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ScienceDirect
Molecular & Biochemical Parasitology 153 (2007) 48–58
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Plasmodium falciparum Na^+/H^+ exchanger activity and quinine resistance☆

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Received 16 November 2006; received in revised form 29 January 2007; accepted 31 January 2007
Available online 8 February 2007

Abstract

Mutations in the *Plasmodium falciparum pfcrt* gene cause resistance to the 4-amino quinoline chloroquine (CQ) and other antimalarial drugs. Mutations and/or overexpression of a *P. falciparum* multidrug resistance gene homologue (*pfmdr1*) may further modify or tailor the degree of quinoline drug resistance. Recently [Ferdig MT, Cooper RA, Mu JB, et al. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 2004;52:985–97] QTL analysis further implicated a region of *P. falciparum* chromosome 13 as a partner (with *pfcrt*) in conferring resistance to the first quinoline-based antimalarial drug, quinine (QN). Since QN resistance (QNR) and CQR are often (but not always) observed together in parasite strains, since elevated cytosolic pH is frequently (but not always) found in CQR parasites, and since the chr 13 segment linked to QNR prominently harbors a gene encoding what appears to be a *P. falciparum* Na^+/H^+ exchanger (PfNHE), we have systematically measured cytosolic pH and PfNHE activity for an extended series of parasite strains used in the QTL analysis. Altered PfNHE activity does not correlate with CQR as previously proposed, but significantly elevated PfNHE activity is found for strains with high levels of QNR, regardless their CQR status. We propose that either an elevated pH_{cyt} or a higher vacuolar pH-to-cytosolic pH gradient contributes to one common route to malarial QNR that is also characterized by recently defined chr 13–chr 9 pairwise interactions. Based on sequence analysis we propose a model whereby observed polymorphisms in PfNHE may lead to altered Na^+/H^+ set point regulation in QNR parasites.
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Keywords: Malaria; PfNHE; Quinine resistance; *P. falciparum*

Abbreviations: CQ, chloroquine; MQ, mefloquine; AQ, amodiaquine; QN, quinine; QD, quinidine; CQR, chloroquine resistance (or resistant); PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; MDR, multidrug resistance; QR, quinoline resistance; Pgp, P-glycoprotein; Pgh-1, Pgp homologue 1; PfMDR1, *Plasmodium falciparum* multidrug resistance protein; QNR, quinine resistance (or resistant); chr, chromosome; NHE, sodium proton exchange; pH_{DV}, steady-state digestive vacuolar pH; pH_{cyt}, steady-state cytosolic pH; DV, digestive vacuole; QTL, quantitative trait loci; PfNHE, *P. falciparum* sodium-proton exchanger; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; SCP, single cell photometry; ROI, region of interest; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; iRBC, infected red blood cell; LSCM, laser scanning confocal microscopy; SDCM, spinning disk confocal microscopy; ps, parasitophorous space; NMDG, *N*-methyl-D-glucosamine; EIPA, ethyl isopropyl amiloride

☆ Supported by NIH grants AI56312 and AI45759 to PDR.
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doi:10.1016/j.molbiopara.2007.01.018

1. Introduction

Antimalarial drug resistance greatly impairs control of malaria [1,2]. Historically, quinolines such as chloroquine (CQ), mefloquine (MQ), amodiaquine (AQ) and the isomers quinine (QN) and quinidine (QD) have been the most widely used class of antimalarial drugs, with oral CQ being the preferred therapy. However, CQ resistance (CQR) in *Plasmodium falciparum* malaria now greatly limits use of the drug. CQR is caused by mutations in a digestive vacuolar membrane protein, PfCRT [3,4]. Various patterns of PfCRT mutations found around the globe may confer variable patterns of resistance to CQ and related quinoline-based drugs, but does not explain "multidrug resistance" (MDR) for many strains and isolates [5]. It is becoming increasingly clear that additional genetic events are required to create MDR malaria (meaning, simultaneous resistance to multiple pharmacophores), and perhaps even malaria resistant to multiple closely related quinolines ("quinoline resistance" or QR[1]). Due to widespread CQR, QN has reemerged as an important antimalarial drug. While the failure of QN to treat severe and complicated malaria *in vivo* is rare [6], evidence of reduced *P. falciparum* susceptibility to the drug *in vivo* as well as QNR concomitant with CQR *in vitro* are cause for concern [1,7–9].

One such additional genetic event may be mutation and/or over expression of the Pgp homologue PfMDRl [5], but the data are somewhat controversial. Regardless, based on available data such a contribution would not fully explain QR or MDR patterns in many strains. Another genetic event appears to involve one or more genes encoded by a segment of chr 13 which appears to be involved in promoting high level QNR via one of two possible genetically defined pathways [10]. This fragment contains a number of hypothetical genes encoding hypothetical proteins with unclear or no homology to known proteins, however, it also encodes homologues to a well known V type ATPase subunit and to the family of Na^+/H^+ exchanger (NHE) proteins. Perhaps not coincidentally, these proteins are involved in trans membrane pH regulation, and a number of changes in digestive vacuolar pH (pH_{DV}) and cytosolic pH (pH_{cyt}), as well as parameters linked to vacuolar pH such as vacuolar Ca^{2+} and volume, have been reported for QR or MDR malaria [11–14].

