Cellular Uptake of Chloroquine Is Dependent on Binding to Ferriprotoporphyrin IX and Is Independent of NHE Activity in Plasmodium falciparum

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Abstract. Here we provide definitive evidence that chloroquine (CQ) uptake in Plasmodium falciparum is determined by binding to ferriprotoporphyrin IX (FPIX). Specific proteinase inhibitors that block the degradation of hemoglobin and stop the generation of FPIX also inhibit CQ uptake. Food vacuole enzymes can generate cell-free binding, using human hemoglobin as a substrate. This binding accounts for CQ uptake into intact cells and is subject to identical inhibitor specificity. Inhibition of CQ uptake by amiloride derivatives occurs because of inhibition of CQ–FPIX binding rather than inhibition of the Na\(^+\)/H\(^+\) exchanger (NHE). Inhibition of parasite NHE using a sodium-free medium does not inhibit CQ uptake nor does it alter the ability of amilorides to inhibit uptake. CQ resistance is characterized by a reduced affinity of CQ–FPIX binding that is reversible by verapamil. Diverse compounds that are known to disrupt lysosomal pH can mimic the verapamil effect. These effects are seen in sodium-free medium and are not due to stimulation of the NHE. We propose that these compounds increase CQ accumulation and overcome CQ resistance by increasing the pH of lysosomes and endosomes, thereby causing an increased affinity of binding of CQ to FPIX.

Key words: Plasmodium falciparum • chloroquine • drug resistance • heme binding • Na\(^+\)/H\(^+\) exchanger

Chloroquine (CQ) has been one of the most successful antimalarial agents ever developed. Unfortunately, the emergence and spread of resistant strains of Plasmodium falciparum has now rendered the drug almost useless in most malaria endemic areas. The immense clinical importance of falciparum malaria and the universal success of CQ before the development of resistance have provided the impetus for investigations about the mode of action of CQ and the basis of resistance at the cellular level. The activity of CQ depends on a high-level accumulation within the malarial parasite and drug resistance stems from reduced drug accumulation. Unfortunately, a definitive mechanistic explanation for these observations has remained elusive (Fitch, 1970; Krogstad et al., 1987; Ginsburg and Stein, 1991; Martiney et al., 1995; Bray et al., 1998).

However, Wünsch and co-workers recently provided compelling evidence for a novel mechanism of CQ resistance based on the differential stimulation of the parasite Na\(^+\)/H\(^+\) exchanger (NHE; Wünsch et al., 1998). Previous work from this group linked altered saturation kinetics of initial CQ uptake to the CQ resistance phenotype (Sanchez et al., 1997). They found that CQ uptake was inhibited competitively by specific inhibitors of NHE, providing evidence that the drug is actively transported through the parasite NHE. They also showed that CQ stimulates the NHE of chloroquine-sensitive (CQS) parasites and suggested that CQ is taken up by the NHE of these parasites in the ensuing rapid burst of sodium–proton exchange (Wünsch et al., 1998). Conversely, it was proposed that the NHE of chloroquine-resistant (CQR) parasites did not transport CQ, since it was constitutively activated and insensitive to further stimulation by CQ (Wünsch et al., 1998). The reversal of CQ resistance by verapamil was proposed to occur by modulating the activity of parasitic NHE via the calcium/calmodulin-dependent pathway (Sanchez et al., 1997).
Since mammalian NHE is incapable of CQ transport (Sanchez et al., 1997; Wünsch et al., 1998), this unusual mechanism of drug uptake should be responsible for the specificity of CQ for malarial parasites. Therefore, this model is incompatible with the notion that the specificity of CQ’s antimalarial action is caused by the formation of a drug-ferriprotoporphyrin IX (FPIX) complex accumulating in the parasite upon exposure to CQ (Chou et al., 1980; Fitch, 1983; Balsubramanian et al., 1984; Sullivan et al., 1996; Ginsburg et al., 1998). In a process unique to the malarial parasite, the FPIX released during proteolysis of hemoglobin is polymerized into an inert crystalline substance called hemozoin (Francis et al., 1997b). CQ inhibits this polymerization process, causing a buildup of free FPIX and/or CQ–FPIX complex that may ultimately kill the parasite (Slater, 1993; Dorn et al., 1995). Our own studies suggest that the specificity, accumulation, and antimalarial activity of CQ are all determined by the saturable equilibrium binding of CQ to FPIX (Bray et al., 1998).

We found that CQR parasites have a reduced apparent affinity of CQ–FPIX binding compared with CQS parasites. We propose that the resistance mechanism acts specifically at the site of FPIX generation to alter the affinity of CQ–FPIX binding rather than changing the active transport of CQ across the parasite plasma membrane (Bray et al., 1998). Here we provide definitive evidence that CQ uptake is determined by the binding of CQ to FPIX. In no part is the uptake of CQ controlled by the differential stimulation of the NHE as proposed (Wünsch et al., 1998). Furthermore, in CQR parasites reduced uptake of CQ, reduced apparent affinity of CQ–FPIX binding, and reversal of these parameters by verapamil are totally independent of NHE activity. We also propose a mechanistic basis for the reversal of CQ resistance in which resistance reversers increase the pH of acid vesicles where FPIX is generated. Therefore, this increases the affinity of CQ–FPIX binding.

Materials and Methods

Reagents

Leupeptin and trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64) were obtained from Boehringer Mannheim. Ro 40-4388, Ro 61-7835, and Ro 61-9379 were provided by Dr. R. G. Ridley and Dr. R. Mon of Hoffmann LaRoche. Triethylamine (TEA), diethylamine (DEA), dibutylyamine (DBA), 1-methyl-piperazine (1-MP) and dipropylamine (DPA) were purchased from Aldrich Chemical Co. Silicon oil was purchased from Duchefa. All other reagents were purchased from Sigma Chemical Co.

Culture of P. falciaparum and Drug Sensitivity Assays

Parasites were cultured and synchronized using standard techniques (Trager and Jensen, 1976; Lambros and Vandenberg, 1979). Drug sensitivity in the presence or absence of potential resistance reversers and hemoglobinase inhibitors was determined according to the method of Desjardins et al. (1979). IC50 values were calculated for each assay using the four parameter logistic method (Graphit program, Erithacus software; Kent Laboratories). The effect of the combination of CQ and Ro 40-4388 on parasite growth was tested by titration of the two drugs at fixed ratios proportional to their IC50 values. The fractional inhibitory concentrations of the resulting IC\textsubscript{50} values were plotted as isobolograms (Berenbaum, 1978).

