate of DV alkalinisation following H^+ pump inhibition increased dramatically in CQR but not CQS strains of *P. falciparum*, and that the pathway underlying the faster alkalinisation was inhibited by the CQ resistance reversing agent verapamil. The data are consistent with the presence in CQR parasites of a (verapamil-sensitive) pathway that mediates the efflux of CQ, together with H^+, from the DV (as represented schematically in Fig. 5). They therefore provide

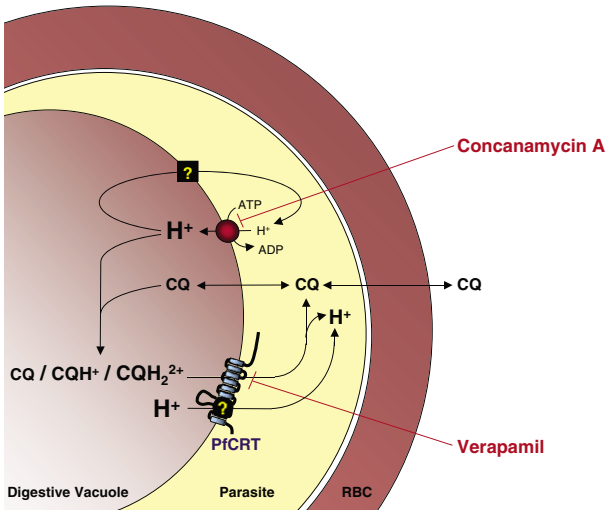

Fig. 5. Representation of the CQ-associated H^+ leak observed in CQR strains. Normal resting pH_{DV} reflects a balance between the inward H^+ pumping of the V-type H^+-ATPase and the outward leak of H^+ from the DV, via as yet uncharacterised leak pathways (indicated by the boxed question mark). On inhibition of the V-type H^+-ATPase with concanamycin A, efflux of H^+ via the leak pathways results in DV alkalinisation. CQ (in its neutral form) diffuses across the membranes separating the extracellular medium from the DV, but on reaching the acidic interior of the DV becomes protonated (mostly diprotonated) and, hence, less membrane-permeant. The efflux of CQ, in its protonated form and/or in symport with H^+, from the DV of CQR parasites, [via the mutated PfCRT (K76T) protein], constitutes a new (verapamil-sensitive) H^+ leak which is responsible for the CQ-associated increase in the rate of alkalinisation following inhibition of the V-type H^+-ATPase. The circled question mark indicates the possibility that the flux of H^+ via mutated PfCRT underlies the higher rate of alkalinisation (following pump inhibition) seen in CQR parasites, as well as the possibility that the efflux of CQ via mutated PfCRT is a H^+-coupled (and therefore secondary active transport) process. RBC, red blood cell.

support for the hypothesis that CQ efflux from the DV in CQR parasites is a major determinant of CQ resistance. The results do not distinguish between a passive leak of protonated CQ and an active transport process involving the transport of protonated or unprotonated CQ in symport with H^+ ions.

The CQ concentration dependence of the initial rate of CQ-associated DV alkalinisation in the CQR 7G8 strain was fitted by a rectangular hyperbola [$y=ax/(b+x)$] with b=23 μM. Although this equates with the Michaelis-Menten equation, with an apparent K_m of 23 μM, it is important to emphasise that this does not represent a true estimate of the K_m of the transport process for the following three reasons. First, b exceeds the highest CQ concentration tested. Second, the concentration of CQ within the DV of CQR parasites at each of the different CQ concentrations tested is not known, and may well differ from that added to the medium. Third, it is possible that, as the CQ concentration and hence the alkalinisation rate is increased, factors other than CQ:H^+ transport (e.g. the permeability of the DV membrane to counterions) will begin to limit the DV alkalinisation rate. Nevertheless, it can be concluded that, if CQ efflux does occur via a saturable process, the K_m is likely to be supramicromolar. This is at odds with a previous study (Sanchez et al., 2003) in which it was proposed, on the basis of trans-stimulation experiments carried out with intact parasitised erythrocytes, that CQ efflux involves a carrier with a high affinity for CQ (in the low nanomolar range). It also contrasts with the findings of a recent study in which PfCRT (containing the key mutation associated with CQ resistance, K76T, though not the other 'compensatory' mutations that accompany the K76T mutation in the field) was expressed in *Pichia pastoris* then reconstituted into proteoliposomes and reported to transport CQ with an apparent K_m of 280 nM (Tan et al., 2006).

