Verapamil increases the net uptake and cytotoxicity of structurally diverse hydrophobic molecules in many multidrug-resistant mammalian cell lines. This compound has also been reported to reverse chloroquine resistance in the human malaria parasite Plasmodium falciparum (Martin, S. K., Oduola, A. M. J., and Mihlous, W. K. (1987) Science 235, 899-901). Although the mechanism of this reversal is unknown, it apparently involves an increase in the amount of chloroquine present in erythrocytes infected with the resistant parasites. Chloroquine is a diprotic weak base that accumulates in acidic organelles as a function of the pH gradient present between the organelle and the external medium. By changing the external medium pH, this property of chloroquine was used to alter the cytotoxicity phenotype of genetically chloroquine-sensitive and -resistant trophozoites. Verapamil was also found to be toxic for malaria trophozoites, although this toxicity was independent of external pH and consistently about 3-4-fold higher against resistant strains. When verapamil was tested for its effects on chloroquine cytotoxicity under conditions of phenotypic reversal, it was still found to exert only a measurable effect on the genetically resistant trophozoites. In short time incubations, verapamil was found to increase net chloroquine accumulation in erythrocytes infected with both chloroquine-sensitive and -resistant organisms, but only to affect the chloroquine susceptibility of the latter. Analysis of our data demonstrates that verapamil works independently of the overall pH gradient concentrating chloroquine into a trophozoite's lysosome. Instead, we propose that it inhibits the activity of a membrane ion channel indirectly responsible for determining chloroquine transit within the parasite's cytoplasm.

Human falciparum malaria (caused by an intracellular parasite of the genus Plasmodium) remains a serious disease in much of the tropical and subtropical world. There are at least 300 million new infections every year causing an estimated 2 million deaths mostly of young children. Due to its specificity, stability, and safety, chloroquine has been one of the most successful and widely used antimalarial drugs (reviewed in Ref. 2). The biological activity of chloroquine is directed against the intraerythrocric stage of Plasmodium infection. During this stage, Plasmodium falciparum trophozoites obtain much of their amino acid requirements by degrading erythrocyte hemoglobin within an acidic food vacuole, a specialized organelle with some resemblance to mammalian lysosomes (3-5). Heme is released during this process and rendered into a nontoxic, crystalline polymer called hemozoin or malaria pigment (6,7). Chloroquine is believed to exert its specific antimalarial effects by inhibiting this polymerization process (8, 9). Accumulation of toxic amounts of heme substrate is probably then ultimately responsible for parasite cell death.

The evolution and geographical spread of P. falciparum trophozoites resistant to chloroquine has greatly reduced the clinical effectiveness of this compound (2, 10, 11). Identification of the biochemical mechanism responsible for chloroquine resistance would therefore assist in the development of alternative chemotherapeutic strategies. The heme polymerizing activity contained in extracts of both chloroquine-resistant and -sensitive trophozoites has similar sensitivity to inhibition by chloroquine (2), while a chemical modification of chloroquine by resistant organisms has never been demonstrated (12). Therefore, the mechanism of resistance must involve either the differential sequestration and/or uptake and transport of chloroquine within the parasite.

Many investigators have associated a reduced accumulation of chloroquine in infected erythrocytes with the acquisition of chloroquine resistance, suggesting that resistant trophozoites lower their lysosomal chloroquine concentration below that necessary to disrupt hemoglobin catabolism. Two mechanisms to account for this reduction in accumulation of chloroquine have been proposed. The weakened proton pump model postulates an increase in vacuolar pH leading to reduced weak base-driven accumulation of chloroquine (a diprotic weak base) into the lysosomes of resistant parasites (13, 14). Although plausible, the central predication of this model concerning changes in vacular pH have not been detected, and it remains controversial. Similarities to mammalian tumor cell multidrug resistance (MDR), chiefly the reversal of chloroquine resistance by drugs such as verapamil (a Ca2+ channel blocker) and phenothiazines lead Martin et al. (1) to propose that the two phenomena were similar (1). However, correlations between either mutation or overexpression of known malaria mdr genes and chloroquine resistance have not been detected in genetic selection experiments.

