The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance

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Summary

Chloroquine resistance in the human malaria parasite, *Plasmodium falciparum*, arises from decreased accumulation of the drug in the 'digestive vacuole' of the parasite, an acidic compartment in which chloroquine exerts its primary toxic effect. It has been proposed that changes in the pH of the digestive vacuole might underlie the decreased accumulation of chloroquine by chloroquine-resistant parasites. In this study we have investigated the digestive vacuole pH of a chloroquine-sensitive and a chloroquine-resistant strain of *P. falciparum*, using a range of dextran-linked pH-sensitive fluorescent dyes. The estimated digestive vacuole pH varied with the concentration and pK_a of the dye, ranging from ~3.7-6.5. However, at low dye concentrations the estimated digestive vacuole pH of both the chloroquine-resistant and chloroquine-sensitive strains converged in the range 4.5-4.9. The results suggest that there is no significant difference in digestive vacuole pH of chloroquine-sensitive and chloroquine-resistant parasites, and that digestive vacuole pH does not play a primary role in chloroquine resistance.

Key words: Chloroquine, Chloroquine resistance, Lysosome, Haem

Introduction

Control of the human malaria parasite *Plasmodium falciparum* is becoming progressively more difficult as resistance to a range of antimalarial drugs spreads throughout parasite populations worldwide. The mechanism of resistance of the intraerythrocytic stage of the parasite to chloroquine (CQ), the most widely used and studied antimalarial, is still not fully understood. During development within human erythrocytes, the malaria parasite endocytoses large quantities of host cell cytosol (Krugliak et al., 2002), breaking down haemoglobin in an acidic compartment known as the digestive vacuole. CQ, a diprotic weak base, accumulates in the digestive vacuole of the parasite (Aikawa, 1972; Saliba et al., 1998; Sullivan et al., 1996; Yayon et al., 1984), where it is thought to exert its antiplasmodial effect by binding to a toxic byproduct of haemoglobin proteolysis, haematin (Bray et al., 1999; Bray et al., 1998). This prevents the incorporation of haematin into a biologically inert crystalline polymer, haemozoin (Pagola et al., 2000; Slater, 1993; Slater et al., 1991; Sullivan et al., 1998; Sullivan et al., 1996), thereby allowing haematin to interfere with enzymatic processes (Surolia, 2000; Surolia and Padmanaban, 1991) and damage parasite membranes (Sugioka et al., 1987).

CQ-resistant (CQR) parasites accumulate much less CQ than CQ-sensitive (CQS) parasites (Fitch, 1970; Yayon et al., 1984), and the 'chemosensitiser' verapamil increases the accumulation of CQ in CQR parasites, attenuating the level of resistance (Martin et al., 1987). Early studies of CQ uptake by parasitised erythrocytes demonstrated the existence of both a saturable and a nonsaturable component (Fitch, 1970). It was demonstrated subsequently that the sensitivity of the parasite to CQ is directly proportional to the saturable component of CQ uptake, and that the apparent affinity of CQ-haematin binding is reduced in CQR parasites (Bray et al., 1999; Bray et al., 1998). However, the mechanism by which differences in apparent binding affinity are mediated is yet to be elucidated, despite recent allelic exchange experiments which link mutations in P-glycoprotein homologue 1 (Pgh1) (Reed et al., 2000) and the *P. falciparum* chloroquine resistance transporter (PfCRT) (Fidock et al., 2000; Sidhu et al., 2002), both digestive vacuole membrane proteins (Cooper et al., 2002; Cowman et al., 1991; Fidock et al., 2000), to the verapamil-reversible CQR phenotype.

It has long been debated whether (and to what extent) the pH of the digestive vacuole (pH_{DV}) might play a role in determining levels of CQ accumulation, and/or affinity of haematin binding, and hence the CQ-sensitivity of the parasite. It is generally assumed that the erythrocyte and parasite membranes are freely permeable to the uncharged species of the weakly basic CQ, that the drug becomes trapped in the acidic digestive vacuole environment upon protonation (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1984), and that CQ uptake into the digestive vacuole is therefore influenced by the pH gradient across the digestive vacuole membrane. It has been postulated that an increased pH_{DV} in CQR parasites will result in a reduction in the trapping of CQ in the vacuole (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1985), and noted that this hypothesis is

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compatible with the reduction in the apparent haematin binding affinity observed in CQR parasites (Bray et al., 1998). Several studies have demonstrated that increasing extracellular pH results in an increase in CQ uptake (Bray et al., 1994; Geary et al., 1990; Hawley et al., 1996; Yayon et al., 1985), an increase in CQ sensitivity of the parasites (Geary et al., 1990; Martiney et al., 1995; Yayon et al., 1985) and an increase in the apparent affinity of CQ-haematin binding (Bray et al., 1998). These observations are consistent with the hypothesis that CQ accumulation is a function of transmembrane pH gradients, although the variation in CQ uptake with extracellular pH could also be a result of the pH dependence of the concentration of the membrane-permeant neutral form of the molecule in the external medium, and consequent changes in the concentration and hence extent of binding of the drug in subcellular compartments.

Using the weakly basic CQ or methylamine as probes, it has been estimated that pH_{DV} in CQS parasites is 4.3-4.7 (Geary et al., 1990; Geary et al., 1986), whereas pH_{DV} in CQR parasites is slightly higher, at around pH 4.7-5.0 (Geary et al., 1990; Geary et al., 1986; Yayon et al., 1984). Experiments using a membrane-impermeant dextran-linked derivative of the pH-sensitive fluorophore Fluorescein suggest that the pH_{DV} of the malaria parasite is between 5.2 and 5.4, but, to our knowledge, no comparisons between CQS and CQR parasites have been made using this dye (Geary et al., 1990; Geary et al., 1986; Krogstad et al., 1985; Yayon et al., 1984).

Ginsburg et al. made use of the membrane-permeant fluorescent weak base Acridine Orange to investigate pH_{DV} in a CQR strain, noting that fluorescence from Acridine Orange in the digestive vacuole was quenched but that uptake could be measured by monitoring extracellular fluorescence (Ginsburg et al., 1989). The pH_{DV} was estimated as ~4.2, although as recognised by the authors, the estimate was confounded by uncertainty as to the extent of Acridine Orange binding within the parasite. More recently Roepe and colleagues have described an alternative use of Acridine Orange as a (membrane permeant) probe of pH_{DV}, involving single-cell fluorescence measurements. On the basis of these they proposed that CQR parasites actually have a decreased pH_{DV} relative to CQS parasites, resulting in a reduction in the amount of soluble haematin available for CQ binding (Bennett et al., 2004; Dzekunov et al., 2000; Ursos et al., 2000). Furthermore, they reported that verapamil increased the pH_{DV} of a CQR strain to a value akin to that of a CQS strain (Ursos et al., 2000), and that the verapamil-reversible increase in digestive vacuole acidification in CQR strains was associated with mutations in PfCRT (Bennett et al., 2004). However, the validity of the pH_{DV} estimates made using Acridine Orange in the manner used in these studies is contentious (Bray et al., 2002a; Bray et al., 2002b; Dzekunov et al., 2002; Spiller et al., 2002; Wissing et al., 2002).