Measurement of the Effect of Proteinase Inhibitors on Parasite Hemoglobin Digestion and Parasite Hemozoin Content

Erythrocytes infected with the K1 strain were synchronized and cultivated to the trophozoite stage and suspended in growth medium at a parasitemia of 10% and a hematocrit of 5%. At time 0, a sample was taken and the infected cells were separated, counted, and analyzed for hemoglobin and hemozoin as described below. The remaining cell suspension was incubated for 3 h at 37°C in the absence or presence of 10 μM Ro 40-4388, 20 μM N-acetyl-L-leucyl-L-leucyl-methional (ALLM), or 20 μM N-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN), or 20 μM leupeptin. A 1-h incubation, each was split into two aliquots. The infected cells from one aliquot were immediately separated from the uninfected cells using a Percoll–alanine gradient and counted as described (Ginsburg et al., 1998). The hemoglobin and hemozoin concentrations of these cells were measured as described below. The infected cells from the remaining aliquot were washed three times in prewarmed complete medium and returned to culture for an additional 3-h incubation period. A 1-h incubation, the infected cells were separated, counted, and the hemozoin concentration was determined.

Hemoglobin concentration was measured using Drabkins reagent as described (Ginsburg et al., 1998). The hemozoin concentration in the pellet was determined after incubation in 2% (wt/vol) SDS in 0.1 M NaHCO\textsubscript{3}, pH 9.1, followed by centrifugation at 14,000 rpm for 5 min. The supernatant was discarded and this step repeated three times to remove all nonhemozoin heme. The remaining hemozoin pellet was dissolved in 1 ml of 0.1 M NaOH and the absorbance was read at 400 nm using a Beckman DU 640 spectrophotometer. The amount of hemozoin/10\textsuperscript{6} cells was calculated from a calibration curve of known amounts of FPIX dissolved in 0.1 M NaOH.

Measurement of the Uptake of [\textsuperscript{3}H]CQ by Intact Parasitized Erythrocytes

Infected erythrocytes were suspended in the appropriate buffer containing [\textsuperscript{3}H]CQ, at a parasitemia of 1-2% and a hematocrit of 0.5%. At the required times, 100-μl samples were removed and the reaction was terminated upon centrifugation of the cells (14,000 rpm for 2 min) through silicon oil and processed for scintillation counting as described in Bray et al. (1996). Parasite specific uptake was determined by subtracting counts because of an equal number of uninfected erythrocytes. Unless otherwise stated, CQ accumulation is expressed as the cellular accumulation ratio (CAR) which is the ratio of the amount of radiolabeled CQ in the parasites to the amount of radiolabeled CQ in a similar volume of buffer after incubation.

The effect of the proteinase inhibitors Ro 40-4388, Ro 61-7835, Ro 61-9379, ALLM, ALLN, leupeptin, and E64, on the steady-state uptake of CQ was measured for 1 h at 37°C, in complete medium containing 1 nM [\textsuperscript{3}H]CQ. The reversibility of Ro 40-4388 (10 μM), ALLM (20 μM), and ALLN (20 μM) was determined as follows: [\textsuperscript{3}H]CQ was added at a concentration of 4 nM to inhibitor-treated and control groups. After 1 h, an aliquot was taken and processed for scintillation counting as described above. The remaining aliquot was washed three times in prewarmed medium (37°C). [\textsuperscript{3}H]CQ was re-introduced to the washed and control cells at a concentration of 4 nM. After 1 h, samples were removed and processed for counting. For the K1 isolate, parallel incubations were performed in the presence of 10 μM verapamil. In the case of ALLM and ALLN, inhibitors were added 45 min before the initial addition of CQ and a 10-min equilibration period was introduced after each wash.

CQ–FPIX binding parameters, in the absence or presence of 10 μM Ro 40-4388, were determined as described in Bray et al. (1998). For the K1 isolate, equilibrium binding parameters were obtained in sodium-free buffer (122.5 mM choline chloride, 5 mM KCl, 1.2 mM CaCl\textsubscript{2}, 0.8 mM MgCl\textsubscript{2}, 5.5 mM d-glucose, 1.0 mM K\textsubscript{2}HPO\textsubscript{4}, 10 mM Hepes, pH 7.4) in the absence or presence of 2 mM NH\textsubscript{4}Cl, 10 μM verapamil, or 100 mM monensin. Saturable uptake of CQ was calculated by subtracting nonsaturable CQ uptake from the total CQ uptake as described previously (Bray et al., 1998). Binding data are presented as Scatchard plots of saturable CQ uptake. The apparent K\textsubscript{s} of binding is given by the reciprocal of the slope and the amount of bound drug is given by the x-intercept.
The effect of resistance modulators was determined over 1 h in growth medium containing 1 mM \(^{3}H\)CQ in the absence or presence of 10 \(\mu\)M verapamil, 100 \(\mu\)M vinblastine, 100 \(\mu\)M monensin, 2 \(\mu\)M NHCl, 2 \(\mu\)M MA, 2 \(\mu\)M TEA, 2 \(\mu\)M DEA, 2 \(\mu\)M DBA, 2 \(\mu\)M 1-MP, or 1 \(\mu\)M DPA. Care was taken to ensure the medium pH was 7.4 throughout. Optimum concentrations of these compounds were obtained in preliminary experiments by measuring the effect of serial 1.3 log dilutions of drugs on CQ uptake (data not shown).

To determine the effect of bicarbonate on the uptake of \(^{3}H\)CQ, growth medium containing various concentrations of bicarbonate was shaken in atmospheric air at room temperature until the pH drift was complete. The pH was adjusted to 7.4 with 1 M HCl. Parasites were incubated in growth medium containing various concentrations of bicarbonate and 3 mM \(^{3}H\)CQ for 1 h and terminated thereafter.