The reversing effect of verapamil on chloroquine resistance...
has been confirmed in numerous studies; however, its biochemical target(s) and mechanism of action in malaria trophozoites are unclear (15, 16). Braissant et al. (15, 16) have shown that verapamil increases chloroquine accumulation in resistant parasites. To further understand the biochemical mechanism behind this observation, we have used the weak base phenelzine to phenotypically alter the chloroquine susceptibility of both genetically chloroquine-sensitive and -resistant trophozoites. Verapamil exerted no effect on the toxicity of chloroquine for sensitive trophozoites under any experimental conditions examined, while it continued to increase the chloroquine susceptibility of resistant organisms even when rendered phenotypically sensitive by weak base manipulation. Verapamil was also found to induce a saturable increase of net chloroquine accumulation into both chloroquine-sensitive and -resistant organisms, while its mechanism of reversing chloroquine resistance is shown to be separable from any direct toxic action. We conclude that verapamil specifically increases chloroquine accumulation in genetically resistant P. falciparum trophozoites independently of weak base processes and phenotypic susceptibility due to external pH manipulations.

EXPERIMENTAL PROCEDURES

Parasites—Cultures of P. falciparum were grown in A+ human erythrocyte suspensions using RPMI 1640 (LifeTechnologies, Inc.) medium supplemented by 25 mM HEPEs (pH 7.35), 0.2% NaHCO3 (23 mM), 0.2% α-glucose, and 10% human A+ plasma and maintained at 37°C in candle jars according to the method of Trager and Jensen (17, 18). Parasites were stage-synchronized by incubating mainly ring-stage infected erythrocytes in 5% sorbitol for 10 min at room temperature (19). Cultures were allowed to undergo one growth cycle (2 days) before use. Strains used were the chloroquine-sensitive strain D10 (SCQ) and the chloroquine-resistant isolate Dd2 (RCQ) (kindly provided by Dr. Michael Lanzer, Sloan Kettering).

Parasite Growth Measurements—Parasite growth was monitored by measuring incorporation of [3H]hypoxanthine into the nucleic acids of the parasite as described previously (20). Effects of chloroquine, verapamil, and external pH on parasite growth was determined by measuring the reduction in incorporation of radiolabeled into complete media and 50 µl of drug dilution or solvent. Media pH was manipulated by the addition of small aliquots of 1 n HCl or 1 n NaOH plus supplemented with 25 mM HEPEs to maintain buffering capacity. The final hematocrit was 1.5%, and percent parasitemias ranged from 1–5%. After 24 h of candle jar incubation at 37°C, 2.5 µCi of [3H]hypoxanthine (17 Ci/mmol, Amersham) was added to each well. After 18 h, the wells were harvested onto glass fiber filters, dried, and placed in scintillation vials with 7 ml of scintillation fluid. Vials were counted in a liquid Beckman LS7800 scintillation counter. Chloroquine and verapamil IC50 values were calculated by extrapolation of the log dose-response curves. IC50 values represent the molar concentration which decreases [3H]hypoxanthine incorporation by 50% as compared to drug-free controls.

Chloroquine Uptake Measurements—Highly synchronized trophozoite-parasitized red blood cell cultures were washed and then incubated in RPMI 1640 medium supplemented by 25 mM HEPEs (pH 7.35), 0.2% NaHCO3, 0.2% α-glucose, 1% human plasma, and 4 mM 3-[3H]chloroquine (254 Ci/mmol; Dupont NEN) at 37°C under continuous stirring and parasitemias of 1–10%. Aliquots were centrifuged through silicon oil (d = 1.050, Aldrich) at various times (0.5–60 min), separating the red blood cell pellet from unincorporated label, terminating chloroquine uptake. For rapid measurements, synchronized trophozoite-stage parasites or control uninfected erythrocytes were layered on an oil cushion of deoxycholate tubes residing in a centrifuge at 37°C. 4 mM radiolabeled chloroquine was added to each tube at 2–3-s intervals. Accumulation was stopped by centrifugation. The cell pellet, now separated from the culture medium by the oil layer, was then processed according to published methods (21) with minor variations. The tube bottom tips containing the cell pellets were cut off and placed in scintillation vials containing 100 µl of ethanol and 50 µl of 25% (v/v) n-butanol (NCS-III Tissue solubilizer; Aldrich). After 18 h, vials were incubated overnight at 37°C. The lysate was then decolorized by the addition of 25 µl of 30% H2O2. Luminescence was blocked by acidification with 25 µl of glacial acetic acid. Vials were counted in a liquid scintillation counter (Beckman LS7800) for 2 min. The amount of label remaining in the aqueous medium was measured throughout the experiment to monitor for depletion. Only time points in which less than 15% of label was depleted from the aqueous layer were used for data analysis. 1 µM verapamil was added to the media in some experiments and preincubated for 10–30 min prior to addition of labeled chloroquine. Uninfected red blood cells were subjected to identical preincubation protocols in verapamil before chloroquine uptake measurements. Data from different experiments was normalized for percent hematoctit, percent parasitemia, and total label added. Chloroquine (7-chloro-4-(4-dimethylamino-1-methylbutylamino)quinoline) diphosphate, verapamil (5-[3,4-dimethoxyphenyl]methylamine)-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile), and all other reagents were obtained from Sigma.