In this study we have investigated the pH_{DV} of a CQS and a CQR strain of *P. falciparum*, using several dextran-linked pH-sensitive dyes in an extension of a technique described previously (Krogstad et al., 1985; Saliba et al., 2003; Spiller et al., 2002). Our results suggest that differences in CQ accumulation which distinguish sensitive and resistant strains are not a consequence of differences in pH_{DV} and, furthermore, raise significant questions about the importance of pH_{DV} to the parasite, and the role of pH_{DV} in antimalarial drug accumulation.

Results

Initial measurements of pH_{DV}

Initial attempts to measure and compare the pH_{DV} of a CQS strain (D10′) and a CQR strain (7G8′) using dextran-linked fluorescent dyes at concentrations similar to those described previously (Krogstad et al., 1985; Saliba et al., 2003; Spiller et al., 2002) indicated that the pH_{DV} of the CQR strain was significantly higher than that of the CQS strain. As shown in Fig. 1A, when measured with Fluorescein ($pK_a \approx 6.4$; ~40 μM), the estimated pH_{DV} of CQR 7G8′ parasites was 5.72±0.10, some 0.2 pH units higher than the value of 5.50±0.14 estimated for CQS D10′ parasites ($P<0.001$). When using Oregon Green 514 ($pK_a \approx 4.7$; ~70 μM) a pH difference of similar magnitude between the two strains (i.e. ~0.2 pH units) was obtained ($P<0.01$; Fig. 1B); however, the pH_{DV} estimated for each strain using Oregon Green 514 (4.66±0.13 for 7G8′, 4.40±0.10 for D10′) was ~1 pH unit lower than that estimated using Fluorescein.

Fig. 2 shows the pH_{DV} of D10′ parasites estimated using a range of different dyes, plotted as a function of the pK_a of the dye. With ~28 μM dextran in the loading solution, the apparent pH_{DV} ranged from ~3.7 to ~6.5, with a positive linear correlation ($R^2>0.99$, $P<0.05$ in all cases) between the estimated pH_{DV} and the pK_a. Despite the large range in the estimated pH_{DV} values, there were no perceptible differences

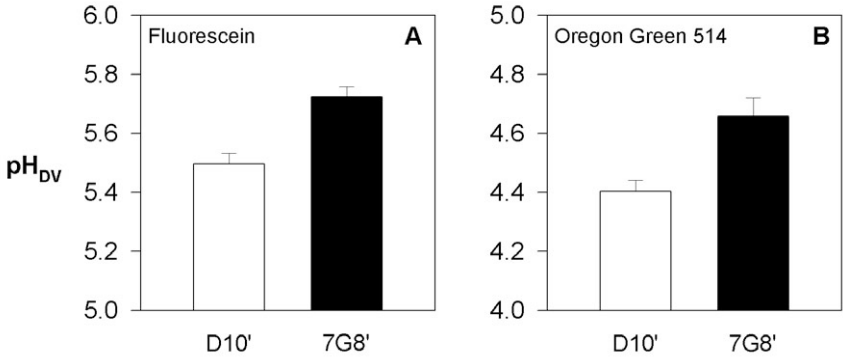

Fig. 1. Estimated digestive vacuole pH (pH_{DV}) of mature trophozoite-stage D10′ (CQS, open bars) and 7G8′ (CQR, filled bars) parasites as determined using either (A) Fluorescein or (B) Oregon Green 514 calibrated with the nigericin/high-K^+ method. The pH_{DV} of 7G8′ was significantly higher than the pH_{DV} of D10′ when measured with either Fluorescein ($P<0.001$) or Oregon Green 514 ($P<0.01$). However, for both strains, the pH_{DV} estimated using Fluorescein was significantly higher than that estimated using Oregon Green 514 ($P<0.001$). The concentration of dextran in each treatment was ~28 μM, resulting in a Fluorescein concentration of ~40 μM and an Oregon Green 514 concentration of ~70 μM as a consequence of the different number of fluorophore molecules per dextran molecule (Haughland, 2002) (see Materials and Methods). The data for each dye represent the means of at least nine (Fluorescein) or at least four (Oregon Green 514) independent experiments, with at least three replicate measurements per experiment. The error bars show s.e.m.

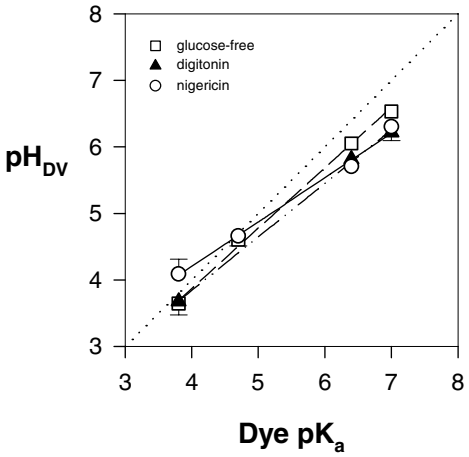

Fig. 2. pH_{DV} of mature trophozoite-stage D10′ parasites as reported by BCECF (pK_a ~7.0), Fluorescein (pK_a ~6.4), Oregon Green 514 (pK_a ~4.7) and CL-NERF (pK_a ~3.8), all at a loading concentration of ~28 μM dextran. The three different pH calibration methods described in Materials and Methods each report a consistent linear relationship between dye pK_a and pH_{DV} (R^2>0.99, P<0.05 in all cases), with the reported pH_{DV} ranging from ~3.7 to ~6.5. The data represent the means (error bars show s.e.m.; where not shown, error bars fall within the symbol) of at least three independent experiments. The dotted line indicates data that would be obtained if pH_{DV} were equal to dye pK_a.

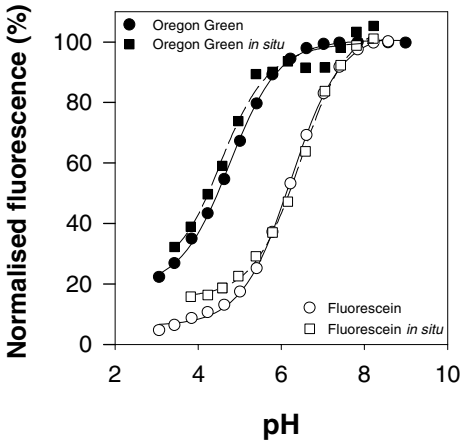

Fig. 3. The normalised ratiometric pH-dependent change in fluorescence of either Fluorescein (open symbols) or Oregon Green 514 (closed symbols). In situ measurements (squares) were made using isolated mature trophozoite-stage D10′ parasites suspended in high-K^+ saline and exposed to 50 μM nigericin. The concentration of dextran in the loading solution was ~28 μM. Cell-free measurements (circles) were made as described in Materials and Methods. The pH range over which the Fluorescein response is linear was ~5.0-7.5, whereas the linear response of Oregon Green 514 was in the pH range ~3.5-5.5.

in the rates of growth of D10′ parasites during the two generations of development in erythrocytes loaded with the different dyes.