**Measurement of the Uptake of \(^{3}H\)CQ Into Isolated Parasites**

Parasites were isolated from the host cell by selective lysis of the host cell compartment (E Ford, 1993). Ringer’s buffer modified to simulate an intracellular milieu was used throughout (Wünsch et al., 1998). In selected experiments, sodium chloride in the buffer system was replaced with molecular equivalents of either choline chloride or N-methyl-D-glucamine chloride. CQ uptake was determined by suspending the cell pellet containing purified free parasites, in 200 \(\mu\)l of the appropriate containing 50 mM \(^{3}H\)CQ, prewarmed to 37°C. Drug uptake was determined in the absence or presence of 100 \(\mu\)M S-N-ethyl-N-isopropyl amiloride (EIPA) or 10 \(\mu\)M verapamil. At the appropriate time points, aliquots were removed and the reaction was terminated by centrifugation (14,000 rpm, 2 min, at 4°C). The sample was placed in an ice-bath, the supernatant was removed, and the pellet was washed once in the appropriate ice-cold buffer without \(^{3}H\)CQ. A minor centrifugation (14,000 rpm, 1 min, at 4°C), the supernatant was carefully removed with a drawn out glass Pasteur pipette and the pellet was processed and counted for infected erythrocytes.

CQ uptake into isolated parasites was ~30% of intact infected erythrocytes’ uptake with over 30 min of CQ exposure. To investigate whether this reduced CQ uptake occurs because of impaired NHE activity, we compared the cytosolic pH of isolated parasites to that of parasites within intact host erythrocytes. In agreement with previous reports (Wünsch et al., 1998), we found no significant differences in cytosolic pH in these preparations. For example, the mean cytosolic pH for the K1 clone in its host cell was 6.902 (SEM = 0.045, \(n = 12\)) compared with 6.902 (SEM = 0.038, \(n = 22\)) when the parasite is liberated from the host cell. In addition, free parasites exhibited a marked cytosolic acidification in sodium-free buffer (0.76 pH unit ± SEM 0.045, \(n = 6\)), in full agreement with previous reports (Bosia et al., 1993). Thus, NHE is functional in isolated parasites.

We also investigated the possibility that the reduced CQ uptake occurs because of a loss of activity. Isolated parasites were found to incorporate radiolabeled hypoxanthine and isoleucine at the same rate as intact infected erythrocytes for at least 6 h (data not shown). Since the parasites are viable and NHE is functioning normally, we assumed that the reduced accumulation of CQ happens because of cessation of hemoglobin trafficking from the host cell at time 0 that reduces the amount of FPIX available for CQ binding.

**Determination of the Effect of Amiloride Analogues and Proteinase Inhibitors on FPIX Polymerization**

Measurement of FPIX polymerization inhibition by amiloride analogues and proteinase inhibitors employed a modification of the procedure described by Bray et al. (1996). A 100 \(\mu\)l aliquot (100 \(\mu\)M) of trophozoite lysate and FPIX (100 \(\mu\)M 3 of \(^{3}H\)CQ in 0.3 M NaCl solution) was mixed with an aliquot of 1 M HCl (10 \(\mu\)l) and sodium acetate (500 mM, pH 5.2) to give a volume of 900 \(\mu\)l in each tube. A series of drug concentrations was prepared in ethanol and 100 \(\mu\)l of each was added to the appropriate samples. The effect of ethanol on the polymerization process was assessed by adding 100 \(\mu\)l of ethanol to the control samples. Samples were mixed and incubated for 12 h, with occasional mixing. A 1 ml incubation, samples were centrifuged (14,000 rpm, 1 min, at 4°C) and the hemoglobin was washed with 2% (w/vol) SDS in 0.1 M sodium bicarbonate, pH 9.0, with sonication (30 min, at 21°C; FS100 bath sonicator; Dicon U Itronics Ltd.) until the supernatant was clear (usually 3–4 times). A 1 ml final wash, the supernatant was removed and the pellet was resuspended in 1 ml of 0.1 M NaOH, incubated for an additional 1 h at room temperature. Next, samples were mixed with a pipette. The hemoglobin content was determined by measuring the absorbance at 400 nm (Beckmann DU 640 spectrophotometer) using a 1-cm quartz cuvette. The amount of hemoglobin formed during incubation was corrected for preformed hemoglobin (the amount of preformed hemoglobin in the parasite extract was determined from a sample containing extract, but no substrate, which was incubated and repeatedly washed for 2% SDS as previously stated). The concentration of drug required to produce 50% inhibition of polymerization (IC50) was determined graphically as described for the drug sensitivity assays.

**Displacement of CQ Bound to FPIX-loaded Ghost Membranes and Parasite Debris**

Erythrocyte ghost membranes were prepared as described previously (Ginsburg et al., 1998). Membranes (0.27 mg protein) were loaded with FPIX (2-5 \(\mu\)M) in 0.2 M Hapes buffer, pH 7.0 (HB7) at 37°C for 7 min followed by centrifugation (14,000 rpm, 10 min). The supernatant was discarded and the pellet was washed once in HB7. Samples of FPIX-loaded membrane (0.01 mg protein) were suspended in 1 ml of HB7 containing 50 mM \(^{3}H\)CQ and incubated in the absence or presence of various concentrations of the proteinase inhibitors, amiloride and the amiloride analogues EIPA, 5-N-N-hexamethylene amidolride (HMA), and 5-N-isobutyryl-N-methyl amidolride (IBMA) for 10 min at 37°C. The membrane suspension was centrifuged (14,000 rpm, 2 min), the supernatant removed, and the pellet washed once in ice-cold HB7 without \(^{3}H\)CQ. The remaining pellet was solubilized and processed for counting, as described above.

To measure CQ-FPIX binding affinity in a parasite-free system, the membranes were loaded in HB7 with 2 \(\mu\)M FPIX as above and samples (0.01 mg protein) were incubated in 1 ml HB7 containing 3 \(\mu\)M \(^{3}H\)CQ and 10 \(\mu\)M nonradioactive CQ for 10 min at 37°C. The reaction was terminated by centrifugation and samples were processed as above. Nonspecific CQ binding was estimated from parallel incubation of FPIX-free membranes and subtracted from the total binding for each CQ concentration. Binding affinity was estimated by a computer fit of the data to the M i cancelis-M ent equation. The displacement of preaccumulated \(^{3}H\)CQ from parasite debris by EIPA was measured as described previously (Bray et al., 1998). Before lysis, erythrocytes infected with the CQS (HB3 strain) were loaded with 50 mM \(^{3}H\)CQ in complete medium for 30 min at 37°C.