The CQR 7G8, K1 and Dd2-PM2-GFP strains used in this study differed significantly in their CQ IC_{50} values, and in the extent to which these IC_{50} values were reduced by verapamil (Table 2); however, the rate of alkalinisation measured in the presence of 10 μM CQ, and the extent to which this was decreased by verapamil, was broadly similar for the three strains (Table 1). It should be emphasised as above that, at the relatively high CQ concentration of 10 μM, the alkalinisation rate is likely to be affected by factors other than CQ:H^+ transport and that, under the conditions used in this study, any relationship between the magnitude or verapamil sensitivity of the CQ-associated H^+ leak and the CQ or verapamil sensitivity of the parasites in growth assays, cannot be quantified precisely.

The basis for differences in CQ response among different CQR strains is not known. Sidhu et al. reported that CQR strains generated by transfecting the CQS GC03 strain with either the Dd2 *pfcrt* allele (which differs from the K1 allele only at amino acid position 356) or the 7G8 *pfcrt* allele (which has a distinct set of 'South American' mutations) had very similar CQ IC_{50} values (Sidhu et al., 2002) . There was a greater difference between the CQ IC_{50} values of the wild-type Dd2 and 7G8 strains (Sidhu et al., 2002), suggesting that mutations in *pfcrt* cannot account fully for differences in CQ response among CQR strains. The CQR strains used in this study all possess different *pfmdr1* alleles (Chen et al., 2002), which may contribute to differences in their CQ IC_{50} values (Duraisingh and Cowman, 2005). However, differences in CQ responses among CQR strains appears to involve multiple genes (Mu et al., 2003), and CQR strains carrying identical *pfcrt* and *pfmdr1* alleles can still differ in their CQ responses (Chen et al., 2002).

In contrast to the situation with CQR parasites, CQ slowed the DV alkalinisation in CQS parasites. The possibility that CQ inhibits a H^+ leak was ruled out using the H^+ ionophore CCCP, which did not negate the (slowing) effect of CQ on DV alkalinisation. Other possible explanations for the effect of CQ on the rate of DV alkalinisation in CQS parasites include a CQ-associated increase in the internal buffering of the DV (most probably because of the accumulated CQ itself), or a CQ-induced decrease in the surface-area-to-volume ratio of the DV. The sigmoidal nature of the time-course for the increase in pH_{DV} following H^+ pump inhibition in the presence of CQ (Fig. 2) is consistent with the former explanation.

It has previously been suggested that there is a leak of protonated CQ from the DV in CQS parasites (Hayward et al., 2006). Our data do not exclude the possibility of there being such a leak that is masked (in the alkalinisation experiment) by the buffering of H^+ by the CQ accumulated in the DV. However, any such leak must be small relative to the native H^+ leaks of the DV and/or insensitive to verapamil, which had no significant effect on the half-times for alkalinisation of the DV in CQS parasites in the presence of CQ.

The CQ concentrations that were necessary to observe the CQ-associated H^+ leak in this study were higher than those required for parasite killing (although it should be noted that there was no evidence for CQ damaging the parasites on the short time-scale of the experiments; microscopic examination of parasites at the conclusion of such experiments revealed that they remained intact and with the fluorescence still localised to their DVs). This is an unavoidable requirement of the method of detection of the H^+ leak. At the sub-micromolar CQ concentrations required to kill the parasite, any CQ-induced H^+ leak is not detectable over the constitutive H^+ leak pathways. Furthermore, the parasites used in this study were isolated from their host erythrocytes and were, therefore, not actively endocytosing haemoglobin during the course of the experiments. This may have consequences for their intravacuolar haem concentrations, which in turn may affect the concentration of free (non-haem-bound) CQ in their DVs and the magnitude of their CQ-associated H^+ leaks. These issues notwithstanding, the significance of this work lies not in the magnitude or consequences for DV physiology of the CQ-associated H^+ leak but in the fact that it provides evidence for the existence of a verapamil-sensitive CQ:H^+ pathway in the DV of CQR parasites.

