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Letters to the Editor

Mutations Conferring Drug Resistance in Malaria Parasite Drug Transporters Pgh1 and PfCRT Do Not Affect Steady-State Vacuolar Ca^{2+}

Resistance to chloroquine (CQ) by the malaria parasite *Plasmodium falciparum* has been observed in every region where *P. falciparum* occurs (20). The exact mode of action of CQ has not been fully elucidated, but it is generally accepted that a crucial step in this process is the binding of the drug to ferriprotoporphyrin IX (heme), a by-product of hemoglobin degradation which occurs in the parasite digestive food vacuole (DV).

A number of studies have contributed to pinpointing the *pfcrt* gene as the major determinant of CQ resistance (1, 3, 4, 6, 14, 15, 17–19). In addition, mutations of the *pfmdr1* gene (expressing Pgh1) have been shown to modulate the level of CQ resistance (11), as well as being partially responsible for the acquired resistance to other drugs such as mefloquine (10). Currently there are two hypotheses as to the function of PfCRT in CQ resistance. The first of these proposes that PfCRT actively removes CQ from the DV, either as an ATP-dependent pump or as a secondary active transporter (12, 13). Alternatively, the "charged drug leak model" proposes that diprotonated CQ (CQ++) leaves the DV via mutated PfCRT passively down its concentration gradient (5). Both theories are in agreement, however, that CQ is transported out of the DV and that this is the key mechanism of CQ resistance.

Little is known of the functional role of the PfCRT transporter in *P. falciparum* physiology. PfCRT is localized to the DV membrane (4), and bioinformatic studies indicate that PfCRT is a member of the drug/metabolite transporter superfamily (7, 16), other members of which are known to transport a variety of substrates, including amino acids, weak bases, and organic cations. Studies which have heterologously expressed PfCRT into yeast (*Pichia pastoris*) (21) and *Xenopus* oocytes (9) have suggested that PfCRT is able to modulate host transport systems. In yeast, PfCRT is reported to function in the passive movement of Cl^- (21), while in the *Xenopus* system, PfCRT-expressing oocytes exhibit a depolarized membrane potential (Ψ_m) and a higher intracellular pH (pH_i) compared to control oocytes (9). One possibility is that PfCRT interferes with second messengers such as Ca^{2+} (9). In addition, Ca^{2+} channel blockers such as verapamil are believed to block PfCRT, resulting in a chemosensitization of CQ-resistant parasites (for examples, see references 5 and 8). Given that the DV of *P. falciparum* has been shown to contain elevated levels of free Ca^{2+} relative to the cytosol (2), this study was undertaken to determine whether PfCRT has a role in DV Ca^{2+} homeostasis.

The resting concentrations of free DV Ca^{2+} ($[Ca^{2+}]_{DV}$) from a number of *P. falciparum* isolates and allelically exchanged *pfcrt* and *pfmdr1* strains (14) were measured. Measurements were carried out using confocal laser-scanning single-cell imaging as described before (2), with the exception that Fluo 5AM was used in preference to Fluo 4AM due to its higher K_d for Ca^{2+}. In addition, in situ $[Ca^{2+}]_{DV}$ calibrations were carried out on erythrocyte-free parasites, as nigericin induces parasites to exit from their host cell. Results shown in Table 1 show that the measured $[Ca^{2+}]_{DV}$ of all the strains was in the region of 2 μM. These values are a little higher than those reported previously (~0.4 μM) and probably reflect the higher external $[Ca^{2+}]$ experienced by free parasites (in RPMI medium) compared to the lower $[Ca^{2+}]$ experienced by the intraerythrocytic parasites measured previously (2). There was, however, no correlation between the steady-state $[Ca^{2+}]_{DV}$ values and the CQ sensitivity status of the various *Plasmodium* strains.

We conclude that mutations in *pfcrt* or *pfmdr1*, conferring drug resistance, do not affect the $[Ca^{2+}]_{DV}$. We further propose that it is unlikely therefore that the functional role of PfCRT is connected to Ca^{2+} homeostasis, unless redundancy in the pathways maintaining DV Ca^{2+} homeostasis masked any effect on this process conferred by PfCRT.

TABLE 1. Resting vacuolar Ca^{2+} concentration of *P. falciparum* strains with various chloroquine sensitivities

Strain[a]	CQ 50% inhibitory concn (nM)[b]	Verapamil effect[c]	Resting vacuolar $[Ca^{2+}]$ (μM)[b]
TM6	93 ± 7	Yes	1.68 ± 0.97
3D7	8 ± 2	No	2.34 ± 0.35
$C3^{Dd2}$	50 ± 3	Yes	1.84 ± 0.71
$C2^{GCO3}$	10 ± 3	No	1.93 ± 0.28
$D10^{D10}$	23 ± 2	No	1.95 ± 0.10
$D10^{7G8}$	16 ± 2	No	2.12 ± 0.60

[a] TM6 is a laboratory-adapted CQ-resistant strain; 3D7 is a laboratory-adapted CQ-sensitive strain. In clone $C3^{dd2}$, the *pfcrt* allele of a CQ-sensitive clone (GCO3) has been replaced by the *pfcrt* allele of the CQ-resistant clone Dd2 (14). In clone $C2^{GCO3}$, the sensitive *pfcrt* allele from a CQ-sensitive clone has been replaced by another CQ-sensitive *pfcrt* allele (14) (as a control for any basal effect of *pfcrt* allelic exchange). In clone $D10^{D10}$, the *pfmdr* allele from a CQ-sensitive isolate (D10) was replaced by the *pfmdr* allele from the same CQ-sensitive clone (11). In clone $D10^{7G8}$, the *pfmdr* allele from a CQ-sensitive clone (D10) was replaced with a *pfmdr* allele from a CQ-resistant clone (7G8) (11).

[b] Values represent means ± standard error from at least three independent measurements.

[c] Ability of verapamil (5 μM) to chemosensitize the parasite clone to CQ (8).

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