**Cell-free Assay for the Generation of CQ Binding Sites from Hemoglobin**

Pure isolated food vacuoles were prepared by modifying the methods of Goldberg et al. (1990) and Saliba et al. (1998). Suspensions of synchronized trophozoites of the HB3 strain (∼15% parasitemia) were washed three times in PBS, pH 7.4. Each 5-ml sample of washed cell-pellet was resuspended in PBS containing 0.1% saponin, incubated for 5 min, and centrifuged (4,500 rpm for 5 min). The isolated trophozoites were washed repetitively in ice-cold PBS until the supernatant was clear. The trophozoite pellet was collected and resuspended in 10 vol of ice-cold trituration buffer (0.25 M sucrose, 10 mM sodium phosphate, 0.5% streptomycin sul fate, pH 7.1) and triturated three times on ice, using a 27-gauge 3/4 in needle. The suspension was centrifuged (13,000 rpm, 2 min, at 4°C), supernatant discarded, and the pellet resuspended in 5 vol of buffer (2 mM magnesium sulfate, 100 mM potassium chloride, 10 mM sodium chloride, 25 mM Hepes, 25 mM sodium bicarbonate, 5 mM sodium phosphate, pH 7.1). To each 1 ml of the suspension 20 \(\mu\)l of 5 mg/ml D Nase I was added and the suspension was incubated at 25°C for 5 min, followed by centrifugation (13,000 rpm, 2 min, at 4°C). The supernatant was discarded and the pellet was resuspended in 5 vol of ice-cold trituration buffer and triturated once. The suspension was layered on top of 7 ml of ice-cold 42% Percoll solution containing 0.25 M sucrose, 1.5 mM magnesium chloride, pH 7.2, and centrifuged (12,000 rpm, 30 min, at 4°C). A minor centrifugation, the purified food vacuoles were harvested from the bottom of the gradient and washed three times in ice-cold buffer (0.25 M sucrose, 10 mM sodium phosphate, 1.5 mM magnesium chloride). Purity was checked by electron microscopy and by using assays of host cell and parasite cytosol marker enzymes as described previously (Saliba et al., 1998). Electron microscopy revealed no contamination with other organelles or membranes and ∼50% of the vacuoles had intact membranes (data not shown). Contamination with the parasite cytosolic enzyme lactate dehydrogenase was <0.7%. Compared with isolated trophozoites and contamination with host cell acetylcholine esterase was below the limits of detection in the assay.
Pellets of pure food vacuoles were added to 20 vol of ice-cold 500 mM sodium acetate, pH 5, and immediately subjected to five cycles of rapid freezing in liquid nitrogen and thawing at room temperature. The suspension was triturated 10 times with a 27-gauge needle and centrifuged (13,000 rpm, 3 min, 4°C). The proteinase-rich supernatant was collected and used for the cell-free assay. Protein content was measured using the bicinchoninic acid assay (Smith et al., 1985). Samples of the enzyme extract (2–10 μl) were added to 200 μl of 500 mM sodium acetate, pH 5, containing [3H]CQ or [3H]JA Q. 100 μl of a 50–μM solution of human hemoglobin was added to the mixture. Purified erythrocyte ghost membranes (0.01 mg protein) were added to act as carriers for the CQ–FPIX complex. The samples were incubated for 1 h at 37°C in the absence or presence of 10 μM Ro 40-4388, or 20 μM Ro 61-7835, or 20 μM Ro 61-9379, or 20 μM leupeptin, or 20 μM E64. After incubation, the samples were centrifuged (14,000 rpm, 1 min) and the supernatant was collected gently with a drawn out glass Pasteur pipette to avoid disturbing the pellet. The pellet was washed once in ice-cold sodium acetate buffer without radiolabel and the remaining pellet was solubilized and processed for counting as described above.

**Measurement of Parasite Cytosolic pH**

Parasite cytosolic pH was estimated using BCECF-AM as described by Wünsch et al. (1998). The parasite suspension preloaded with BCECF was incubated at 37°C for 15 min in a perfusion chamber and the cells were allowed to settle on a glass coverslip coated with poly-L-lysine. The experimental chamber was transferred to the stage of an inverted Diaphot microscope (Nikon). At appropriate time points, 10 μM Ro 40-4388, or 20 μM Ro 61-7835, or 20 μM Ro 61-9379, or 20 μM leupeptin, or 20 μM E64. After incubation, the samples were centrifuged (14,000 rpm, 1 min) and the supernatant was removed gently with a drawn out glass Pasteur pipette to avoid disturbing the pellet. The final piece of experimental evidence that proves the central role of FPIX binding in the uptake of CQ was obtained using a cell-free system. The binding characteristics of intact CQS parasites can be reconstituted using an erythrocyte ghost membrane preparation preloaded with FPIX. The capacity (Bmax) of saturable CQ binding to these membranes was dependent on the FPIX concentration, although at higher loading concentrations the apparent binding affinity was reduced due to aggregation of FPIX (data not shown). We measured an affinity (Kd) for this interaction of 25 nM (Fig. 3A). This value is the same as the apparent Kd of saturable CQ uptake in CQS P. falciparum, again indicating that FPIX binding is all that is required to account for the saturable uptake of CQ (Bray et al., 1998 and see below). Other experiments indicate that CQ binding sites can be generated from native hemoglobin by an extract from purified food vacuoles (Fig. 3B). These data show that the concentration of FPIX is reduced when hemoglobin digestion is blocked. The inhibition was much more pronounced with ALLM, ALLN, and Ro 40-4388 than with leupeptin and was found to be reversible. Hemoglobin production resumed at the normal rate when the proteinase inhibitors were removed by washing (Fig. 2). All these results are in agreement with the effect of the same inhibitors on CQ uptake (Fig. 1, A and B) and provide compelling evidence that CQ uptake results from binding of the drug to FPIX generated inside the infected erythrocyte.

**Results**

**Saturable CQ Uptake in P. falciparum Is Driven by Binding to FPIX**

We determined the effect of a range of proteinase inhibitors on CQ uptake. In addition to Ro 40-4388, two other specific plasmpsin inhibitors, Ro 61-7835 and R 61-9379, inhibited CQ uptake at low micromolar concentrations (Fig. 1A). Similar inhibition was observed with the tripeptide aldehydes, ALLM and ALLN, which are known inhibitors of the processing of proplasmepsins I and II into active mature enzymes (Francis et al., 1997a). The other cysteine proteinase inhibitors tested, E64 and leupeptin, were much less effective. Inhibition of proplasmepsin processing by the tripeptide aldehydes and the direct inhibition of the plasmpsin with Ro 40-4388 are reversible (Francis et al., 1997a; Berry, C., personal communication).