To establish that these observations were not the result of an anomalous fluorescence response by the dyes in the vacuole, we compared the fluorescence profile of the dyes either in situ or in cell-free conditions over a range of extracellular pH values (Fig. 3). Both dyes displayed a characteristic sigmoidal response to changes in pH, regardless of whether fluorescence was measured in situ (in isolated dye-loaded parasites suspended in high-K^+ saline containing 50 μM nigericin) or under cell-free conditions, with the points of inflexion corresponding well with the given pK_a for each dye. For parasites grown in normal, unloaded erythrocytes autofluorescence was negligible and showed no systematic variation with pH.

Effect of parasite age on pH_{DV}

Fig. 4 shows the effect of parasite age on the estimated pH_{DV} values obtained for the CQS strain, as determined using either Fluorescein (~40 μM) or Oregon Green 514 (~70 μM). The estimated pH_{DV} of early trophozoite-stage parasites (16-22 hours post invasion) was 4.89±0.17 when measured with Fluorescein, and 4.70±0.10 when measured with Oregon Green 514. These values were not significantly different from one another (P>0.3) but were, for each dye, significantly different (P<0.05) from those obtained for mature trophozoites (32-38 hours post invasion).

Effect of dye concentration on pH_{DV}

A range of concentrations of both Fluorescein and Oregon Green 514 was tested in mature trophozoite-stage parasites in

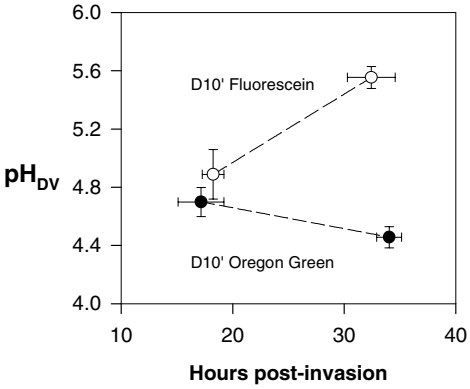

Fig. 4. Comparison of pH_{DV} in D10′ parasites at the early trophozoite stage (16-22 hours post-invasion) and at the mature trophozoite stage (32-38 hours post-invasion) as determined using either ~40 μM Fluorescein (open symbols) or ~70 μM Oregon Green 514 (closed symbols) (i.e. ~28 μM dextran in the loading solution in each case), calibrated using the nigericin/high-K^+ method. For both dyes, the reported pH_{DV} changed significantly as the intraerythrocytic parasites developed from the early to the mature stage (P<0.05). The data represent the means (± s.e.m.) of at least three independent experiments, each derived from at least three replicate measurements per experiment.

order to determine the lowest concentrations which could provide reproducible estimates of pH_{DV}. Reproducible baseline pH_{DV} and calibration traces were obtained using Fluorescein concentrations as low as ~10 μM (Fig. 5B) and Oregon Green 514 concentrations as low as ~4 μM (Fig. 5D). For both dyes at these low concentrations, the addition of concanamycin A, a potent inhibitor of the digestive vacuole V-type H^+-ATPase, resulted in a pronounced increase in the fluorescence ratio,

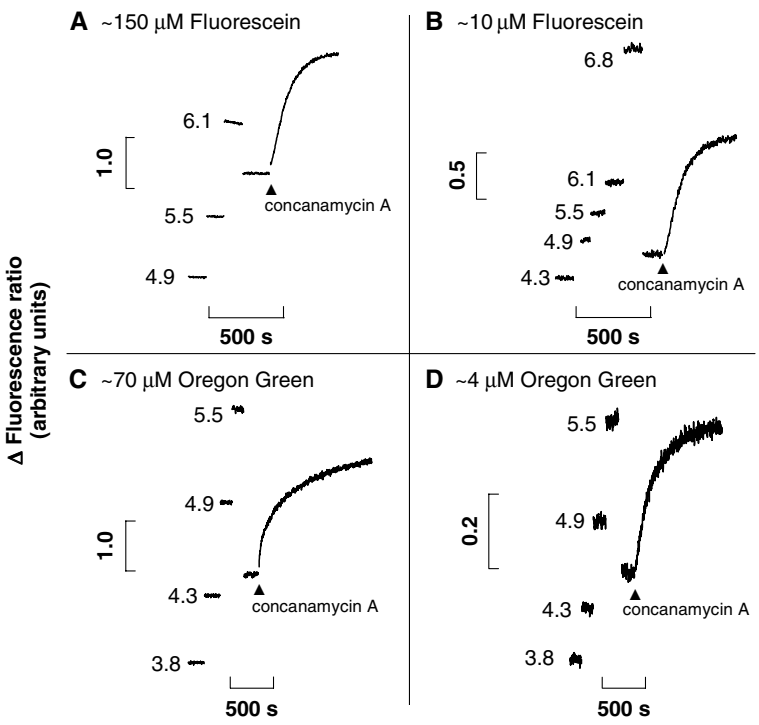

Fig. 5. Fluorometer traces of mature trophozoite-stage D10′ parasites isolated from erythrocytes loaded with (A) ~150 μM Fluorescein, (B) ~10 μM Fluorescein, (C) ~70 μM Oregon Green 514, or (D) ~4 μM Oregon Green 514. Cells were suspended in minimal saline solution at pH 7.1. Black arrowheads indicate the point of addition of concanamycin A (75 nM), a potent and specific inhibitor of the digestive vacuole V-type H^+ pump (Saliba et al., 2003). Numbered traces indicate the pH of calibration samples (nigericin/high-K^+ method). The traces are from single experiments, and are representative of those obtained in at least three separate experiments for each dye concentration. Note that the dynamic range of Oregon Green 514 (see Fig. 3) precluded accurate measurement of the endpoint of digestive vacuole alkalinisation by concanamycin A.