RESULTS

Verapamil Reversal of Chloroquine Resistance Is Specific for Resistant Parasites—Under standard conditions, the IC50 values for D10 and Dd2 parasite strains are 22 ± 5 and 131 ± 25 nM chloroquine, respectively; these two strains are henceforth designated as chloroquine-sensitive (SCQ) and resistant (RCQ). Yayon et al. (22) showed that raising external medium pH (pHm) increases chloroquine toxicity for FCR3TC P. falciparum trophozoites, as predicted by the weak base effect. We used this method to make SCQ trophozoites phenotypically resistant (for example, a chloroquine IC50 of 25 nM at pHm of 7.35 is increased to >50 nM at pHm below 6.60). Similar experiments with RCQ demonstrated that they become hyper-resistant at pHm below 7.0 (i.e., the efficiency of their resistance mechanism is enhanced at low pHm) but phenotypically sensitive above pHm 8.20 (chloroquine IC50 < 50 nM). The weak base effect can therefore be used to switch the chloroquine susceptibility of sensitive and resistant organisms. This phenotypic reversal is not due to interstrain differences in parasite growth. Measurements of growth at various pHm values revealed no difference between SCQ and RCQ parasites (data not shown).

Martin et al. (1) reported that nontoxic verapamil concentrations reverse chloroquine resistance and yet have no effect on chloroquine susceptibility of sensitive strains. By using pHm manipulations to reverse phenotype, we found that the verapamil reversal effect is specific for chloroquine-resistant genotype, but independent of actual chloroquine sensitivity. For RCQ parasites, 1 µM verapamil reduced chloroquine IC50 regardless of whether their phenotypic susceptibility was hyper-resistant (for example, 584 nM at pHm 6.55) or sensitive (for
example, 29 nM at pH 7.8; 15 nM at pH 8.50 (Fig. 1). The verapamil-induced increase in RCQ chloroquine susceptibility is therefore independent of whether the parasites are phenotypically hyper-resistant, resistant, or susceptible to chloroquine. On the other hand, identical verapamil concentrations have no effect on genetically chloroquine-sensitive parasites, even under conditions in which they are phenotypically resistant. For example, at pH 6.35, the SCQ strain has an IC$_{50}$ of 210 nM independent of the presence of verapamil (Fig. 1). Furthermore, the decrease in RCQ chloroquine IC$_{50}$ by verapamil is remarkably similar at all pH 6 values, regardless of actual IC$_{50}$, 1 µM verapamil reduced RCQ chloroquine IC$_{50}$ an average of 73 ± 6% across all pH 6 tested. On the other hand, verapamil had no effect on SCQ chloroquine IC$_{50}$ (average change of −4 ± 9%). These data demonstrate that the reversal of chloroquine resistance by verapamil is specific for parasite genotype and not phenotype.