The QNR associated chr 13 locus was recently isolated [10] by subjecting the progeny of a *P. falciparum* genetic cross [15] to genome-wide scans for quantitative trait loci (QTL). In addition to finding additive QTLs on chromosomes 7 and 5 as expected (the identified fragments contain *pfcrt* and *pfmdr1*, respectively) these investigators found a strong (but not perfect) association between QNR and a fragment of chr 13. Pairwise effects were detected between this chr 13 fragment and an additional locus on chr 9. Of the potential genes found within chr 13, one encodes a putative Na^+/H^+ exchanger, *pfnhe-1*. The encoded PfNHE protein is 1920 aa in length with 12 predicted transmembrane segments and a signal peptide cleavage site [10,16]. It is the second largest eukaryotic NHE yet identified (surpassed only by a NHE for the related apicomplexan *T. gondii*), and has a number of additional unusual features. Polymorphisms that led to variable DNNND repeat units in the predicted PfNHE protein sequence were found in progeny of the Dd2 × HB3 cross as well as a range of field isolates and additional laboratory strains showing variable QNR.

Ginsburg and colleagues provided the first functional evidence for a NHE activity in *P. falciparum* and suggested that its primary role was the removal of the increased cytosolic acid burden caused by anaerobic glycolysis [17]. Prior to the availability of the *P. falciparum* genome (which facilitated identification of *pfnhe*) Lanzer and colleagues suggested that the *cg2* gene found close by *pfcrt* on Pf chr 7 encoded this NHE activity [18]. These workers suggested that mutations in *cg2* altered NHE activity and pH_{cyt} and caused CQR by a combination of effects including direct transport of CQ [19]. These conclusions were strongly disputed, since the encoded CG2 protein is actually peri vacuolar [20], not plasma membrane localized, since a different chr 7 gene (now called *pfcrt*) was subsequently found to cause CQR [3] and because the putative CG2-NHE homology [18] was based on sequence analysis that did not account for very high AT content in malarial genes [21].

Regardless, in our hands single cell photometry (SCP) analysis of pH_{cyt} for intra erythrocytic parasites under continuous physiologic perfusion did indeed show elevated pH_{cyt} for some CQR parasites [22], but not all [3,4]. A corollary suggested early on [4] is that the relative size of the net cytosolic-digestive vacuolar (DV) pH gradient might be a more important parameter for CQR or QR, rather than steady-state pH_{cyt} or pH_{DV} values alone. Overall, a range of pH phenomena and genetic changes consistent with perturbations in pH regulation have been associated with QR, but the precise contributions of pH_{cyt} to CQR versus QNR versus QR remain unclear [11–14,18–24].

In this study, we first optimize localization of the ratiometric pH probe BCECF exclusively to the parasite cytosol to avoid confusion related to operator dependent definition of ROIs. We use nigericin–BSA "pH clamping" techniques to set parasite pH_{cyt} at uniform values before quantifying NHE activity for Dd2/HB3 cross progeny with a range of chr 5, 6, 7, 9, and 13 inheritance patterns. We measure cytosolic buffering capacity at a range of pH_{cyt} using ammonium pulse, and quantify recovery of pH_{cyt} after acid pulse that is either Na^+-dependent or Na^+-independent. We find that elevated pH_{cyt} is well correlated with increased PfNHE activity and also associated with high level QNR due to chr 9, 13 loci (but not necessarily QNR associated with chr 5, 6 loci). We thus suggest there are at least two physiological signatures for QNR that segregate with the different genetic descriptions. We propose a biochemical model that

[1] As the list of drugs in MDR profiles have grown, and as the variety of different MDR genotypes and phenotypes for a given cell type or micro-organism has expanded enormously, the term "MDR" has become imprecise or confusing in most settings. Here, "MDR" means resistance to multiple antimalarials with distinctly different pharmacophore backbones (e.g. anti folates and quinolines), whereas "QR" means cross resistance to multiple quinolines (e.g. chloroquine, quinine, mefloquine, etc.). In malaria, MDR is clearly multigenic (e.g. antifolate and quinoline resistance requires mutations in both *pfdhfr* and *pfcrt* genes for *P. falciparum*, respectively). Surprisingly, different forms of QR also appear to be multigenic. The best data currently available suggest multigenic pathways to QR versus MDR differ, and therefore that the molecular mechanism(s) of QR versus MDR are likely not identical.

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