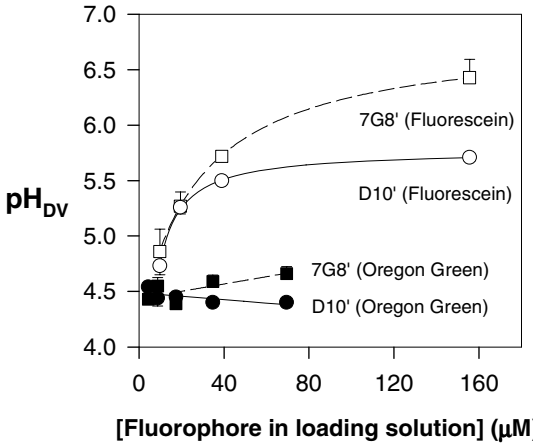

Fig. 6. pH_{DV} of mature trophozoite-stage D10′ (circles) and 7G8′ (squares) parasites as determined using either Fluorescein (open symbols) or Oregon Green 514 (closed symbols) over a range of concentrations and calibrated with the nigericin/high-K^+ method. For both D10′ and 7G8′ parasites, the pH_{DV} reported by Fluorescein was significantly lower (P<0.01) at the lowest concentration tested than at the highest (~10 μM and ~156 μM, respectively). However, there was no significant change in reported pH_{DV} for either strain over the range of concentrations of Oregon Green 514 tested (~4-70 μM). At Fluorescein loading concentrations of ~40 μM and above, the reported pH_{DV} of 7G8′ parasites was significantly higher than that of D10′ parasites (P<0.01). Similarly, at Oregon Green 514 loading concentrations of ~35 μM and above, the reported pH_{DV} of 7G8′ parasites was significantly higher than that of D10′ parasites (P<0.05). The data represent the means (± s.e.m.; where not shown, error bars fall within the symbol) of at least three independent experiments, each derived from at least three replicate measurements per experiment. Curves (rectangular hyperbolic and simple linear) were fitted by least-squares regression with SigmaPlot 2001 (SPSS).

consistent with the expected alkalinisation (Saliba et al., 2003). However, for Fluorescein (but not for Oregon Green 514) the resting pH_{DV} estimated using the lowest dye concentration (Fig. 5B) was significantly lower than that estimated using the highest concentration (Fig. 5A).

Estimated pH_{DV} values were obtained for the CQS and CQR strains over a range of concentrations of Fluorescein and Oregon Green 514 (Fig. 6). Decreasing the concentration of Fluorescein-dextran in the loading solution resulted in an apparent 1 pH unit decrease in the pH_{DV} of D10′ parasites, from 5.72±0.07 at ~150 μM fluorophore to 4.73±0.18 at ~10 μM fluorophore. Over the same range of Fluorescein concentrations, the pH_{DV} of 7G8′ parasites decreased more than 1.5 pH units, from 6.43±0.33 to 4.86±0.35. However, changing the concentration of Oregon Green 514 in the loading solution did not produce the same effect. For both strains, the difference in pH_{DV} reported by the highest and lowest concentration of Oregon Green 514 fluorophore was not significant (P>0.3).

The observation that as the dye concentration decreased, the reported pH_{DV} of both strains, using both dyes, converged at a point in the range 4.5-4.9 (with no significant difference in the apparent pH_{DV} of the strains at the lowest fluorophore concentration tested; P>0.2) is consistent with this being the 'true' physiological resting pH_{DV} of both strains.

CQ accumulation in CQR 7G8′ and CQS D10′ parasites

As has been shown previously (Reed et al., 2000), and as is shown in Fig. 7 (filled bars) CQR 7G8′ parasites accumulate CQ to a much lesser extent than CQS D10′ parasites. A similar difference was observed for the accumulation of CQ by parasitised erythrocytes that had been subjected to the lysing-and-resealing protocol used to preload the fluorescent dyes (Fig. 7, open bars); for both the 'normal' erythrocytes and the lysed-and-resealed erythrocytes, those infected with CQS D10′ parasites accumulated eight to nine times more [^{3}H]CQ than those infected with 7G8′ parasites.

Apparent changes in pH_{DV} do not affect drug sensitivity or CQ uptake

It has been proposed that CQ accumulates in the acidic digestive vacuole as a result of its protonation and consequent trapping (Ferrari and Cutler, 1991; Homewood et al., 1972; Yayon et al., 1984). We therefore investigated whether alterations in the

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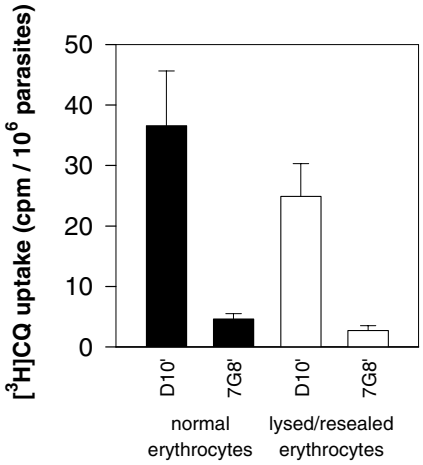

Fig. 7. Total uptake of [^{3}H]CQ into parasites in intact normal erythrocytes (i.e. not subjected to the lysing/resealing process; filled bars) and in intact lysed/resealed erythrocytes (open bars). For both types of erythrocytes [^{3}H]CQ uptake by cells infected with D10′ (CQS) parasites was significantly (>eightfold) higher than [^{3}H]CQ uptake by cells infected with and 7G8′ (CQR) parasites (P<0.05). The data represent the means (+ s.e.m.) of four independent experiments, each carried out in triplicate.

apparent pH_{DV}, arising from pre-loading the digestive vacuole with either Fluorescein or Oregon Green 514 affected: (1) the uptake of CQ into the parasite, and (2) the sensitivity of the parasites to CQ and to the monoprotic weak base mefloquine.

Erythrocytes were loaded with ~40 μM Fluorescein-dextran, ~70 μM Oregon Green 514-dextran, or dextran alone (the dextran concentration was, in each case, ~28 μM), then invaded by D10′ parasites. Despite there being a difference of ~1 pH unit in the estimated pH_{DV} for the parasites loaded with the two different dyes (Fig. 1) there was, as shown in Table 1, no significant difference between any of the three differentially-loaded parasites in the IC_{50} for either CQ or mefloquine, or in the level of CQ accumulation.

Discussion

For both the CQS *P. falciparum* strain D10′ and the CQR strain 7G8′, the estimated pH_{DV} varied systematically with both the concentration and the pK_a of the fluorescent indicator used (Figs 1-2, 5-6). There are two alternative interpretations of these data. The first is that the apparent variation of pH_{DV} with the concentration and pK_a of the fluorescent indicator used is due not to a genuine variation in pH_{DV}, but to an artefact of the technique; i.e. the pH_{DV} values obtained here are inaccurate. The second is that the pH_{DV} values reported by the dyes under the different conditions tested are accurate, that the pH_{DV} did vary in the manner indicated, and that the parasite is therefore able to grow under conditions in which pH_{DV} varies over a wide range.

