Evidence for Activation of Endogenous Transporters in Xenopus laevis Oocytes Expressing the Plasmodium falciparum Chloroquine Resistance Transporter, PfCRT*

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A large body of genetic, reverse genetic, and epidemiological data has linked chloroquine-resistant malaria to polymorphisms within a gene termed pfcrt in the human malarial parasite Plasmodium falciparum. To investigate the biological function of the chloroquine resistance transporter, PfCRT, as well as its role in chloroquine resistance, we functionally expressed this protein in Xenopus laevis oocytes. Our data show that PICRT-expressing oocytes exhibit a depolarized resting membrane potential and a higher intracellular pH compared with control oocytes. Pharmacological and electrophysiological studies link the higher intracellular pH to an enhanced amiloride-sensitive H+ extrusion and the low membrane potential to an activated nonselective cation conductance. The finding that both properties are independent of each other, together with the fact that they are endogenously present in X. laevis oocytes, supports a model in which PICRT activates transport systems. Our data suggest that PfCRT plays a role as a direct or indirect activator or modulator of other transporters.

Malaria remains one of the most important infectious diseases, causing an estimated 300–500 million clinical cases and 1–3 million deaths annually (1). The current situation is largely due to the spread of chloroquine-resistant Plasmodium falciparum strains and a lack of alternative drugs that can replace chloroquine as a first-line antimalarial drug. Chloroquine is believed to exert its antimalarial activity by interfering with endogenous heme detoxification pathways (2). During intraerythrocytic development, P. falciparum degrades the host cell hemoglobin in its acidic food vacuole, releasing large quantities of heme, which are detoxified, presumably through peroxidative degradation (3), glutathione-dependent decomposition (4), and/or crystallization to inert hemoxizin (5). Chloroquine prevents heme detoxification pathways by forming complexes with heme (6–9). The buildup of toxic membrane-associated heme-chloroquine complexes eventually destroys the integrity of the parasite’s membranes (10–14). The consensus of evidence indicates that chloroquine-resistant strains control access of chloroquine to its intracellular binding site heme (15, 16), through an active drug efflux carrier system (17, 18), novel heme-binding proteins (15, 16), or changes in vacuolar pH, which would affect heme detoxification processes (19–21) or acidotropic drug accumulation (22, 23).

Chloroquine resistance has been linked to polymorphisms within a gene termed pfcrt (for P. falciparum chloroquine resistance transporter) residing on chromosome 7 of the human malarial parasite P. falciparum (24–27). pfcrt encodes a protein of 421 amino acids with 10 predicted membrane-spanning domains located in the membrane of the parasite’s food vacuole (24). BLAST analysis has revealed no obvious homologies of PICRT to proteins of known function. However, one study has reported a vague structural resemblance of PICRT to aqueous chloride channels (28), whereas another study has suggested that PICRT belongs to the drug/metabolite transporter family (29).

Thus far, only one study has attempted to investigate the function of PICRT. Heterologous expression of PICRT fusion proteins in Pichia pastoris and the subsequent investigation of transport properties displayed by inside-out membrane vesicles suggested a function for PICRT in passive transmembrane Cl− movement (30). However, it is not clear from these data whether PICRT itself mediates Cl− movement, e.g., by functioning as a chloride channel, or whether the observed effects are of a secondary nature due to the activation of endogenous channels. Thus, there is very little information available regarding the biological function of PICRT during parasite development or regarding its role as a mediator of chloroquine resistance. A better understanding of the properties of PICRT would be an important step toward novel intervention strategies, which may either circumvent the chloroquine resistance mechanism or inactivate it, thereby revitalizing chloroquine as a first-line drug.