The Reversal and Toxic Effects of Verapamil Are Not Related—When directly tested for cytotoxicity, verapamil has been shown to have weak intrinsic antimalarial properties (1). The two strains used in this study differ in their sensitivity to verapamil, RCQ parasites being more susceptible to verapamil toxicity than SCQ parasites under normal growth conditions (Fig. 2). There was little variation in verapamil IC$_{50}$ within each strain throughout the pH 6 range of 6.4 to 7.9 (7.2 ± 2.1 µM for RCQ and 2.5 ± 0.7 µM for SCQ), suggesting that verapamil does not act as a lysosomal weak base. The average fold difference in verapamil toxicity between strains was 3.2.

These findings raise the following questions. 1) Is reversal of chloroquine resistance an indirect consequence of differential verapamil toxicity (as concentrations of verapamil used in these and other published studies are primarily within toxic ranges for Dd2 parasites)? 2) Can the chloroquine IC$_{50}$ of sensitive parasites be lowered in the presence of more verapamil? To address these issues we measured chloroquine IC$_{50}$ in the presence of increasing verapamil concentrations (Fig. 3). RCQ chloroquine IC$_{50}$ decreases with increasing verapamil concentrations until it plateaus at 10 nM in the presence of 5 µM verapamil. Although verapamil toxicity continues to increase, suppressing parasite growth by 90% at 15 µM, no further changes in chloroquine IC$_{50}$ are detected. The chloroquine-sensitive D10 parasites, on the other hand, undergo no changes in chloroquine IC$_{50}$ as a function of verapamil concentration, even at highly toxic verapamil concentrations (20–30 µM). Therefore, the chloroquine resistance reversing property and direct toxic actions of verapamil are not related. Furthermore, reversal of chloroquine resistance is not an indirect outcome of further stressing a “verapamil-weakened” parasite, as toxic concentrations of verapamil do not alter chloroquine sensitivity in the SCQ strain.

Verapamil Affects Chloroquine Accumulation in Both Strains—Krogstad et al. (23) and Bray et al. (15, 16) demonstrated that verapamil increases chloroquine accumulation in resistant parasites providing a possible mechanistic explanation of its reversal activity. We examined the effects of verapamil on chloroquine accumulation under our experimental conditions. As expected, RCQ parasites accumulate less chloroquine than SCQ trophozoites (Fig. 4A). 1 µM verapamil increased chloroquine accumulation into RCQ parasites about 2-fold (Fig. 4B). This increase was similar at all pH 6 tested (an average of 2.2 ± 0.5-fold after 15 min, data not shown). There was no change in background chloroquine accumulation into uninfected erythrocytes in the presence of verapamil (data not shown). Interestingly, chloroquine accumulation seems to slightly increase in D10 parasites in the presence of 1 µM verapamil. Therefore, we measured chloroquine accumulation into trophozoites in the presence of increasing verapamil concentrations (Fig. 5). Chloroquine accumulation increases rapidly in Dd2 trophozoites as verapamil concentration is increased up to 2 µM, after which only minor fluctuations in uptake are observed. This verapamil dose-response curve correlates with the IC$_{50}$ data illustrated in Fig. 3, in which chloroquine sensitivity decreases rapidly up to 2 µM, and then saturates. Therefore, we were able to measure chloroquine accumulation into resistant parasites at normally toxic verapamil concentrations because this assay lasts for only 10–30 min. In SCQ trophozoites, a similar range of verapamil concentrations results in a significant rise in chloroquine accumulation between 0.5 and 1 µM verapamil before saturating (Fig. 5). The total increase in chloroquine accumulation is similar to that observed in RCQ parasites (about a 2-fold increase from baseline levels). This result is significant in that it is not accompanied by any changes in chloroquine sensitivity (see Fig. 3). We conclude that verapamil affects a saturable mechanism regulating the net accumulation of chloroquine in both parasite strains, but that sensitivity to chloroquine is altered only in genetically resistant parasites. Interestingly, chloroquine accumulation does not correlate...
Verapamil Reversal of Malarial Chloroquine Resistance

perfectly with chloroquine sensitivity. At pH 7.35, RCQ parasites are 4–5-fold less sensitive to the toxic effects of chloroquine but accumulate only about 2–3-fold less drug. Manipulation of the weak base effect by alteration of pH results in chloroquine accumulation variations. Even under conditions in which resistant parasites accumulate more chloroquine than sensitive trophozoites (Fig. 6), chloroquine sensitivity is still markedly higher in SCQ trophozoites (chloroquine IC_{50} of 35 ± 5 at pH_{o} of 6.7 versus RCQ IC_{50} of 90 ± 10 at pH_{o} of 7.6).