The fact that the verapamil concentrations required to inhibit the CQ-associated H^+ leak (50 μM for complete inhibition) are higher than those required to reverse CQ resistance in parasite proliferation assays might reflect the fact that the transport experiments described here were performed on a time-scale of a few minutes whereas the growth assays were carried out over two days. Furthermore, as noted above, the CQ concentration (10 μM) used in these experiments is higher than those used to achieve parasite killing in proliferation assays and, if verapamil and CQ compete for binding to PfCRT, it might follow that higher verapamil concentrations are required under these conditions. The verapamil concentrations used to lower the IC_{50} values of CQ against CQR parasites in vitro are approximately fivefold to 20-fold higher than the CQ IC_{50} values in the absence of verapamil. Similarly, the concentrations of verapamil required here to inhibit the CQ-associated H^+ leak were some fivefold higher than the CQ concentration used.

In summary, in this study, measurements of the alkalinisation of the DV following inhibition of the V-type H^+-ATPase of the parasite (Krogstad et al., 1985; Saliba et al., 2003) allowed us to test a hypothesis for the mechanism of CQ resistance. The results are consistent with the presence in CQR (but not CQS) parasites of a

verapamil-sensitive pathway that mediates the efflux of CQ together with H^+ from the DV. The presence of such a pathway would account for the reduced accumulation of CQ by (and hence the reduced CQ sensitivity of) CQR parasites. The approach used here may also be applicable to investigating other H^+-coupled transport processes of interest in the DV of the malaria parasite.

Materials and Methods

Culture conditions

The CQS *P. falciparum* strains 3D7, D10 and 3D7-PM2-GFP and the CQR strains 7G8, K1 and Dd2-PM2-GFP were cultured and synchronised as described elsewhere (Allen and Kirk, 2004). Their CQ sensitivities are shown in Table 2.

Loading fluorescein-dextran into the digestive vacuole of the parasite

The pH_{DV} in the 3D7, D10, 7G8 and K1 strains was monitored by loading the membrane-impermeant pH indicator fluorescein-dextran (pK_a ~6.4; ~10×10^3 M_r) into the DV. The dye was loaded into uninfected erythrocytes by a lysing-then-resealing process as described previously (Krogstad et al., 1985; Saliba et al., 2003), and the dye-loaded erythrocytes were then inoculated with trophozoite-infected erythrocytes. The parasites invaded the erythrocytes and, as they matured, endocytosed the fluorescein-containing erythrocyte cytosol into their DVs (Krogstad et al., 1985; Saliba et al., 2003). Typically, 1 ml of infected erythrocyte culture (~20% parasitaemia, 4% haematocrit) was added per ml of loaded erythrocytes, and experiments were performed after two complete life cycles. For some experiments, trophozoite-infected erythrocytes were separated from uninfected erythrocytes using a Miltenyi Biotec VarioMACS magnet (Paul et al., 1981; Staalsoe et al., 1999), allowing the addition of more infected erythrocytes with fewer uninfected erythrocytes. When this was performed, experiments could often be performed after just one complete life cycle.

Unless stated otherwise, the concentration of fluorescein in the loading solution was ~110 μM. It has been reported that this concentration of fluorescein raises the resting pH_{DV} (Hayward et al., 2006). Key results were therefore confirmed using a lower dye concentration (~10 μM), at which the pH_{DV} is thought to be close to its 'physiological' value (Hayward et al., 2006). The lower concentration was not used throughout because of difficulties associated with obtaining an adequate signal and the need to use much higher concentrations of dye-loaded cells.