In search of an alternative, more amenable heterologous expression system to study the function of PICRT, we have expressed, in Xenopus laevis oocytes, the wild-type pfcrt allele from the chloroquine-sensitive P. falciparum clone HB3. Successful membrane expression of the protein was associated with changes in oocyte properties. Compared with control cells, PICRT-expressing oocytes exhibited a more alkaline intracellular pH and a less negative transmembrane potential difference. The alkaline intracellular pH reverted to control values in the presence of Na+-H+ exchanger inhibitors. The membrane potential hyperpolarized upon removal of extracellular...
Na⁺ or in the presence of diphenylamine-2-carboxylic acid. We conclude from our data that PCRT expression activates the endogenous Na⁺-H⁻ antiporter and an endogenous nonselective cation conductance, both present in the plasma membrane of *X. laevis* oocytes.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were purchased from Sigma, except Hoechst 694 ((3-methyl-sulfonfonyl-4-piperidinoenobenzoylguanidine methanesulfonate), which was a gift from Dr. H. J. Lang (Aventia). Biological Material—*X. laevis* adult females were purchased from CNRS (Montpellier, France) and NSK and kept as described (31). Adults were anesthetized by immersion in ice-cold water supplemented with 2 mM ethyl n-aminozoate methanesulfonate. Ovarian fragments were incubated in collagenase A-containing Ringer’s solution as described previously (32). After rinsing, stage V–VI oocytes were defolliculated using fine forceps.

Expression of *pfcrt* cRNA in *X. laevis* Oocytes—To obtain PCRT expression in *X. laevis* oocytes, the pCRT6 clone HB3 was reconstructed on the basis of the yeast codon usage (Geneart, Regensburg, Germany). The resulting fragment was subcloned into pGEM-3zf(+) and transfected into *E. coli*. Subsequent in vitro transcription was performed using a MegaScript kit (Ambion Inc.). cRNA was dissolved in RNase-free water, stored at ~80 °C, and diluted to a concentration of 100 ng/μl immediately prior to injection. 50 nl (containing 20 ng of cRNA or water for the controls) were injected into defolliculated stage V–VI *X. laevis* oocytes using a Nanoject automatic injector (Drummond Science Inc., Broomall, PA).

**Immunodetection of PCRT**—2–3 days after cRNA injection into oocytes, membranes were prepared from *X. laevis* oocytes (35) and solubilized in SDS-PAGE loading buffer. Membrane proteins were size-fractionated on a 12% SDS-polyacrylamide gel and transferred to a polyvinyldene difluoride membrane. PCRT was detected using a guinea pig anti-peptide antiserum (1:1000 dilution) raised against the N terminus of PCRT (MKF ASK KNN QKN SSK; Eurogentec). As a secondary antibody, we used a peroxidase-conjugated donkey anti-guinea pig antiserum (1:10,000 dilution) purchased from Dianova (Dianova, Hamburg, Germany).

**Immunofluorescence**—2 days after cRNA injection, oocytes were embedded in Tissue-Tek cryo-embedding compound (Miles Inc.); frozen in liquid propane; which was cooled by liquid nitrogen; and stored at −80 °C until used. Sections (5 μm) were cut at −26 °C with a cryotome and mounted on glass slides. For immunofluorescence staining, sections were fixed for 15 min with 4% paraformaldehyde in phosphate-buffered saline (PBS). PCRT was detected using the guinea pig anti-PCRT antiserum. As a secondary antibody, a Cy3-conjugated donkey anti-guinea pig antiserum (1:50 dilution; Dianova) was used. Images were acquired using a CLSM 510 confocal laser scanning microscope (Leica Microsystems). For immunofluorescence staining, sections were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min and blocked with 1% BSA (Sigma-Aldrich) for 1 h. Sections were incubated with primary antibodies overnight at 4 °C. Primary antibodies were diluted in PBS/5% BSA. As a primary antibody, we used a mouse anti-PfCRT (MKF ASK KNN QKN SSK; Eurogentec) and rabbit anti-guinea pig antiserum (1:10,000 dilution) purchased from Dianova. After rinsing, sections were incubated with a secondary antibody. As a secondary antibody, we used a fluorophore conjugated to a secondary antibody. After rinsing, sections were mounted on glass slides. For immunofluorescence staining, sections were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min and blocked with 1% BSA (Sigma-Aldrich) for 1 h. Sections were incubated with primary antibodies overnight at 4 °C. Primary antibodies were diluted in PBS/5% BSA. As a primary antibody, we used a mouse anti-PfCRT (MKF ASK KNN QKN SSK; Eurogentec) and rabbit anti-guinea pig antiserum (1:10,000 dilution) purchased from Dianova. After rinsing, sections were incubated with a secondary antibody. As a secondary antibody, we used a fluorophore conjugated to a secondary antibody. After rinsing, sections were mounted on glass slides.