Kinetic Analysis of Chloroquine Uptake—We performed detailed kinetic studies on the initial rate of chloroquine uptake by infected erythrocytes (0–2 min). Addition of radiolabeled chloroquine to infected and uninfected red blood cells results in an instantaneous (≤2 s) uptake of label, probably the initial partitioning of chloroquine across membranes. Infected erythrocytes continue to accumulate label and by 10–15 s there is a clear difference between infected and uninfected red blood cell. By 45–60 s, there is a clear difference in chloroquine accumulation between resistant and sensitive parasites (Fig. 7). However, the increased chloroquine accumulation observed in verapamil-treated Dd2 trophozoites (versus untreated Dd2) takes >5 min to develop (Fig. 4B). Therefore, verapamil does not affect chloroquine accumulation during the first 60 s of uptake, the time period during the accumulation phase that differences between SCQ and RCQ are first evident.

DISCUSSION

Chloroquine-sensitive and -resistant P. falciparum trophozoites develop apparently identically within human erythrocytes and produce similar amounts of hemoglobin pigment. As chloroquine resistance does not appear to involve a change in the process of hemoglobin proteolysis, heme polymerization, or drug metabolism, resistance can only be achieved by lowering the effective concentration of chloroquine within the trophozoite's food vacuole (2). Chloroquine and related antimalarial quinolines are hydrophobic weak bases known to concentrate within low pH vacuoles (24). For example, Ginsburg and Geary (25) estimate that at nanomolar external concentrations (pH 7.2) diprotic chloroquine can reach millimolar levels within a trophozoite lysosome (estimated pH in the range 4.8–5.2).

Three different mechanisms by which chloroquine-resistant trophozoites could reduce the amount of chloroquine that accumulates in this organelle can be envisaged. 1) Reducing the magnitude of the weak base effect by alteration of pH results in chloroquine accumulation variations. Even under conditions in which resistant parasites accumulate more chloroquine than sensitive trophozoites (Fig. 6), chloroquine sensitivity is still markedly higher in SCQ trophozoites (chloroquine IC_{50} of 35 ± 5 at pH_{o} of 6.7 versus RCQ IC_{50} of 90 ± 10 at pH_{o} of 7.6).

Kinetic Analysis of Chloroquine Uptake—We performed detailed kinetic studies on the initial rate of chloroquine uptake by infected erythrocytes (0–2 min). Addition of radiolabeled chloroquine to infected and uninfected red blood cells results in an instantaneous (≤2 s) uptake of label, probably the initial partitioning of chloroquine across membranes. Infected erythrocytes continue to accumulate label and by 10–15 s there is a clear difference between infected and uninfected red blood cell. By 45–60 s, there is a clear difference in chloroquine accumulation between resistant and sensitive parasites (Fig. 7). However, the increased chloroquine accumulation observed in verapamil-treated Dd2 trophozoites (versus untreated Dd2) takes >5 min to develop (Fig. 4B). Therefore, verapamil does not affect chloroquine accumulation during the first 60 s of uptake, the time period during the accumulation phase that differences between SCQ and RCQ are first evident.
We and others have shown that the toxicity of chloroquine for both chloroquine-sensitive and -resistant trophozoites is dependent on $pH_o$ as predicted by the weak base theory of drug uptake. Assuming that the vacuolar $pH$ of chloroquine-sensitive trophozoites is about 4.8, then changing the pH gradient from 3 ($pH_o = 7.8$) to 2 ($pH_o = 6.8$) results in a 4-fold increase in chloroquine IC$_{50}$. Under physiological conditions ($pH_o = 7.35$), chloroquine-resistant trophozoites typically display a 5-10-fold decrease in their chloroquine sensitivity. To account for this by a direct change in the pH gradient from external medium to lysosome would clearly require a vacuolar pH in excess of 6.0. However, attempts to directly measure even a small difference in lysosomal pH between chloroquine-sensitive and -resistant trophozoites have so far been unsuccessful (21). The results of Goldberg et al. (3, 4) also indicate that the rate of lysosomal hemoglobin degradation would be markedly slower if vacuolar pH values were above 6.0, again inconsistent with the equal rates of hemozoin formation observed in chloroquine-sensitive and -resistant trophozoites.