Fluorescence measurements

Fluorometry experiments were performed, essentially as described previously (Saliba et al., 2003), on mature trophozoite-stage parasites (fluorescein-loaded in the case of 3D7, D10, 7G8 and K1 but not in the case of 3D7-PM2-GFP or Dd2-PM2-GFP) that had been functionally isolated by permeabilising the host erythrocyte and parasitophorous vacuole membranes with saponin (0.05% w/v yielding a 0.005% w/v solution of the active agent sapogenin) (Saliba and Kirk, 1999). Parasites isolated using this technique retain an intact plasma membrane and are able to maintain large transmembrane ion gradients (Alleva and Kirk, 2001; Saliba and Kirk, 1999), as well as an inwardly negative membrane potential (Allen and Kirk, 2004). They maintain a physiological ATP concentration (Saliba and Kirk, 1999), and synthesise proteins (Martin and Kirk, 2007) and metabolise the essential vitamin pantothenic acid (Saliba et al., 1998b) at the same rates as parasites within intact erythrocytes. The integrity of the DV membrane in isolated parasites was confirmed in this study using fluorescence microscopy, which revealed that fluorescein was localised exclusively to the DV, and by performing pH calibrations at the conclusion of a number of experiments with fluorescein-loaded parasites, which yielded acidic pH values comparable with those obtained by Hayward et al. (Hayward et al., 2006).

The isolated parasites were washed several times and resuspended in a saline solution (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glucose and 25 mM HEPES; pH 7.1) at a density of ~10^7 cells/ml. For each trace, an aliquot (1 ml) of the parasite suspension was transferred to a cuvette and fluorescence measurements were performed at 37°C using a PerkinElmer Life Sciences LS-50B spectrofluorometer with a dual excitation Fast Filter accessory. For fluorescein-loaded parasites, the ratio of the fluorescence intensity measured at 520 nM using two excitation wavelengths (490 nM and 450 nM) varies systematically with pH and was used throughout this study as an indicator of pH_{DV}. For 3D7-PM2-GFP and Dd2-PM2-GFP parasites, the fluorescence intensity obtained using excitation and emission wavelengths of 480 nm and 520 nm, respectively, was used as an indicator of pH_{DV}. Unless stated otherwise, compounds tested for their effect on DV alkalinisation were added 1 minute prior to the addition of the H^+-pump inhibitor concanamycin A. The relevant solvent controls were performed in each case.

To determine half-times of DV alkalinisation, a sigmoidal curve $\{y=y_0+a/[1+(t/t_{1/2})^b]\}$ was fitted to the data by regression analysis using SigmaPlot, where y is the fluorescence ratio, y_0 is the initial fluorescence ratio, t is time, $t_{1/2}$ is the half-time for DV alkalinisation, a is the maximal change in fluorescence ratio and b is a fitted constant. y_0 was set to the resting fluorescence ratio averaged over the 20 seconds immediately prior to the opening of the fluorometer chamber to add concanamycin A, and the next reading above this baseline was assumed to occur 4 seconds after the addition of concanamycin A to the cuvette (because of the time taken to close the chamber). Initial rates of DV alkalinisation were determined by fitting a line through the initial linear portion of the fluorescence traces.

Parasite proliferation assays

Parasite proliferation assays were performed in 96-well plates over 48 hours using the [^{3}H]hypoxanthine incorporation assay (Desjardins et al., 1979).

This work was supported by Australian National Health and Medical Research Council Grant 418055. We are grateful to Simon Cobbold for the independent testing of key findings, to the Canberra Branch of the Australian Red Cross Blood Service for the provision of blood, and to Michael Klemba and Leann Tilley for the generous gift of the 3D7-PM2-GFP and Dd2-PM2-GFP parasite strains.

References

Allen, R. J. W. and Kirk, K. (2004). The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* **279**, 11264-11272.