**Electrophysiological Approaches**—For electrophysiological experiments, oocytes were placed in a Plexiglas microchamber and superfused with a conventional barrel measured the electrochemical potential of the ion under investigation (V_m) across the cell membrane. The electrometer output displayed on a multichannel recorder (Sefram, Servofram, Saint Etienne, France) gave both the V_m signal and the algebraic sum of V_m and V_i.

The intracellularionic activity of the ion i (A_i) was calculated using the following general relationship: A_i = A_ext × 10^V_m / V_i, where A_ext is the ionic activity for i in the reference solution (using an activity coefficient of 0.75) and S is the slope. For experiments with pH-sensitive microelectrodes, this general equation reduces to the simplified relationship pH = pH_ext - (V_m - V_i) / S, where pH_ext is the pH of the Ringer’s solution, and V_m is the proton electrochemical potential across the cell membrane. In experiments in which only V_m was needed, conventional microelectrodes filled with 1 or 3 M KCl were used. Preliminary experiments showed that the measured V_m was similar when using 1 or 3 M KCl to backfill conventional microelectrodes. The electrical circuit was closed by a KCl (1 or 3 M)–agarose Ag/AgCl macroelectrode placed in the bath.

To obtain oocyte-specific membrane conductances (G_m) and current-voltage relationships, cells were punctured with two separate microelectrodes filled with 3 M KCl (resistance of ~5 MΩmho). Experiments were controlled using a GeneClamp 500 amplifier (Axon Instruments, Inc., Foster City, CA) (37, 38). To avoid bath error potentials induced by superfusate changes, the bath potential was virtually clamped to 0 mV using a two-electrode virtual ground circuit (37). Electrode tip potentials were adjusted to 0 mV in the bulk solution prior to penetration of oocytes. Membrane capacitance was compensated for by ~90% using the capacitance compensation circuit of the amplifier. To account for resistance errors due to electrode tip polarizations, the tip potential was measured at the end of an experiment after electrode withdrawal from the oocyte, and experiments during which the tip potentials varied by ~2 mV were discarded. The G_m obtained from current clamp experiments as follows. The V_m was set to ~70 mV by injection of a constant current from which current pulse steps ranging from ~10 to ~30 nA in 10-nA increments were applied for 8–10 s, and the change in V_m was recorded. The input conductance was calculated from the slope of the linear steady-state V_m–current relationship. G_m = i / (V_m – iR_m) was determined by relating the input conductance to the membrane surface of the oocytes, calculated after optical measurement of the oocyte diameter.

For voltage clamp recordings, the holding potential was adjusted to ~70 mV, and potential step pulses lasting for 15 s were applied from ~120 to ~20 mV in 20-mV increments while the membrane current (I_m) was recorded. The current-voltage relationship was determined from the steady-state I_m values.

**Artificial Solutions**—Prior to the experiments, oocytes were kept at 18 °C in an amphibian-adapted Ringer’s solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, and 5 mM HEPES adjusted to pH 7.5 with NaOH) supplemented with penicillin/streptomycin. Experiments were performed at room temperature. In Na⁺-free solutions, NaCl was replaced with an equimolar concentration of KCl in the bathing solution, with NaCl replaced with 30 mM KCl. In experiments in which only NaCl was replaced with KCl, the solution was equilibrated with CO₂ at a pressure of 3 kilopascals to pH 7.5. In ammomium-containing solutions, 20 mM NH₄Cl were used at the expense of NaCl. In cell acid loading experiments, a 40 mM sodium salicylate-containing solution (NaCl was replaced by an equivalent amount) was used. Where indicated, the Ringer’s solution was supplemented with diethylamino-2-carboxylic acid (DPC; 1 mM), ouabain (0.1 mM), amiloride (1 or 0.1 mM), Hoechst 694 (50 μM), or ethylisopropylamiloride (EIPA; 50 μM).

**Statistics**—Results are expressed as means ± S.E., with n = number of oocytes investigated from at least three independent experiments. Significance of the results was assessed by paired or unpaired Student’s t test, and results were considered significantly different for p < 0.05.

**RESULTS**

Expression of PCRT in *X. laevis* Oocytes—Translation of *pfcrt* cRNA in Xenopus oocytes was verified by Western analysis using a guinea pig antiserum specific to PCRT, which identified a protein with the expected size of 48.6 kDa (Fig. 1a).