We have established that the inhibition of CQ uptake by both classes of compounds is also reversible, as predicted by our hypothesis (Fig. 1, B and C). In CQS parasites, CQ uptake is inhibited almost completely by the proteinase inhibitors (Fig. 1, A and B). In CQR parasites, the effect was smaller but the inhibition of the resistance-reversing effect of verapamil was spectacular (Fig. 1C). This observation is in agreement with our hypothesis that verapamil increases CQ uptake by increasing the affinity of CQ–FPIX binding (Bray et al., 1998). Our data show that CQ–FPIX binding determines the amount of drug that is taken up by the parasite. The marked antagonism of CQ activity in the presence of Ro 40-4388 (Fig. 1D; Moon et al., 1997) suggests that binding to FPIX also determines the antimarial activity of CQ.

Our model of the mechanism of CQ uptake is supported by the demonstration that proteinase inhibitors efficiently inhibit the degradation of hemoglobin and liberation of FPIX under the same conditions used to inhibit CQ uptake (Fig. 2). Hemoglobin digestion is almost completely inhibited by ALLM, ALLN, and Ro 40-4388. On the other hand, the effect of leupeptin was smaller. It is very difficult to accurately measure the concentration of free FPIX in parasites, so we have measured the concentration of hemoglobin (polymerized FPIX). At the concentrations used, none of the compounds had any direct effect on the polymerization of FPIX in vitro (data not shown). However, all four compounds inhibited the production of hemoglobin by intact parasites to some extent (Fig. 2). These data show that the concentration of FPIX is reduced when hemoglobin digestion is blocked. The inhibition was much more pronounced with ALLM, ALLN, and Ro 40-4388 than with leupeptin and was found to be reversible. Hemoglobin production resumed at the normal rate when the proteinase inhibitors were removed by washing (Fig. 2). All these results are in agreement with the effect of the same inhibitors on CQ uptake (Fig. 1, A and B) and provide compelling evidence that CQ uptake results from binding of the drug to FPIX generated inside the infected erythrocyte. The final piece of experimental evidence that proves the central role of FPIX binding in the uptake of CQ was obtained using a cell-free system. The binding characteristics of intact CQS parasites can be reconstituted using an erythrocyte ghost membrane preparation preloaded with FPIX. The capacity (Bmax) of saturable CQ binding to these membranes was dependent on the FPIX concentration, although at higher loading concentrations the apparent binding affinity was reduced due to aggregation of FPIX (data not shown). We measured an affinity (Kd) for this interaction of 25 nM (Fig. 3A). This value is the same as the apparent Kd of saturable CQ uptake in CQS P. falciparum, again indicating that FPIX binding is all that is required to account for the saturable uptake of CQ (Bray et al., 1998 and see below). Other experiments indicate that CQ binding sites can be generated from native hemoglobin by an extract from purified food vacuoles (Fig. 3B). There is little doubt that CQ binding is absent when either hemoglobin or vacuole extract is omitted, or when the extract has been heat-treated (data not shown). The same proteinase inhibitors that block the uptake of CQ into intact cells block CQ binding in the cell-free system (Fig. 3B). In fact, the percent inhibition of CQ binding in the cell-free system is almost perfectly correlated with the inhibition of CQ uptake into intact cells by similar concentrations of these compounds (R2 = 0.99, P < 0.001). A Q binding sites can be generated in a similar fashion and have a similar in-

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These data suggest that the mechanism of AQ uptake is the same as that of CQ, i.e., the uptake of both drugs is driven by their binding to FPIX.

The initial rate of uptake of CQ into CQS parasites is rapid, prompting some authors to suggest that CQ is actively transported (Warhurst, 1986; Ferrari and Cutler, 1991; Sanchez et al., 1997). However, our data suggest that the initial rate of CQ uptake is determined by the intraparasitic binding of CQ to FPIX rather than active transport. We found that after only a 5-min exposure of H B3 (CQS) parasites to CQ, the number of CQ binding sites ($B_{max}$) was reduced from 17.2 μmol/liter to 6.9 μmol/liter by the specific hemoglobinase inhibitor Ro 40-4388 (Fig. 4).
The Uptake of CQ Is Independent of the Activity of the NHE

In contrast to our explanation, others believe that CQ is transported through the NHE of CQS parasites (Sanchez et al., 1997; Wünsch et al., 1998). The NHE of CQR isolates was activated constitutively, incapable of CQ transport, and responsible for an elevated cytoplasmic pH (Wünsch et al., 1998). We were not able to reproduce these observations. In our hands, cytosolic pH is subject to a greater degree of variation and is actually lower in the resistant isolate (HB3, pH 7.183 vs. pH 6.902). We found that the binding of CQ to FPIX in ghost membranes is sensitive to buffer pH, but this effect is probably not significant across the range of reported cytosolic pHs (Fig. 5A). We have also tested the effect of hemoglobinase inhibition on cytosolic pH although such an effect seems unlikely. We found that concentrations of Ro 40-4388, sufficient to block CQ uptake, had no effect on cytosolic pH, indicating that blocking hemoglobin degradation does not affect the cytosolic pH and that this compound does not block CQ uptake by interacting with the parasite NHE (Fig. 5B).

It is proposed that CQ is driven through the NHE of CQS parasites in a rapid burst of sodium-proton exchange stimulated by CQ itself (Wünsch et al., 1998). We tested this proposal directly by replacing the sodium ions in the media with nonexchangeable cations and monitoring the uptake of CQ into isolated parasites. This maneuver resulted in a marked inhibition of the NHE of isolated parasites, as evidenced by the inability to recover from an acid load (0.76 pH unit ± SEM 0.045, n = 6). It follows that sodium-free buffer should produce a very marked effect on CQ uptake if the NHE is involved in this process. It was observed that the initial rate of CQ uptake and steady-state CQ accumulation into isolated CQS parasites (HB3 strain) remained constant regardless of the concentration of sodium ions in the buffer (Fig. 6A and B). These data demonstrate that CQ uptake is independent of the parasite’s NHE activity status. The reported ability of CQ to stimulate the NHE of CQS parasites (Wünsch et al., 1998) is interesting but it appears to have no involvement in the mechanism of CQ uptake.