Alleva, L. M. and Kirk, K. (2001). Calcium regulation in the intraerythrocytic malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **117**, 121-128.

Bennett, T. N., Kosar, A. D., Ursos, L. M. B., Dzekunov, S., Sidhu, A. B. S., Fidock, D. A. and Roepe, P. D. (2004). Drug resistance-associated PfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol. Biochem. Parasitol.* **133**, 99-114.

Bray, P. G., Mungthin, M., Ridley, R. G. and Ward, S. A. (1998). Access to hematin: the basis of chloroquine resistance. *Mol. Pharmacol.* **54**, 170-179.

Bray, P. G., Janneh, O., Raynes, K. J., Mungthin, M., Ginsburg, H. and Ward, S. A. (1999). Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. *J. Cell Biol.* **145**, 363-376.

Bray, P. G., Martin, R. E., Tilley, L., Ward, S. A., Kirk, K. and Fidock, D. A. (2005). Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Mol. Microbiol.* **56**, 323-333.

Bray, P. G., Mungthin, M., Hastings, I. M., Biagini, G. A., Saidu, D. K., Lakshmanan, V., Johnson, D. J., Hughes, R. H., Stocks, P. A., O'Neill, P. M. et al. (2006). PfCRT and the trans-vacuolar proton electrochemical gradient: regulating the access of chloroquine to ferriprotoporphyrin IX. *Mol. Microbiol.* **62**, 238-251.

Chen, N., Russell, B., Fowler, E., Peters, J. and Cheng, Q. (2002). Levels of chloroquine resistance in *Plasmodium falciparum* are determined by loci other than *pfcrt* and *pfmdr1*. *J. Infect. Dis.* **185**, 405-407.

Cooper, R. A., Ferdig, M. T., Su, X. Z., Ursos, L. M. B., Mu, J., Nomura, T., Fujioka, H., Fidock, D. A., Roepe, P. D. and Wellems, T. E. (2002). Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol. Pharmacol.* **61**, 35-42.

Demaurex, N. (2002). pH homeostasis of cellular organelles. *News Physiol. Sci.* **17**, 1-5.

Demaurex, N., Furuya, W., D'Souza, S., Bonifacino, J. S. and Grinstein, S. (1998). Mechanism of acidification of the trans-Golgi network (TGN). In situ measurements of pH using retrieval of TGN38 and furin from the cell surface. *J. Biol. Chem.* **273**, 2044-2051.

Desjardins, R. E., Canfield, C. J., Haynes, J. D. and Chulay, J. D. (1979). Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob. Agents Chemother.* **16**, 710-718.

Duraisingh, M. T. and Cowman, A. F. (2005). Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Trop.* **94**, 181-190.

Dzekunov, S. M., Ursos, L. M. B. and Roepe, P. D. (2000). Digestive vacuolar pH of intact intraerythrocytic *P. falciparum* either sensitive or resistant to chloroquine. *Mol. Biochem. Parasitol.* **110**, 107-124.

Ferrari, V. and Cutler, D. J. (1991). Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with *Plasmodium falciparum*. Evidence for a drug-importer in chloroquine-sensitive strains. *Biochem. Pharmacol.* **42**, S167-S179.

Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M. B., Sidhu, A. B., Naude, B., Deitsch, K. W. et al. (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell* **6**, 861-871.

Fitch, C. D. (1970). *Plasmodium falciparum* in owl monkeys: drug resistance and chloroquine binding capacity. *Science* **169**, 289-290.

Fitch, C. D. (2004). Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life Sci.* **74**, 1957-1972.

Francis, S. E., Sullivan, D. J., Jr and Goldberg, D. E. (1997). Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* **51**, 97-123.

Gligorijevic, B., Bennett, T., McAllister, R., Urbach, J. S. and Roepe, P. D. (2006). Spinning disk confocal microscopy of live, intraerythrocytic malarial parasites. 2. Altered vacuolar volume regulation in drug resistant malaria. *Biochemistry* **45**, 12411-12423.