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1. The abbreviations used are: DPC, diphenylamine-2-carboxylic acid; EIPA, ethylisopropylamiloride; NHE, Na⁺-H⁺ exchanger.
The bulk of PICRT was found associated with the total membrane fraction (Fig. 1a). Immunofluorescence revealed co-localization of PICRT over the oolemma. In addition, a dotted staining pattern was observed within the yolk (Fig. 1b).

**PICRT Expression Alters Oocyte pH**

To determine whether PICRT expression modifies basic properties of the oocytes, we measured the resting $V_m$, $pH_i$, and $a_{Na}$ in control oocytes. Compared with control oocytes, for which measured values were consistent with published data (32, 39), PICRT-expressing oocytes exhibited a higher $pH_i$ and a lower $V_m$ (Table I). $a_{Na}$ and $a_{Cl}$ were similar in control and PICRT-expressing oocytes (Table I). Using a CO$_2$/HCO$_3^-$-free and a HEPES-buffered Ringer's solution had no effect on the $V_m$ values (data not shown), yet slightly reduced $pH_i$, while maintaining the pH differences observed between PICRT-expressing and control oocytes (Table I). This finding suggests that the effects of pfert expression on the oocyte $V_m$, and $pH_i$, are HCO$_3^-$-dependent. For this reason, we performed further investigations in a nominal CO$_2$/HCO$_3^-$-free buffer.

To better define the temporal changes in $V_m$ and $pH_i$, we measured both parameters every 6 h after the injection of the pfert cRNA. Both the $V_m$ and $pH_i$ began to diverge from the control values, determined in mock-transfected oocytes, 16 h after cRNA injection and approached steady-state values after 42 h (Fig. 2, a and b). Subsequent experiments were performed 46–72 h after cRNA (or water) injection.

**PICRT Expression Enhances Proton-equivalent Extrusion**

We next sought to investigate the cause of the higher resting $pH_i$ observed in PICRT-expressing oocytes compared with control oocytes. Previous studies have shown that the main $pH_i$ regulatory mechanism in oocytes is Na$^+-H^+$ exchange (39). We therefore reasoned that altered Na$^+-H^+$ exchanger (NHE) activity may be responsible for the higher $pH_i$ observed in PICRT-expressing oocytes. To verify this hypothesis, we incubated oocytes in the presence of amiloride (1 and 0.1 mM), Hoechst 33342 (50 µM), and EIPA (50 µM). These compounds are established inhibitors of NHFs (40), including that of *X. laevis* oocytes (39, 41). Although no immediate changes in $pH_i$ were observed, incubations for 4 h in the presence of NHE inhibitors affected the $pH_i$ in both control and PICRT-expressing oocytes (Fig. 3a). However, the observed intracellular acidification was significantly stronger in PICRT-expressing oocytes, with $pH_i$ values now approaching those of control oocytes (Fig. 3a). Incubating oocytes in the presence of amiloride or other NHE inhibitors for 4 h did not alter the $V_m$ in either control or PICRT-expressing oocytes (data not shown). These data are consistent with amiloride and the other compounds inhibiting an electroneutral H$^+$ extrusion system, viz. NHE.

To verify the conclusion that PICRT-expressing oocytes have an activated NHE, we determined the rate of $pH_i$ recovery after an acid load, brought about by superfusing the oocytes in a salicylate-containing solution. Exposure to the salicylate-containing solution acidified the cells to similar $pH_i$ values (7.03 ± 0.03 (n = 6) and 6.97 ± 0.02 (n = 6) for PICRT-expressing and control oocytes, respectively). Upon salicylate withdrawal, the $pH_i$ recovery rates were then determined in the presence and absence of amiloride in a paired fashion. As shown in Fig. 3b, in the absence of amiloride, recovery of the $pH_i$ to its resting value was faster in PICRT-expressing oocytes than in control oocytes. The amiloride-sensitive component of the $pH_i$ recovery rates was $(1.27 ± 0.2) \times 10^{-2}$ pH units/min (n = 3) for PICRT-expressing oocytes and $(0.24 ± 0.15) \times 10^{-2}$ pH units/min (n = 3) for control oocytes (p < 0.001) (Table I).