We also tested the effect of the sodium ion concentration on the uptake of CQ into isolated CQR parasites (K1 strain). The results, presented in Fig. 6C, again show that CQ uptake is unchanged when sodium ions are removed from the buffer. In addition, the reversal of CQ resistance by verapamil was independent of NHE activity, as it is retained in sodium-free medium (Fig. 6C). CQ accumulation of CQR parasites is reduced by a similar ratio compared with CQS parasites regardless of the sodium ion concentration. Moreover, it is independent of the presence or absence of the host cell (Fig. 6, A and C; Bray et al., 1998). Therefore, all of the phenotypic features of CQ uptake that distinguish CQR parasites from CQS parasites are retained in sodium-free medium thereby providing compelling evidence that the parasite NHE is not involved in CQ resistance.

Amiloride Derivatives Bind to FPIX and Displace CQ

EIPA significantly inhibits the uptake of CQ even though there are no sodium ions in the buffer and NHE is inactive (Fig. 6, A and B). These results are interesting because they indicate that EIPA inhibits CQ uptake by a mechanism distinct from the inhibition of NHE. One possibility is that EIPA inhibits the binding of CQ inside the parasite. This assessment is supported by the data presented in Fig. 7, showing EIPA displacing prebound CQ from parasite debris in a concentration-dependent manner. This interaction is not due to displacement of CQ bound to the NHE.
protein as this could only account for ~5% of the measured CQ binding (calculated from binding data given in Sanchez et al., 1997).

A miloride and its derivatives were shown to competitively inhibit the saturable uptake of CQ (Wünsch et al., 1998). Since we attribute saturable uptake of CQ to CQ–FPIX binding, we have looked for an interaction of these compounds with FPIX. Such an interaction is indicated by the data in Fig. 8, showing amilorides inhibiting FPIX polymerization. Indeed, HMA is almost as efficient an inhibitor of FPIX polymerization as CQ itself. These data suggest that amiloride derivatives might inhibit the uptake of CQ into P. falciparum by preventing CQ–FPIX binding. We tested this possibility directly and demonstrated that these compounds inhibit CQ–FPIX binding in loaded ghost membranes in a dose-dependent manner (Fig. 9). Interestingly, the 50% inhibitory concentration for EIPA was within the range reported for 50% inhibition of CQ uptake into P. falciparum by EIPA (Sanchez et al., 1997). Furthermore, the rank order of inhibition of CQ–FPIX binding by the amiloride analogues (HMA > EIPA > IBMA > amiloride) is the same as the rank order of their inhibition of CQ uptake into intact parasitized erythrocytes (Wünsch et al., 1998). These data indicate that amiloride analogues do inhibit CQ uptake by blocking CQ–FPIX binding rather than inhibiting the parasite NHE. We also hypothesized that verapamil reverses CQ resistance by increasing the apparent affinity of CQ binding to FPIX (Bray et al., 1998). If our working model is correct and if EIPA binds to FPIX at the expense of CQ, then EIPA should ablate the verapamil effect; this is clearly demonstrated in Fig. 6C.

Lysosomotropic Compounds Selectively Increase the Apparent Affinity of CQ–FPIX Binding in CQR Isolates

Although CQ is not actively transported through NHE
(Fig. 6, A–C) it is still possible that CQ resistance may arise from changes in the cytosolic pH (Martiney et al., 1995). Accordingly, we examined the effect of various agents known to perturb intracellular pH on the uptake of CQ by CQS and CQR parasites. We found that ammonium chloride, methylamine, a variety of alkylamines, and the carboxylic ionophores, monensin and nigericin, selectively increase the CQ accumulation of CQR parasites. The maximum effect was found at concentrations of 1–2 mM for amines and 100 nM for ionophores. In contrast, CQ accumulation by CQS parasites was either unaffected or reduced (Fig. 10 A). In this respect, the amines and ionophores mimic the effect of verapamil (Krogstad et al., 1987). A ammonium chloride (Fig. 10 B) and methylamine were also capable of reversing CQ resistance in the same way as verapamil (although the effect was not as pronounced). The remaining weak bases could not be assessed for their resistance reversal potential because of their inherent cytotoxicity.

Ammonium chloride, methylamine, and verapamil were selected to investigate the possibility that resistance reversers alter cytosolic pH of CQR parasites. We found that these compounds had no significant effect on cytosolic pH at the low concentrations required to reverse resistance (data not shown). Therefore, the ability of these compounds to increase CQ accumulation is unlikely caused by any interaction with cytosolic pH regulators like NHE. Indeed, any possible contribution of the NHE in the process of resistance reversal is negated by the data showing that ammonium chloride, monensin, and verapamil all increase the apparent affinity of CQ binding to FPIX in sodium-free medium, when the NHE is inactivated (Fig. 11, A–D).

We were also interested to see if any other cytosolic pH regulators could influence CQ uptake. In common with other eukaryotic cells, it is possible that P. falciparum parasites possess a chloride/bicarbonate exchange mechanism (Bosia et al., 1993). If so, and if this protein can influence CQ uptake as suggested (Martiney et al., 1995), then CQ uptake should be influenced by the bicarbonate concentration of the medium. It was found that CQ uptake into CQS and CQR parasites was not effected by the concentration of bicarbonate in the medium (Fig. 12). Taken to-
together, our data suggest that none of the major cytosolic pH regulators play a major role in the uptake of CQ or in the mechanism of CQ resistance.

**Discussion**

The specificity of antimalarial activity of CQ stems from the potential of malaria parasites to accumulate more CQ than any other type of eukaryotic cell. CQR parasites of some species, including P. falciparum, have evolved ways to reduce the extent of CQ accumulation. A better understanding of the mechanism of CQ uptake in P. falciparum would be a significant step forward since it should explain the specificity of the drug and may also offer clues to the basis of resistance. CQ undoubtedly penetrates the parasite by passive diffusion as observed in other eukaryotic cells (MacIntyre and Cutler, 1993). CQ is a diprotic weak base and, as such, undoubtedly accumulates to some extent inside the acidic compartments of the parasite by a proton trapping mechanism (Yayon et al., 1985). This mechanism may account for a considerable concentration of CQ inside cells (Geary et al., 1986). However, it probably does not account for the full extent of CQ accumulation by P. falciparum, which is notably greater than CQ accumulation by other eukaryotic cells with large acidic compartments (MacIntyre and Cutler, 1993). Pioneering studies by Fitch in the 1970s demonstrated that CQ uptake into P. falciparum is saturable, energy-dependent, and possibly inhibited by various compounds (Fitch, 1970; Fitch et al., 1974). These studies suggest that malaria parasites possess an additional CQ concentrating mechanism acting in concert with passive diffusion and proton trapping. Two putative CQ concentrating mechanisms are currently under consideration: active CQ import (Warhurst, 1986) mediated by the parasite NHE (Sanchez et al., 1997; Wünsch et al., 1998); and intracellular binding of CQ to FPIX (Chou et al., 1980; Bray et al., 1998).