Grabe, M. and Oster, G. (2001). Regulation of organelle acidity. *J. Gen. Physiol.* **117**, 329-344.

Hayward, R., Saliba, K. J. and Kirk, K. (2006). The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. *J. Cell Sci.* **119**, 1016-1025.

Homewood, C. A., Warhurst, D. C., Peters, W. and Baggaley, V. C. (1972). Lysosomes, pH and the anti-malarial action of chloroquine. *Nature* **235**, 50-52.

Kirk, K. and Saliba, K. J. (2001). Chloroquine resistance and the pH of the malaria parasite's digestive vacuole. *Drug Resist. Updat.* **4**, 335-337.

Klonis, N., Tan, O., Jackson, K., Goldberg, D., Klemba, M. and Tilley, L. (2007). Evaluation of pH during cytostomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*. *Biochem. J.* **407**, 343-354.

Krogstad, D. J., Schlesinger, P. H. and Gluzman, I. Y. (1985). Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J. Cell Biol.* **101**, 2302-2309.

Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A. M., Martin, S. K., Milhous, W. K. and Schlesinger, P. H. (1987). Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science* **238**, 1283-1285.

Kuhn, Y., Rohrbach, P. and Lanzer, M. (2007). Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin. *Cell. Microbiol.* **9**, 1004-1013.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95**, 6803-6808.

Lukacs, G. L., Rotstein, O. D. and Grinstein, S. (1991). Determinants of the phagosomal pH in macrophages. In situ assessment of vacuolar H^+-ATPase activity, counterion conductance, and H^+ "leak". *J. Biol. Chem.* **266**, 24540-24548.

Martin, R. E. and Kirk, K. (2004). The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. *Mol. Biol. Evol.* **21**, 1938-1949.

Martin, R. E. and Kirk, K. (2007). Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *Blood* **109**, 2217-2224.

Martin, S. K., Oduola, A. M. and Milhous, W. K. (1987). Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science* **235**, 899-901.

Martinoia, E., Massonneau, A. and Frangne, N. (2000). Transport processes of solutes across the vacuolar membrane of higher plants. *Plant Cell Physiol.* **41**, 1175-1186.

Meredith, D. (2004). Site-directed mutation of arginine 282 to glutamate uncouples the movement of peptides and protons by the rabbit proton-peptide cotransporter PepT1. *J. Biol. Chem.* **279**, 15795-15798.

Mu, J., Ferdig, M. T., Feng, X., Joy, D. A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R. A., Wootton, J. C. et al. (2003). Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Mol. Microbiol.* **49**, 977-989.

Naude, B., Brzostowski, J. A., Kimmel, A. R. and Wellems, T. E. (2005). *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. *J. Biol. Chem.* **280**, 25596-25603.

Orjih, A. U., Ryerse, J. S. and Fitch, C. D. (1994). Hemoglobin catabolism and the killing of intraerythrocytic *Plasmodium falciparum* by chloroquine. *Experientia* **50**, 34-39.

Paul, F., Roath, S., Melville, D., Warhurst, D. C. and Osisanya, J. O. (1981). Separation of malaria-infected erythrocytes from whole blood: use of a selective high-gradient magnetic separation technique. *Lancet* **2**, 70-71.

Reeves, D. C., Liebelt, D. A., Lakshmanan, V., Roepe, P. D., Fidock, D. A. and Akabas, M. H. (2006). Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms. *Mol. Biochem. Parasitol.* **150**, 288-299.

Saliba, K. J. and Kirk, K. (1999). pH regulation in the intracellular malaria parasite, *Plasmodium falciparum*. H^+ extrusion via a V-type H^+-ATPase. *J. Biol. Chem.* **274**, 33213-33219.

Saliba, K. J., Folb, P. I. and Smith, P. J. (1998a). Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance. *Biochem. Pharmacol.* **56**, 313-320.

Saliba, K. J., Horner, H. A. and Kirk, K. (1998b). Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* **273**, 10190-10195.