The results suggest that, in PICRT-expressing oocytes, the alkaline resting $pH_i$ is due to an increase in H$^+$ extrusion mediated by an amiloride-sensitive NHE. Moreover, the high resting $pH_i$ is apparently not directly related to the low $V_m$ in PICRT-expressing oocytes.

**PICRT Expression Activates a Nonselective Cation Conductance**

To investigate the low $V_m$ values observed in PICRT-expressing oocytes, we first determined the $G_m$ in control and PICRT-expressing oocytes. From an initial holding $V_m$ of −70 mV, step currents of −10, +10, +20, and +30 nA were applied to the oocytes, and temporal changes in $V_m$ were monitored (Fig. 4a). A significantly higher $G_m$ value of 48.10 ± 6.7 microsiemens/cm$^2$ (n = 24) was observed in PICRT-expressing oocytes compared with control oocytes, which had a $G_m$ of 15.09 ± 0.97 microsiemens/cm$^2$ (n = 23; p < 0.001) (Fig. 4b). Removing Na$^+$ (substituted with an equimolar amount of choline$^+$) or Cl$^-$ (substituted with an equimolar amount of gluconate$^-$) from the bath had virtually no effect on the $G_m$ in control oocytes, but substantially reduced the $G_m$ in PICRT-expressing oocytes (Fig. 4b).

To further characterize the enhanced $G_m$ observed in PICRT-expressing oocytes, current-voltage relationships were established over a range from −120 to +20 mV. Fig. 5a shows representative recordings of membrane currents of oocytes bathed in Ringer’s solution. Consistent with measured steady-state $V_m$ values (zero current), the current-voltage relationship of PICRT-expressing oocytes was shifted by about +20 mV compared with that of control cells (Fig. 5b). Moreover, a slowly activating inward rectification was observed at negative voltage (Fig. 5b). Cl$^-$ removal had little effect on the current-voltage relationships of control oocytes (Fig. 5b). In PICRT-expressing oocytes, however, Cl$^-$ removal considerably reduced the inward rectification observed at negative voltage without significantly affecting the potential at which currents inverted polarity ($E_{rev}$). In comparison, Na$^+$ removal shifted the $E_{rev}$ to a more negative value (from −19.99 ± 0.83 to −34.23 ± 1.86 mV) (Fig. 5c). In the range of $E_{rev}$ (−40 to −20 mV), Na$^+$ removal also induced a reduction in $G_m$ (from 36.04 ± 2.87 microsiemens/cm$^2$ (n = 40) to 23.12 ± 2.73 microsiemens/cm$^2$ (n = 11; p < 0.01)) in PICRT-expressing oocytes as determined.
from the current-voltage relationships (Fig. 5c). At $V_m$ values more negative than $-40$ mV, the reduction in $G_m$ was even more pronounced, consistent with the data shown in Fig. 4. 

Na$^+$ removal had no significant effect on control oocytes (Fig. 5c). These findings suggest that, in PICRT-expressing oocytes, the low resting $V_m$ is associated with an increased $G_m$ and a change in ion selectivity of the membrane, which, in turn, is consistent with the activation of a cation conductance.

To determine the nature of this conductance, the effects of extracellular ion substitutions on the $V_m$ were monitored. Consistent with results from the previous protocol (zero current conditions), changing the superfusate from Ringer’s solution to a Cl$^-$-free solution did not significantly alter the $V_m$ in both control and PICRT-expressing oocytes (Fig. 6a and Table I). (The slight change in $V_m$ observed was due to the liquid junction potential.) This finding suggests that the activation of a Cl$^-$ conductance is not predominantly responsible for the altered resting $V_m$ observed in PICRT-expressing oocytes. Switching the superfusate from Ringer’s solution to an Na$^+$-free solution had no major effect on the $V_m$ in control oocytes. In the case of PICRT-expressing oocytes, however, this experimental maneuver resulted in a dramatic membrane hyperpolarization, with the $V_m$ now approaching the values measured in control oocytes (Fig. 6b and Table I). These data, together with the results obtained under voltage clamp conditions, strongly support the idea that PICRT expression activates an Na$^+$- or nonselective cation conductance ($G_{cat}$) in X. laevis oocytes.