The accuracy of the pH_{DV} estimates

The difficulties associated with alternate methods of measuring the pH_{DV}, which might provide independent means of verification, make it difficult to exclude completely the possibility that the pH estimates obtained in this study are inaccurate. Previous attempts to measure the pH of the parasite digestive vacuole using the diprotic weak base CQ (Bray et al., 1992; Geary et al., 1990; Geary et al., 1986; Yayon et al., 1984; Yayon et al., 1985) as a probe have proved problematic. Some studies suggested that CQ accumulation by the parasite could be fully accounted for by the diffusion of the uncharged form of the molecule across the various membranes separating the interior of the digestive vacuole from the extracellular medium, with the protonated form accumulating in each compartment in accordance with the Henderson-Hasselbach equation and pH_{DV} ~4.2-4.5 (Geary et al., 1990; Geary et al., 1986; Ginsburg et al., 1989; Hawley et al., 1996; Yayon et al., 1985). However, other studies suggested that some parasites accumulated much more CQ than could be predicted by this model (Krogstad et al., 1992; Krogstad and Schlesinger, 1986; Krogstad and Schlesinger, 1987; Krogstad et al., 1985). Furthermore, one of the key assumptions underlying the simple passive diffusion/'proton trapping' model of CQ accumulation is that there is no intracellular binding of the drug, an assumption falsified by the finding that the saturable component of CQ uptake, the component thought to underlie the antimalarial activity of the drug, is due to binding to haematin (Bray et al., 1999; Bray et al., 1998; Fitch, 1970). This finding, subsequently extended to a range of weakly basic antimalarial drugs (Bray et al., 1999; Mungthin et al., 1998), suggests that changes in accumulation due to binding to haematin (and possibly other cellular components) may potentially mask any changes in accumulation mediated by alterations in pH_{DV}, rendering such drugs ineffective as tools for measuring pH_{DV}. These findings argue against CQ accumulation being determined simply by its properties as a weak base.

Using Acridine Orange, another weak base, Ginsburg et al. estimated the pH_{DV} in a CQR strain to be ~4.2, but recognised that this estimate was confounded by the difficulties in quantifying the extent of Acridine Orange binding within the parasitised erythrocyte (Ginsburg et al., 1989). More recent measurements, using Acridine Orange in conjunction with single cell fluorescence imaging (Bennett et al., 2004; Cooper et al., 2002; Dzekunov et al., 2000; Mehlotra et al., 2001; Ursos

Table 1. Drug sensitivity of, and CQ uptake by, D10′ parasites

Erythrocyte loading treatment	Estimated pH_{DV}	CQ IC_{50} (nM)*	CQ uptake (cpm/10^6 parasites)*	Mefloquine IC_{50} (nM)*
Dextran	~4.5†	28±3	94±17	34±7
Oregon Green 514 (pK_a ~4.7)	4.40±0.10	31±3	104±11	35±8
Fluorescein (pK_a ~6.4)	5.50±0.14	29±5	98±22	34±6

*Values are the means (± s.e.m.) of four independent experiments, each carried out in triplicate. The concentration of dextran in each case was ~28 μM. There were no significant differences in IC_{50} for either CQ or mefloquine, or in CQ uptake (P>0.4). Nor was there any significant difference in [^{3}H]hypoxanthine incorporation between parasites growing in erythrocytes loaded with the different dyes (P>0.9). CQ uptake was measured at a CQ concentration of 1 nM.

†Approximation derived from the converging D10′ pH_{DV} data presented in Fig. 6 and extrapolated to a dye concentration of zero.

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et al., 2000; Waller et al., 2003) have been interpreted as inferring that CQR parasites have a lower pH_{DV} than CQS parasites, and that verapamil restores the pH_{DV} of CQR parasites to levels similar to those observed in CQS parasites (Dzekunov et al., 2000; Ursos et al., 2000). In attempting to reconcile these results with the CQ accumulation data it was proposed that because CQ does not bind as efficiently to insoluble haematin (Dzekunov et al., 2000), and because haematin becomes less soluble as pH_{DV} is reduced (Dzekunov et al., 2000), a reduction in pH_{DV} in CQR parasites produces a concomitant reduction in the availability of the drug target. Further studies using Acridine Orange (Bennett et al., 2004) showed that the verapamil-reversible decrease in the estimated pH_{DV} in CQR strains was associated with CQ resistance-associated mutations in the digestive vacuole membrane protein PfCRT (Cooper et al., 2002; Fidock et al., 2000; Sidhu et al., 2002). However, the appropriate interpretation of the Acridine Orange fluorescence data is contentious (Bray et al., 2002a; Bray et al., 2002b; Dzekunov et al., 2002; Spiller et al., 2002; Wissing et al., 2002). A more likely explanation of the data is, perhaps, that the alterations in Acridine Orange distribution associated with mutations in PfCRT may be a result of Acridine Orange being a substrate for PfCRT, as Acridine Orange bears close structural similarity to the antimalarial quinacrine, which shows cross-resistance with CQ (Spiller et al., 2002; Warhurst et al., 2002; Zhang et al., 2002).

Complications such as these make it difficult to gain independent verification of the pH_{DV} estimates made in the present study using ratiometric fluorescent indicators. However, the data presented in Fig. 3 do show that the characteristics of the fluorescence response of Fluorescein and Oregon Green 514 in situ are very similar to those obtained under cell-free conditions, with the points of inflexion corresponding well with the given pK_a for each dye. This indicates that these two dyes at least are behaving according to specifications (Haughland, 2002) when loaded into the digestive vacuole. Additionally, data presented in Fig. 5 confirm that the vacuoles alkalinise in response to the presence of concanamycin A, a potent inhibitor of V-type H^+-ATPases, in a predictable manner, as described previously in a range of cell types (Nishi and Forgac, 2002; Saliba et al., 2003). These observations provide at least some support for the view that the dyes used in this study provide an accurate measure of pH_{DV} under the different conditions tested.

It should be noted that these experiments were conducted in the nominal absence of bicarbonate and/or CO_2 and that the conditions therefore do not accurately reflect those found in vivo. The presence of bicarbonate may well influence the pH in the subcellular compartments of the parasitised erythrocyte. However, this limitation does not affect the conclusions of this in vitro study.

The flexibility of pH_{DV}

Assuming that the dyes used here do provide an accurate measure of pH_{DV}, our results indicate that, in dye-loaded parasites, pH_{DV} varied systematically with the concentration and pK_a of the fluorescent indicator used (Figs 1-2, 5-6) and that the indicators altered at least some of the processes that determine pH_{DV}. Even more strikingly, these results indicate that variations in pH_{DV} over a range of two pH units had no discernible effect on parasite growth.

The digestive vacuole is thought to be the site of breakdown of host cell haemoglobin, and the crystallisation and storage of the haem byproduct. There is abundant evidence that the proteases implicated in haemoglobin catabolism have a broad pH activity profile in vitro (Banerjee et al., 2002; Dorn et al., 1998; Francis et al., 1994; Gluzman et al., 1994; Goldberg et al., 1991; Goldberg et al., 1990; Moon et al., 1997; Murata and Goldberg, 2003; Shenai et al., 2000; Sijwali et al., 2001; Tyas et al., 1999; Westling et al., 1999) and there is also evidence for there being considerable redundancy among the various proteases (Malhotra et al., 2002; Sijwali et al., 2004; Liu et al., 2005; Omara-Opyene et al., 2004). Thus, our observation that the pH_{DV} can apparently vary by at least 2 pH units without significant detriment to the parasite might be accounted for by the considerable functional overlap amongst the proteases present in this organelle permitting haemoglobin degradation to occur even when the pH_{DV} was outside the optimum range for one or more of the vacuolar enzymes.