The NHE hypothesis initially appears plausible and many of the supporting data are robust. However, a close scrutiny of the available literature reveals some intriguing inconsistencies that call into question the mechanistic explanation offered by Wünsch et al. (1998). Particularly problematic is the uptake of AQ, a close structural analogue of CQ. Early studies showed that AQ competitively inhibits the uptake of CQ in P. falciparum, suggesting that the same mechanism drives the uptake of both drugs.

Figure 6. The uptake of CQ into isolated parasites is not affected by the buffer Na+ concentration. Cell suspensions were incubated with 50 nM [3H]CQ at 37°C and the amount of CQ taken up was determined at the indicated time points. (A) Initial velocity of CQ uptake into isolated CQS (HB3) parasites in buffer containing sodium (open circle), or choline (closed circle), or glucamine (open square), or choline plus 100 μM EIPA (closed triangle). Data points are means of duplicate observations. (B) Uptake of [3H]CQ into isolated CQS (HB3) parasites after 30 min at varying ratios of sodium ions to choline ions in the absence (light shading) and presence (dark shading) of 100 μM EIPA. Data are means ± SD from five experiments, each performed in duplicate. (C) Uptake of [3H]CQ into isolated CQR (K1) parasites after 30 min in the absence (light shading) and presence (dark shading) of 10 μM verapamil in buffer containing either sodium, choline, glucamine, or choline plus 100 μM EIPA. Data are means ± SD from five experiments, each performed in duplicate.
We reported that the uptake of AQ into CQR parasites is equivalent to the uptake of CQ into CQS parasites (Bray et al., 1996). It is hard to see how the extensive uptake of AQ into CQR parasites can be driven by an NHE that apparently is activated constitutively and incapable of drug transport (Wünsch et al., 1998).

A further difficulty is encountered when the effect of verapamil is considered. Many studies have highlighted the ability of verapamil to selectively increase the uptake of CQ into CQR parasites (Krogstad et al., 1987; Wellems et al., 1990; Bray et al., 1992; Walter et al., 1993; Bray et al., 1996). Furthermore, this property of verapamil was linked perfectly to CQ resistance in the progeny of a genetic cross of CQR and CQS clones (Wellems et al., 1990). If NHE is responsible for CQ uptake, then verapamil would somehow have to selectively stimulate NHE of resistant parasites that is constitutively activated and incapable of CQ transport. However, this is an unlikely scenario, doubly so, when one considers the large quantity of literature demonstrating verapamil functioning to inhibit rather than stimulate drug transporters and ion channels (Gottesman and Pastan, 1993; Bray and Ward, 1998). The active transport model is inconsistent with our own data showing a role for CQ–FPIX binding in the uptake of CQ, in verapamil-sensitive CQ resistance and in the uptake of AQ (Bray et al., 1998). We rigorously tested these two hypotheses and found that the FPIX binding model remains valid, whereas the altered NHE model failed.

We have tested the assertion that a rapid burst of sodium–proton exchange is required to drive uptake of CQ into CQS parasites. We used isolated parasites for these experiments and assumed that NHE activity is not impaired under such conditions. We show here as others have shown that parasitic NHE is fully functional after the parasite has been isolated from its host cell (Bosia et al., 1993). We found that replacing sodium ions in the buffer with nonexchangeable cations, such as choline or N-methyl-D-glucamine, inactivated NHE but had no effect on the amount of CQ taken up at steady-state by isolated parasites of CQ into CQR parasites (Krogstad et al., 1987; Wellems et al., 1990; Bray et al., 1992; Walter et al., 1993; Bray et al., 1996). Furthermore, this property of verapamil was linked perfectly to CQ resistance in the progeny of a genetic cross of CQR and CQS clones (Wellems et al., 1990). If NHE is responsible for CQ uptake, then verapamil would somehow have to selectively stimulate NHE of resistant parasites that is constitutively activated and incapable of CQ transport. However, this is an unlikely scenario, doubly so, when one considers the large quantity of literature demonstrating verapamil functioning to inhibit rather than stimulate drug transporters and ion channels (Gottesman and Pastan, 1993; Bray and Ward, 1998). The active transport model is inconsistent with our own data showing a role for CQ–FPIX binding in the uptake of CQ, in verapamil-sensitive CQ resistance and in the uptake of AQ (Bray et al., 1998). We rigorously tested these two hypotheses and found that the FPIX binding model remains valid, whereas the altered NHE model failed.

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CQS parasites (Fig. 6 B). Moreover, sodium-free buffer had no effect on the initial velocity of CQ uptake into CQS parasites (Fig. 6 A). This was measured over the first 5 min after the addition of CQ, which coincides with the reported period of maximal stimulated NHE activity (Wünsch et al., 1998). Since there can be no rapid burst of sodium–proton exchange in sodium-free buffer and CQ uptake is not altered by these conditions, our results strongly suggest that any ability of CQ to stimulate the NHE of CQS parasites (Wünsch et al., 1998) is unrelated to the mechanism of CQ uptake. In addition, we found that stimulation of CQ uptake into CQR parasites, produced by verapamil and other resistance reversers, was unrelated to the activity of the NHE because these effects are retained in sodium-free buffer (Figs. 6 C and 11, A – D). Consequently, the effect of verapamil on the uptake of CQ cannot be attributed to modulation of NHE activity via the calcium-calmodulin regulatory pathway, as postulated by Sanchez et al. (1997). Hence, the clone-specific phenotypic characteristics of CQ uptake into CQS and CQR parasites are retained in conditions which completely inactivate the NHE (Figs. 6, A – C, and 11, A – D). Therefore, it is difficult to see how these characteristics can be related in any way to the activity of the parasite NHE.