Evidence for the existence of a rapid efflux mechanism for the selective expulsion of chloroquine from erythrocytes infected with chloroquine-resistant but not chloroquine-sensitive trophozoites has been presented, although the finding is controversial (summarized in Refs. 2 and 11). Using the D10 and Dd2 strains of chloroquine-sensitive and chloroquine-resistant P. falciparum, we have only been able to detect a small and inconsistent difference in the rate of chloroquine efflux between the two strains. For example, after loading trophozoites for 45-60 min at $pH_o$ 7.35 with 4 nM chloroquine, average drug efflux after 10 min was 32 ± 14% from SCQ trophozoites ($n = 6$) and 43 ± 3% from RCP trophozoites ($n = 4$). This agrees with the results of Bray and colleagues (13-16) that chloroquine resistance correlates with net chloroquine uptake rather than rapid drug efflux from infected erythrocytes. Furthermore, our observation that 2 $\mu$M verapamil increases net chloroquine uptake about 2-fold in both SCQ and RCP trophozoites also argues against the importance of a verapamil-inhibitable chloroquine efflux mechanism specific for chloroquine-resistant trophozoites.

By manipulating $pH_o$, we have found that the chloroquine cytotoxicity phenotype of genetically chloroquine-sensitive and -resistant trophozoites can be reversed. Verapamil lowered the chloroquine IC$_{50}$ of RCP trophozoites about 75% irrespective of whether the trophozoites were forced to be phenotypically sensitive ($pH_o > 7.8$) or hyper-resistant ($pH_o > 6.8$). In marked contrast, the chloroquine susceptibility of SCQ trophozoites was completely refractory to coincubation with verapamil, even when they were made phenotypically resistant by lowering $pH_o$ below 6.5. Therefore, the verapamil-inhibitable mechanism of chloroquine resistance is specific for genetically chloroquine-resistant parasites and does not simply result from a change in the net chloroquine gradient from external medium to lysosome.

In RCP trophozoites exposed to verapamil, the decrease observed in chloroquine IC$_{50}$ only depends on verapamil concentration and not on the rate of chloroquine accumulation. A likely explanation for this observation is that a given amount of verapamil inhibits a constant fraction of the activity of some undefined protein. If verapamil acted to reduce vacuolar pH (thereby increasing the rate of chloroquine accumulation by directly raising the magnitude of the pH gradient between external medium and lysosome), it would be expected to proportionately exert a more pronounced effect on chloroquine cytotoxicity when the pH gradient was small. However, this is clearly not consistent with our data. It is much more likely that the protein target for verapamil modulates chloroquine accumulation and intracellular partitioning independent of vacuolar pH, and that the reversing agent blocks the activity of the same fraction of this putative protein irrespective of the pH gradient present. Consistent with this proposal, in the presence of 1 $\mu$M verapamil, the chloroquine IC$_{50}$ of RCP trophozoites responds to changes in $pH_o$ in a similar way to SCQ parasites cultured without the drug (Fig. 1). We conclude that the overall weak base pH gradient driving chloroquine concentration from the external medium into the lysosome of a trophozoite is not the site for verapamil reversal of chloroquine resistance.

Given the greater sensitivity of chloroquine-resistant trophozoites to the direct toxic effects of verapamil, it has been proposed that the synergism observed between chloroquine and verapamil is due to a nonspecific "weakening" of the resistant parasite by the later compound, indirectly making them more sensitive to chloroquine. However, ultrastructural studies have shown that the early morphological changes characteristic of chloroquine toxicity in a sensitive strain of P. falciparum are also apparent in resistant trophozoites upon simultaneous exposure to verapamil and chloroquine, arguing against two different mechanisms for cell killing (26). Our data confirm this by demonstrating that verapamil-weakened SCQ trophozoites remain completely refractory to any changes in their susceptibility to chloroquine. The direct toxic effects of verapamil therefore appear to be unrelated to its reversal properties. However, it would be interesting to test whether other chloroquine resistance reversal agents, such as desipramine and cymetidine, also demonstrate differential toxicity between strains.