An alternative, or perhaps additional, explanation for the apparent lack of effect of marked variations in pH_{DV} on parasite growth might lie in a proposal made previously (Hempelmann et al., 2003; Spiller et al., 2002), that much of the haemoglobin degradation actually occurs in the vesicles in which the haemoglobin is trafficked from the parasite surface to the digestive vacuole (i.e. before they fuse with the digestive vacuole). With a much greater surface-area-to-volume ratio than the digestive vacuole, these vesicles might exert a much tighter control over their internal pH than the digestive vacuole, allowing the proteolytic enzymes involved to function within their optimal pH range. Measuring the pH within these vesicles is beyond the capability of technology presently available to us. However, our observations in Fig. 4 that the early trophozoite (16-22 hours post-invasion) has an estimated pH_{DV} similar to that deduced to be the 'true' physiological resting pH_{DV} in the mature trophozoite-stage parasite (i.e. in the range 4.5-4.9; Fig. 6) invite speculation that the pH of the trafficking vesicles may also be in this range. At the very least, our results suggest that the digestive vacuole of the young trophozoite is less susceptible to the influence of the dyes than the digestive vacuole of the mature trophozoite, perhaps as a consequence of the digestive vacuole of the young trophozoite having a greater surface-area-to-volume ratio, and/or a lower concentration of endocytosed dye, than the digestive vacuole of the mature trophozoite.

The basis of the variation of the estimated pH_{DV} under the different conditions tested

The mechanism by which the dyes used here might influence pH_{DV} is not clear. One possibility is that the dyes (at the higher concentrations used here) are simply buffering pH_{DV} to a value close to their pK_a values. According to the generally accepted 'pump-and-leak' model of pH regulation in subcellular compartments, however, the steady-state pH in an acidic organelle is determined primarily by a balance between the rate of protons being pumped into the organelle, and the rate of protons leaving (i.e. leaking from) the organelle via the various endogenous H^+ permeability pathways (Demaurex, 2002; Grabe and Oster, 2001; Poole and Ohkuma, 1981). In this model, alterations in the steady-state pH of an organelle should be attributable to a perturbation of this balance either by influencing the rate of H^+ influx (via the H^+ pumps) or the rate

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of H^+ efflux (H^+ leak). The internal buffering capacity is believed to be a much less important determinant of the steady-state pH of an organelle (Demaurex, 2002; Grabe and Oster, 2001).

The digestive vacuole of the malaria parasite is known to have two types of H^+ pump: a V-type H^+-ATPase and a H^+-pyrophosphatase (Saliba et al., 2003). Fluorescein and several of its derivatives, including BCECF, have been shown to inhibit both V-type H^+-ATPase and H^+-pyrophosphatase activity in cultured plant cells (Pfeiffer and Hoftberger, 2000). One possibility therefore is that Fluorescein, BCECF, Oregon Green 514 (a fluorinated analogue of Fluorescein) (Haughland, 2002) and CL-NERF (a chlorinated derivative of Rhodol Green, considered to be a hybrid of Fluorescein and Rhodamine) (Haughland, 2002; Lin et al., 1999) likewise interfere with one of, or both, the V-type H^+-ATPase and pyrophosphatase on the malaria parasite digestive vacuole, doing so via a pK_a-dependent mechanism. We were unable to test this hypothesis directly, although preliminary investigations (data not shown) indicated that parasite plasma membrane potential (Allen and Kirk, 2004) did not change in the presence of either extracellular or cytosolic BCECF, suggesting that the V-type ATPase on the plasma membrane is not affected by this fluorescent indicator.

For both Fluorescein and Oregon Green 514, at fluorophore concentrations of ~35 μM and above, the estimated pH_{DV} of 7G8′ parasites was significantly higher than that of D10′ parasites, and was, in each case, closer to the pK_a of the dye (Figs 1 and 6, Fig. 2). Since there was no significant difference detected in the average fluorescence per parasite between these two strains, and therefore no evidence for the dye concentrations in the two strains being significantly different (data not shown), we propose that 7G8′ parasites are more susceptible than D10′ to the pH-altering effects of the fluorophores Fluorescein and Oregon Green 514. Whether or not this is a characteristic associated with CQ resistance requires further investigation. At concentrations of Fluorescein and Oregon Green 514 below ~35 μM, the differences in apparent pH_{DV} between D10′ and 7G8′ diminished, and at ~5-10 μM there was no significant difference in apparent pH_{DV} between either strain, regardless of fluorophore (Fig. 6). It should be stressed that at these low fluorophore concentrations the fluorometry traces were still distinct and straightforward to interpret (Fig. 5), and that calibration of pH_{DV} as reported by Fluorescein was reproducible, despite the fact the reported pH_{DV} was below the linear range of the pH-dependent response of this dye (Fig. 3). We also note that: (1) these fluorophore concentrations are substantially lower than the concentration of dextran-linked DM-NERF used to corroborate the pH_{DV} reported by Acridine Orange in a recent study by Bennett and colleagues (23 μM) (Bennett et al., 2004); and (2) in three of the four strains Bennett et al. investigated using dextran-linked DM-NERF, the reported pH_{DV} was close to the pK_a of DM-NERF, 5.4 (Bennett et al., 2004).

The effect of pH_{DV} on CQ uptake and sensitivity?

At low concentrations of both a high- and a low-pK_a dye, the estimated pH_{DV} of the CQ-resistant and CQ-sensitive strains converged at a value in the range 4.5-4.9, prompting the hypotheses that: (1) the normal resting pH_{DV} is within this range; and (2) there is no significant difference between the resting pH_{DV} of CQS D10′ parasites and CQR 7G8′ parasites. As shown in Fig. 7, erythrocytes lysed and resealed in the same manner as those loaded with fluorescent dyes, then infected with D10′ parasites, accumulated eight to nine times more [^{3}H]CQ than those infected with 7G8′ parasites. The available data are therefore consistent with the view that the marked difference in the CQ accumulation by the CQR and CQS strains studied here is due to something other than a difference in the resting pH_{DV}.

Furthermore, the results summarised in Table 1 call into question the extent to which variations in pH_{DV} do actually influence drug accumulation and sensitivity. Significant (dye-induced) changes in the apparent pH_{DV} had no effect on either CQ uptake by CQS D10′ parasites, or sensitivity of D10′ parasites to two weak-base antimalarials, CQ and mefloquine. These data are consistent with the view that accumulation of these weakly basic antimalarials is not a simple consequence of the trapping of the protonated forms of the drug in the digestive vacuole (Bray et al., 1999; Bray et al., 1998). If CQ were accumulated as a consequence of the charged forms of the molecule being totally membrane-impermeant, then the ~1 pH unit difference between the estimated pH_{DV} of cells loaded with Fluorescein and that of cells loaded with Oregon Green 514 (Fig. 1) should have resulted in a >150-fold difference in CQ accumulation. The data therefore argue against the simple weak base trapping model and raise the possibility that the DV membrane of the CQ-sensitive D10′ strain has a significant permeability to the charged forms of the drug.