Saliba, K. J., Allen, R. J. W., Zissis, S., Bray, P. G., Ward, S. A. and Kirk, K. (2003). Acidification of the malaria parasite's digestive vacuole by a H^+-ATPase and a H^+-pyrophosphatase. *J. Biol. Chem.* **278**, 5605-5612.

Sanchez, C. P., Stein, W. and Lanzer, M. (2003). Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. *Biochemistry* **42**, 9383-9394.

Sanchez, C. P., McLean, J. E., Stein, W. and Lanzer, M. (2004). Evidence for a substrate specific and inhibitable drug efflux system in chloroquine resistant *Plasmodium falciparum* strains. *Biochemistry* **43**, 16365-16373.

Sanchez, C. P., McLean, J. E., Rohrbach, P., Fidock, D. A., Stein, W. D. and Lanzer, M. (2005). Evidence for a *pfcrt*-associated chloroquine efflux system in the human malarial parasite *Plasmodium falciparum*. *Biochemistry* **44**, 9862-9870.

Sanchez, C. P., Stein, W. D. and Lanzer, M. (2007a). Is PfCRT a channel or a carrier? Two competing models explaining chloroquine resistance in *Plasmodium falciparum*. *Trends Parasitol.* **23**, 332-339.

Sanchez, C. P., Rohrbach, P., McLean, J. E., Fidock, D. A., Stein, W. D. and Lanzer, M. (2007b). Differences in *trans*-stimulated chloroquine efflux kinetics are linked to PfCRT in *Plasmodium falciparum*. *Mol. Microbiol.* **64**, 407-420.

Schapiro, F. B. and Grinstein, S. (2000). Determinants of the pH of the Golgi complex. *J. Biol. Chem.* **275**, 21025-21032.

Schuldiner, S., Shirvan, A. and Linial, M. (1995). Vesicular neurotransmitter transporters: from bacteria to humans. *Physiol. Rev.* **75**, 369-392.

Sidhu, A. B., Verdier-Pinard, D. and Fidock, D. A. (2002). Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. *Science* **298**, 210-213.

Staalsoe, T., Giha, H. A., Dodoo, D., Theander, T. G. and Hviid, L. (1999). Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. *Cytometry* **35**, 329-336.

Tan, W., Gou, D. M., Tai, E., Zhao, Y. Z. and Chow, L. M. (2006). Functional reconstitution of purified chloroquine resistance membrane transporter expressed in yeast. *Arch. Biochem. Biophys.* **452**, 119-128.

Tran, C. V. and Saier, M. H., Jr (2004). The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. *Microbiology* **150**, 1-3.

Trape, J. F. (2001). The public health impact of chloroquine resistance in Africa. *Am. J. Trop. Med. Hyg.* **64**, 12-17.

Ursos, L. M. B., Dzekunov, S. M. and Roepe, P. D. (2000). The effects of chloroquine and verapamil on digestive vacuolar pH of *P. falciparum* either sensitive or resistant to chloroquine. *Mol. Biochem. Parasitol.* **110**, 125-134.

van Schalkwyk, D. A. and Egan, T. J. (2006). Quinoline-resistance reversing agents for the malaria parasite *Plasmodium falciparum*. *Drug Resist. Updat.* **9**, 211-226.

Warhurst, D. C., Craig, J. C. and Adagu, I. S. (2002). Lysosomes and drug resistance in malaria. *Lancet* **360**, 1527-1529.

Wu, M. M., Grabe, M., Adams, S., Tsien, R. Y., Moore, H. P. and Machen, T. E. (2001). Mechanisms of pH regulation in the regulated secretory pathway. *J. Biol. Chem.* **276**, 33027-33035.

Yayon, A., Cabantchik, Z. I. and Ginsburg, H. (1984). Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine. *EMBO J.* **3**, 2695-2700.

Yayon, A., Cabantchik, Z. I. and Ginsburg, H. (1985). Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc. Natl. Acad. Sci. USA* **82**, 2784-2788.