The site and molecular identity of the verapamil-sensitive target in chloroquine-resistant trophozoites is unknown. One way in which a trophozoite could exploit the weak base effect to become chloroquine-resistant is to reduce the pH of its cytoplasm, analogous to an experimental reduction in external pH. This would increase the fraction of protonated chloroquine molecules present and thereby reduce the rate of further passage of the unprotonated form into the lysosome. For example, a reduction of cytoplasmic pH by 0.1 unit would decrease uncharged chloroquine by 22%; a further 0.1 change would result in 37% less uncharged chloroquine available for entry into the vacuole. Several published reports have documented cytoplasmic pH ($pH_i$) alterations in mammalian tumor MDR cell lines. For example, the steady state efflux of the weak base doxorubicin has been shown to strongly correlate with $pH_i$ (27). Possible mechanisms of $pH_i$ alteration include modifications in $Cl^-$, $H^+$, or $HCO_3^-$ transport and other indirect changes in

\[ 3 J. A. Martiney, unpublished data. \]
membrane potential and/or cell volume. Overexpression of murine MDR1 in CHO fibroblasts results in an inhibition of Cl\textsuperscript{−} and HCO\textsubscript{3}−-dependent pH homoeostasis concomitant with the drug resistance phenotype (28, 29). A Cl\textsuperscript{−} ion conductance associated with the MDR protein has been observed (30, 31), while others have shown that a monoclonal antibody raised against MDR1 inhibits chloride conductance in pancreatic zymogen granules, but recognizes a different protein of 65 kDa (32). The transmembrane movement of Cl\textsuperscript{−} ions is an important component in the regulation of pH\textsubscript{i}, volume, and membrane potential in mammalian cells, and it has recently been proposed that tumor multidrug resistance mediated by P-glycoprotein occurs secondary to perturbations in these parameters (33). Verapamil could thus act by “correcting” an anomalous Cl\textsuperscript{−} conductance. Indeed, evidence that verapamil reverses the altered Cl\textsuperscript{−}-dependent pH\textsubscript{i} behavior of MDR-transfected mammalian cell lines has been presented (34).

Members of the ATP-binding cassette (ABC) family of transmembrane transporters such as the multidrug resistance (MDR) and cystic fibrosis transmembrane regulator (CFTR) proteins have been associated with increased Cl\textsuperscript{−} channel activity. This channel regulator function is separate from any putative transport activities (35). We therefore hypothesize that the mutation conferring chloroquine resistance in P. falciparum up-regulates a Cl\textsuperscript{−} channel regulator protein, which alters ion conductances that indirectly control drug transit within the parasite’s cytoplasm by setting the cytoplasmic pH. It is this chloride channel regulator which is inhibited by verapamil during reversal of resistance. Interestingly, we have recently found that changes in the ionic composition of the external medium, as well as the presence of specific blockers of several known pH and volume regulators, alters chloroquine uptake into erythrocytes infected with chloroquine-resistant trophozoites.\textsuperscript{1} Resolution of the role of ion channels in drug transport is critical both to the further understanding of chloroquine resistance and to the ultimate goal of pharmacological intervention intended to overcome this problem.

Acknowledgments—We thank Gary Latortue and Angel Ferrer for technical help, and Barbara Sherry, Kevin Tracey, and Kirk Manogue for helpful discussions.

\textsuperscript{1} J. A. Martiney, A. Cerami, and A. F. G. Slater, manuscript in preparation.