Indirect support for this view comes from a recent study of *Dictyostelium discoidium*. *D. discoidium* has internal acidic vesicles that are estimated from published fluorescence micrographs (Naude et al., 2005) to have a similar total volume and also have a similar pH to that of the digestive vacuole of the mature intraerythrocytic *P. falciparum* parasite. However, the acidic vesicles of *D. discoidium* do not contain haematin, a major site of CQ binding in the intraerythrocytic parasite (Bray et al., 1999; Bray et al., 1998). The accumulation of CQ by *D. discoidium* (Naude et al., 2005) is several hundredfold less than would be predicted on the basis of the Henderson-Hasselbach model, assuming that protonated CQ is membrane impermeant. This is again consistent with the hypothesis that the *D. discoidium* vesicle membrane is permeant to one or both of the charged forms of CQ.

The permeation of charged CQ across the digestive vacuole membrane would provide a mechanism for the shuttling of H^+ from the digestive vacuole, down its electrochemical gradient. Ginsburg et al. (Ginsburg et al., 1989) have reported alkalinisation of the parasite digestive vacuole by a range of aminoquinoline drugs, including chloroquine. However this was only seen at drug concentrations one to two orders of magnitude higher than therapeutic levels, indicating that at lower (therapeutic) concentrations any drug-mediated H^+ shuttling out of the digestive vacuole is effectively counteracted by the action of the digestive vacuole H^+ pump(s).

The results of this study highlight the limitations of methodology on which the majority of previous estimates of pH_{DV} in the malaria parasite have been based. However by using multiple dyes over a range of concentrations, we have estimated the resting pH_{DV} of the malaria parasite to be in the range 4.5-4.9 and, furthermore, shown there to be no difference between the apparent resting pH_{DV} of a CQS and CQR parasite

strain. The data are inconsistent with the hypothesis that differences in the resting pH_{DV} of the parasite underlie the differences in CQ accumulation seen between the CQS and CQR parasites studied here. Our observations do not exclude the possibility that the CQ-resistance phenotype in other CQR strains could be caused by alterations in pH_{DV}. Genetic analysis indicates that there have probably been at least four independent CQ resistance 'founder events' worldwide (Wootton et al., 2002). However, given that these all entailed polymorphisms in *pfcrt*, a gene that has a near-ubiquitous association with in vitro CQ resistance, the possibility that there are multiple causes of the CQR phenotype seems perhaps unlikely. Furthermore, the observation that variations in the apparent resting pH_{DV} had no perceptible effect on either CQ accumulation or the CQ- or mefloquine-sensitivity of CQS D10′ parasites calls into question the extent to which variations in pH_{DV} can actually influence drug uptake and sensitivity.

The fact that there were no perceptible differences in rates of parasite growth (measured over two generations) under conditions in which the estimated pH_{DV} ranged from ~3.7 to ~6.5 raises significant questions about the extent to which the parasite needs to maintain pH_{DV} within a narrow range. The lack of effect on parasite growth of these marked variations in pH_{DV} might be accounted for by the considerable functional overlap amongst the proteases present in the digestive vacuole and/or the possibility that proteolysis actually occurs in the vesicles that carry haemoglobin from the parasite surface to the digestive vacuole (and for which the internal pH is unknown). In either case, the implication is that maintenance of pH_{DV} within a narrow range is not a high priority for the parasite.

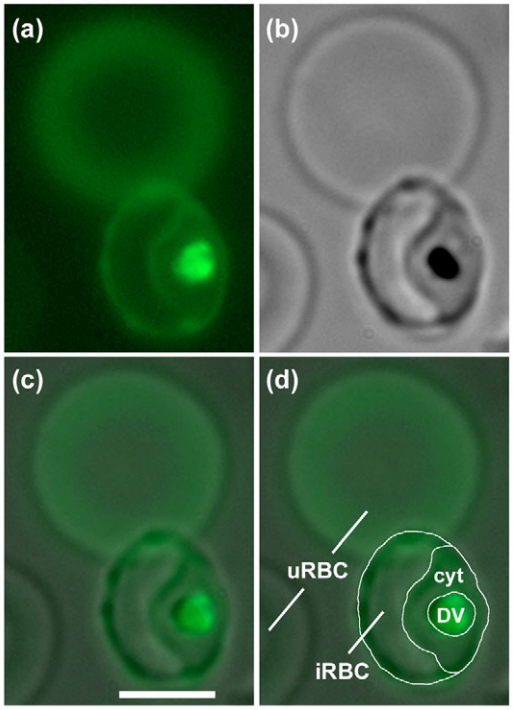

Fig. 8. Dextran-linked pH indicator fluorescence localises to the *Plasmodium falciparum* digestive vacuole, as demonstrated by this representative set of fluorescence and brightfield images. (a) False-colour image of the fluorescence emitted at >515nm when dextran-Fluorescein-loaded mature trophozoite-stage parasitised erythrocytes, together with dye-loaded uninfected erythrocytes, were excited at 420-490 nm. (b) Brightfield image of the same field, collected immediately after the fluorescent image. (c,d) Composite images with uninfected red blood cells (uRBC), infected red blood cell (iRBC), parasite cytosol (cyt) and digestive vacuole, as indicated. As described previously, fluorescence within the dye-loaded parasite emanates predominantly from a restricted area that coincides with the haemozoin crystals within the digestive vacuole (Bray et al., 2002a; Spiller et al., 2002). Bar, 5 μm.

Materials and Methods

Culture conditions

CQ-sensitive D10-mdr^{D10} and CQ-resistant 7G8-mdr^{7G8} *P. falciparum* parasites (Reed et al., 2000), referred to as D10′ and 7G8′, respectively, were obtained from Alan Cowman (Walter and Eliza Hall Institute, Melbourne, Australia) and cultured under conditions described previously (Allen and Kirk, 2004; Hayward et al., 2005). Synchrony of cultures was maintained by elimination of trophozoite-stage parasites from 'ring-stage' cultures by suspension of the cells in an iso-osmotic (5% w/v) sorbitol solution (Lambros and Vanderberg, 1979).