We have demonstrated that amiloride analogue inhibition of CQ uptake occurs because of inhibition of CQ–FPIX binding by these compounds, rather than the inhibition of the parasite NHE. In a critical series of experiments, we were able to demonstrate that the inhibition of CQ uptake by EIPA was distinct from any activity of this drug against NHE. CQ uptake is undiminished in sodium-free buffer when NHE is inactive, yet it is effectively inhibited when EIPA is present in this buffer (Fig. 6, A – C). These data strongly suggest that blocking CQ uptake by EIPA is caused by inhibition of a process that does not require sodium–proton exchange for CQ uptake. This directly violates the fundamental requirement of the NHE hypothesis.

There is solid experimental support for the FPIX model (Balasubramian et al., 1984; Sullivan et al., 1996; Bray et al., 1998). Therefore, the demonstration that EIPA displaces prebound CQ from parasite debris (Fig. 7), binds to FPIX (Fig. 8), and inhibits the binding of CQ to FPIX-loaded ghost membranes (Fig. 9) provides direct evidence that EIPA inhibits CQ uptake by binding to FPIX. Furthermore, the rank order of activity of the amiloride analogues (HMA > EIPA > IBMA > amiloride) is the same for the inhibition of CQ–FPIX binding (Figs. 8 and 9) as it is for the inhibition of CQ uptake (Wünsch et al., 1998). Examination of the chemical structure of amiloride reveals that either of the two terminal amino groups of the guanidine function has the poten-
tial to coordinate with the iron center of the porphyrin as an axial ligand (Rocha Gonslaves et al., 1991). Hoe 370, a specific NHE inhibitor that is structurally unrelated to the amiloride analogues, also has been shown to inhibit CQ uptake (Wünsch et al., 1998). Note, this compound also contains a guanidine function and may also bind to FPIX. Inhibition of CQ uptake by these specific NHE inhibitors provided the best evidence of active import of CQ through the NHE. However, in the light of the data reported here, we believe that this property of NHE inhibitors must now be considered to support the alternative theory that CQ uptake is governed by its binding to FPIX.

The central theme of the studies presented here is the definitive proof, after some thirty years of controversy, that saturable CQ uptake is driven by its binding to FPIX. We recently demonstrated that Ro 40-4388, a potent and specific inhibitor of the parasite proteolytic enzyme plasmepsin I (Moon et al., 1997), produces a concentration-dependent reduction in the number of CQ binding sites of intact parasitized erythrocytes (Bray et al., 1998). Here we have extended this observation blocking hemoglobin degradation by two distinct mechanisms. The plasmepsins are thought to initiate hemoglobin degradation by cutting the Phe33-Leu34 bond of the alpha chain. This unfolds the hemoglobin tetramer, allowing further proteolysis and the release of FPIX (Goldberg et al., 1991; Gluzman et al.,...
Perhaps the most convincing support for our hypothesis comes from the observation that a purified food vacuole extract can generate CQ binding sites in a cell-free system using human hemoglobin as a substrate (Fig. 3 B). Published estimates of the rate of hemoglobin digestion adequately defend this argument. It is estimated that each parasite digests 0.06 fmol of hemoglobin per hour (Goldberg et al., 1990). This would liberate 50 μmol FPIX per liter of parasites in 5 min, i.e., more than enough to account for initial bound CQ (17.2 μmol per liter in 5 min, Fig. 4) even at a stoichiometry of 2 FPIX:1 CQ. If so, this indicates that CQ uptake may limit itself by stopping hemoglobin digestion since the steady-state Bmax is only 30–40 μmol per liter (Bray et al., 1998). CQ uptake might be limited by CQ–FPIX inhibition of the hemoglobinase enzymes. It is possible that sufficient CQ–FPIX complex remains within the vacuole to inhibit the proteolytic enzymes (Vander Jagt et al., 1987; Gluzman et al., 1994).

To quote Chou et al. (1980), “Unequivocal identification of an isolated substance as a drug receptor requires (a) that affinities and specificities of binding of the drug match those of the receptor, (b) that the drug is ineffective when the putative receptor is absent from the organism, and (c) that drug effectiveness returns when the receptor is reintroduced into the organism.” The specificity of hemoglobinase inhibitors that inhibit cellular CQ uptake is identical to their specificity in the cell-free enzymatic CQ binding assay (Figs. 1 A and 3 B). These drugs are specific and reversible inhibitors of FPIX generation (Fig. 2). This biochemical knockout has permitted us to show that FPIX can be reversibly removed, causing a reversible inhibition of drug uptake (Figs. 1 B and 2). In addition to governing the uptake of the drug, CQ–FPIX binding determines antimalarial activity since the combination of CQ and hemoglobinase inhibitors is markedly antagonistic (Fig. 3 C; Moon et al., 1997). Furthermore, we were able to match the affinity of binding of CQ to CQS parasites to the affinity of CQ–FPIX binding in a parasite-free system (Fig. 3 A). Thus, all of the above criteria have been satisfied and identify FPIX as the CQ receptor in P. falciparum.

Our data indicate that NHE has no involvement in the mechanism of CQ resistance (Figs. 6 and 11). Indeed, any involvement of cytosolic pH regulation seems unlikely. Instead, our data suggest that CQ resistance stems from an alteration in the local environment of FPIX generation in acid vesicles. We found that a wide range of lysosomotropic compounds mimics the effects of verapamil (Fig. 10 A). This could indicate the inhibition of a drug transporter similar to P-glycoprotein. However, since many of these compounds are not known to interact with P-glycoprotein, we suggest an alternative mode of resistance reversal. The concentrations required to reverse resistance produced no alkalinization of the parasite cytosol but might be expected to produce a significant alkalinization of lysosomes and endosomes (Millot et al., 1998). There is evidence in the literature that hemoglobin digestion begins in hemoglobin delivery vesicles, before they fuse with the food vacuole (Slomianny and Prensier, 1990). To protect the parasite, the resistance mechanism must be operational throughout the endocytic pathway. The intracellular localization of CQ resistance gene CG2 throughout the endocytic pathway is certainly consistent with this hypothesis (Su et al., 1997). It is our belief that CQ resistance results from a selective change in vesicular function within rel-
evant hemoglobin processing acidic compartments. This reduces the affinity of CQ–FP IX binding that can be reversed by lysosomotropic agents. CG 2 and related pro-

cells could potentially alter the binding of CQ to FP IX by directly binding to FP IX or by altering vesicular pH or buffering capacity. A ll of these mechanisms could be mod-

ulated by vesicle alkalization and are currently under in-

vestigation in our laboratory.

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