REFERENCES

1. Martin, S. K., Oduola, A. M. J., and Milhous, W. K. (1987) Science 235, 899–901
2. Slater, A. F. G. (1993) Pharmacol. Ther. 57, 203–235
3. Goldberg, D. E., Slater, A. F. G., Cerami, A., and Henderson, G. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2893–2895
4. Goldberg, D. E., Slater, A. F. G., Beavis, R., Chait, B., Cerami, A., and Henderson, G. B. (1991) J. Exp. Med. 173, 961–965
5. Gluzman, I. Y., Francis, S. E., Oksman, A., Smith, C. E., Duffin, K. L., and Goldberg, D. E. (1994) J. Clin. Invest. 93, 1602–1608
6. Slater, A. F. G., Swiggard, W. J., Orton, B. R., Fletter, W. D., Goldberg, D. E., Cerami, A., and Henderson, G. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 325–329
7. Slater, A. F. G. (1992) Exp. Parasitol. 74, 362–365
8. Slater, A. F. G., and Cerami, A. (1993) Nature 365, 167–169
9. Dorn, A., Stoffel, R., Matthi, H., Rubendorf, A., and Ridley, R. G. (1995) Nature 374, 269–271
10. White, N. J. (1992) J. Antimicrob. Chemother. 30, 571–585
11. Foote, S. J., and Cowman, A. F. (1994) Acta Trop. 56, 157–171
12. Gluzman, I. Y., Schlesinger, P. H., and Krosgstad, D. J. (1987) Antimicrob. Agents Chemother. 31, 32–36
13. Bray, P. G., Howells, R. E., and Ward, S. A. (1992) Biochem. Pharmacol. 43, 1219–1227
14. Bray, P. G., and Ward, S. A. (1993) FEBS Microbiol. Lett. 113, 1–8
15. Bray, P. G., Boulter, M. K., Ritchie, G. Y., Howells, R. E., and Ward, S. A. (1994) Mol. Biochem. Parasitol. 63, 87–94
16. Bray, P. G., Howells, R. E., Ritchie, G. Y., and Ward, S. A. (1992) Biochem. Pharmacol. 44, 1317–1324
17. Trager, W., and Jensen, J. B. (1976) Science 193, 673–675
18. Jensen, J. B., and Trager, W. (1977) J. Parasitol. 63, 883–886
19. Lambros, C., and Vanderberg, J. P. (1979) Antimicrob. Agents Chemother. 15, 11042–11056
20. Desjardins, R. E., Canfield, C. J., Haynes, J. D., and Chulay, J. D. (1979) J. Clin. Invest. 64, 2495–2531
21. Krogstad, D. J., Gluzman, I. Y., Herwaldt, B. L., Schlesinger, P. H., and Wellems, T. E. (1992) Biochem. Pharmacol. 43, 57–62
22. Luyon, A. C., Beckthir, Z. I., and Ginsburg, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2748–2788
23. Krogstad, D. J., Gluzman, I. Y., Kyle, D. E., Oduola, A., Martin, S. K., Milhous, W. K., Martin, S. K., and Schlesinger, P. H. (1987) Science 233, 1283–1285
24. DeDuve, C., De Barro, P., Boile, B., Trouet, A., Tulkens, P., and Van Hooft, F. (1974) Biochem. Pharmacol. 23, 2495–2531
25. Ginsburg, H., and Geary, T. G. (1987) Biochem. Pharmacol. 36, 1567–1576
26. Jacs, G. H., Oduola, A. M. J., Kyle, D. E., Milhous, W. K., Martin, S. K., and Aikawa, M. (1988) Am. J. Trop. Med. Hyg. 36, 15–20
27. Roepe, P. D. (1992) Biochemistry 31, 12595–12604
28. Liu, T., Wei, L., Basi, S., and Roepe, P. D. (1994) Biochemistry 33, 7239–7249
29. Roepe, P. D., Wei, L., Cruz, J., and Carlson, D. (1993) Biochemistry 32, 11042–11056
30. Valverde, M. A., Diaz, M., Sepulveda, F. V., Gill, D. R., Hyde, S. C., and Higgins, C. F. (1992) Nature 355, 830–833
31. Gill, D. R., Hyde, S. C., Higgins, C. F., Valverde, M. A., Mintenig, G. M., and Sepulveda, F. V. (1992) J. Exp. Med. 173, 23–32
32. Thevenod, F., Anderie, I., and Schulz, I. (1994) J. Biol. Chem. 269, 24410–24417
33. Roepe, P. D. (1994) Trends Pharmacol. Sci. 15, 445–446
34. Roepe, P. D., Weisburg, J. H., Luz, J. G., Hoffman, M. M., and Wei, Y. (1994) Biochimie 76, 7239–7249
35. Valverde, M. A., Hardy, S. P., and Sepulveda, F. V. (1995) FASEB J. 9, 509–515