Loading fluorescent pH indicators into the parasite's digestive vacuole

Membrane-impermeant dextran-linked forms of various fluorescent pH indicators (Molecular Probes, Eugene, OR) dissolved in sterile distilled water were loaded into uninfected erythrocytes essentially as described previously (Krogstad et al., 1985; Saliba et al., 2003). The loaded erythrocytes were invaded by parasites which, as they grew, endocytosed the dye-loaded erythrocyte cytosol, depositing it in their digestive vacuole, thereby selectively labelling the digestive vacuole with fluorescent indicator (Fig. 8). The ratiometric indicators used included 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-Fluorescein (BCECF, pK_a ~7.0), Fluorescein (pK_a ~6.4), Oregon Green 514 (pK_a ~4.7), and 5-(and-6)-carboxy-2-chloro-3′-hydroxy-1,2,3,4-tetrahydropropyridino[5,6]-spiro[isobenzofuran-1(3H),9'-(9H)xanthen]-3-one (CL-NERF, pK_a ~3.8). Experiments were performed on parasites which had undergone two cycles of invasion of dye-loaded erythrocytes, resulting typically in a parasitaemia of 10-20%, with ≤2% of parasites in the second cycle inhabiting residual non-loaded erythrocytes from the inoculating culture. Note that unless specified otherwise all dye concentrations are described in terms of the concentration of the fluorophore in the loading solution. For example, 2.25 ml of loading solution containing 25 μl of 25 mg/ml Fluorescein-dextran (~10×10^3 M_r) is described as having a concentration of ~28 μM dextran but ~40 μM Fluorescein, as there are, on average, 1.4 fluorophore molecules attached to each dextran molecule (Haughland, 2002). By contrast, 2.25 ml of loading solution containing 25 μl of 25 mg/ml Oregon Green 514-dextran (also ~10×10^3 M_r) has the same dextran concentration but the fluorophore concentration is ~70 μM, as there are, on average, 2.5 molecules of dye per dextran molecule (Haughland, 2002). In experiments designed to test the effect of varying the dye concentration in the digestive vacuole, unlabelled dextran of the same relative molecular mass as that of the dye conjugate was added to the loading solution to ensure that the concentration of dextran remained constant (~28 μM, except in the case of ~150 μM Fluorescein and ~280 μM Oregon Green, where the dextran concentration was ~110 μM).

Fluorometric pH measurements

All fluorometry experiments were performed on parasites that had been isolated from their host erythrocytes by saponin-permeabilisation of the host erythrocyte membrane (Saliba and Kirk, 1999). The parasites were resuspended in a minimal saline solution comprising 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM glucose and 25 mM HEPES, at pH 7.1, and measurements of pH were then carried out at 37°C using a PerkinElmer Life Sciences LS-50B spectrofluorometer. Using the dual-excitation 'Fast Filter' accessory, the samples were excited and fluorescence emission was measured at the specified wavelengths for each dye (Haughland, 2002). The ratio of the fluorescence intensities measured using the two excitation wavelengths for each dye provides a measure of pH. All experiments were conducted using late-stage parasites (mature trophozoites, 32-38 hours after invasion). The experiments giving rise to Fig. 4 also included measurements performed on early trophozoites (16-22 hours after invasion).

For any given cell treatment, at least three replicate measurements of baseline fluorescence at an extracellular pH of 7.1 were obtained in each experiment. Calibration of the relationship between the fluorescence ratio and pH_{DV} was initially performed using three different techniques. The first was the nigericin calibration technique described previously (Saliba and Kirk, 1999; Wunsch et al., 1997), in which parasites were suspended in a saline solution containing 130 mM K^+ (a concentration assumed to approximately equal that of the parasite cytosol) (Lee et al., 1988) and treated with the proton ionophore nigericin (50 μM), which catalyses the exchange of K^+ for H^+. This causes all parasite compartments to equilibrate

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rapidly with the pH of the external solution, which was varied as required. The second calibration method involved washing and resuspending cell aliquots in glucose-free saline (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 25 mM HEPES), then incubating at 37°C for 1 hour. Deprived of glucose, and hence ATP (Sherman, 1998), the parasite loses the ability to regulate its cytosolic (Saliba and Kirk, 1999) and digestive vacuole (Saliba et al., 2003) pH, causing it to equilibrate to approximately that of the external solution, which was varied as required. The third calibration method followed on from the second. Once a measurement was obtained from ATP-depleted parasites in a defined extracellular pH, the parasite membranes were permeabilised with 200 ng/ml digitonin (Krogstad et al., 1985) to ensure that the pH gradients between the digestive vacuole, the parasite cytosol and the external medium had indeed collapsed in the absence of ATP. Attempts to calibrate pH_{DV} using a fourth approach, the 'null method' described by Eisner et al. (Eisner et al., 1989), were unsuccessful, perhaps because of the complexities arising from the presence of the cytosolic compartment separating the digestive vacuole from the extracellular medium. As early experiments confirmed that there was good agreement between the first three methods of pH calibration (see Fig. 2), subsequent experiments relied primarily on the nigericin/high-K^+ method, for reasons of expediency. Calibration curves were fitted by least-squares regression with either first or second order polynomial functions, depending on whether measurements were inside or outside the linear range of the characteristic sigmoidal response for each particular dye (see Fig. 3).

pH dependence of the dextran-linked dyes

In order to test whether the pK_a values of the dyes used were altered in the environment of the digestive vacuole, the fluorescence of Fluorescein (70 nM fluorophore) and Oregon Green 514 (120 nM fluorophore) in high-K^+ saline was measured at a range of pH values and normalised, so that the resulting cell-free curve could be compared with that generated by the dye in situ; i.e. in the digestive vacuoles of parasites suspended in nigericin-treated high-K^+ saline over a similar pH range.

In vitro testing of CQ sensitivity

The sensitivity of parasites in dye-loaded erythrocytes to CQ and to mefloquine (another aminoquinoline antimalarial) was determined by a modification of the standard microdilution technique described previously (Desjardins et al., 1979). Briefly, at the beginning of the second cycle of invasion of dye-loaded erythrocytes the parasitaemia and haematocrit was adjusted to 5% and 1%, respectively. The culture was then incubated in RPMI medium in 96-well plates containing 18 different CQ or mefloquine concentrations (from 200 to 1.5 nM, and 500 to 1.2 nM, respectively) for 24 hours at 37°C. [^{3}H]hypoxanthine (Amersham) was added to each well to a final concentration of 2 μCi/ml, and the plates incubated at 37°C for a further 18-20 hours. Parasite DNA and RNA was subsequently harvested onto glass fibre filter paper (Packard) using a Filtermate 196 Harvester (Packard). Incorporation of [^{3}H]hypoxanthine was measured using a Topcount Scintillation Counter (Packard). Sigmoidal curves were fitted to the data by least-squares regression with SigmaPlot 2001 (SPSS).

[^{3}H]CQ Uptake measurements

Measurements of the uptake of [^{3}H]CQ by *P. falciparum*-infected erythrocytes were carried out essentially as described previously (Bray et al., 1996; Reed et al., 2000). Late-stage parasites (mature trophozoites, 32-38 hours after invasion) in intact erythrocytes were incubated at a density of ~1-2×10^8 cells/ml and a parasitaemia of ~4-8% in bicarbonate-free RPMI medium containing 1 nM [^{3}H]CQ at 37°C for 1 hour, to determine total uptake.

Statistical analysis

Statistical significance (*P* value) was tested using the appropriate two-tailed *t*-test.

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