The change in $V_m$ induced by the Na$^+$-free solution in PICRT-expressing oocytes was fully reversible when the superfusate was switched back to an Na$^+$-containing solution or an Na$^+$-free solution containing Li$^+$ as a substitute (Fig. 6, b and c; and Table II). In both cases, the $V_m$ returned to its original value measured in Ringer’s solution superfusate (Fig. 6, b and c; and Table II). This finding suggests that the conductance poorly discriminates between Na$^+$ and Li$^+$. The data are consistent with the activation of a $G_{cat}$ rather than an Na$^+$ conductance.

Previous studies have shown that $G_{cat}$ can be inhibited by DPC in X. laevis oocytes (32, 42, 43). Although adding 1 mM DPC to the superfusate had only a marginal effect on the $V_m$ in control oocytes, a strong membrane polarization was observed in the presence of DPC in PICRT-expressing oocytes (Fig. 6d and Table II). This result is consistent with the activation of a DPC-sensitive $G_{cat}$ in PICRT-expressing oocytes. To further confirm this conclusion, we measured the changes in $V_m$ ($\Delta V_m$) induced by an increase in bath K$^+$ concentration ([K$^+$]$_b$), affects the equilibrium potentials of K$^+$ and of the combined cationic species ($E_{cat}$). Experiments were performed in the presence of ouabain (0.1 mM) to inhibit the endogenous Na$^+$,K$^+$ pump activity, which is known to be sensitive to [K$^+$]$_b$ (32), and under Na$^+$-free conditions to remove the major substrate of $G_{cat}$. As shown in Fig. 7, under such experimental conditions, a 5-fold increase in [K$^+$]$_b$ induced a significant membrane depolarization in control oocytes, which was insensitive to DPC. These findings suggest that, in control oocytes, the K$^+$-induced membrane depolarization results from a membrane partial conductance to K$^+$ ($G_K$) rather than from a $G_{cat}$. In comparison with control oocytes, the high K$^+$ solution induced a significantly lower $\Delta V_m$ in PICRT-expressing oocytes, which may indicate a reduced $G_K$ and/or an enhanced $G_{cat}$. The latter assumption was supported by the significant increase in high K$^+$-induced $\Delta V_m$ in the presence of DPC. As the $E_{cat}$ was shifted to negative values under the Na$^+$-free conditions used in this experiment, the $\Delta V_m$ increased in the presence of DPC if the $G_{cat}$ was enhanced. Thus, the lower $\Delta V_m$ induced by high K$^+$ in PICRT-expressing oocytes compared with that in control oocytes reveals the presence of an enhanced $G_{cat}$ in addition to a $G_K$.
Previous studies have shown that the $G_{cat}$ allows for an increased $\text{NH}_4^+$ influx in oocytes (31, 32). We therefore investigated the effect of 20 mM NH$_4$Cl on the pH$_i$. A significantly faster NH$_4$Cl-induced intracellular acidification (expressed as pH$_i$ change as a function of time, $\Delta$P$_H$/$\Delta t$) was observed in PfCRT-expressing oocytes compared with control oocytes ($\Delta$P$_H$/$\Delta t = 0.22 \pm 0.02$ (n = 17) versus $\Delta$P$_H$/$\Delta t = 0.08 \pm 0.01$ (n = 27); $p < 0.001$). The faster cell acidification measured in PfCRT-expressing oocytes is consistent with an accelerated NH$_4^+$ influx, followed by the partial intracellular dissociation of NH$_4^+$ into NH$_3$ and H$^+$ (31, 44). Taken together, our data provide strong evidence for activation of the $G_{cat}$ in PfCRT-expressing $X$. laevis oocytes.

To investigate whether the lower $V_m$ and the more alkaline pH$_i$ observed in PfCRT-expressing oocytes are related, we measured the effect of external ion substitutions on the pH$_i$. As shown in Fig. 6, the pH$_i$ was not affected by Na$^+$ or Cl$^-$ removal from the bath or by the addition of DPC, at least within the timeframe measured (15 min). These data support the view that the changes in $V_m$ and pH$_i$ observed in PfCRT-expressing oocytes are two independent events.

**DISCUSSION**

Here, we have reported the successful expression of the $P$. falciparum chloroquine resistance transporter, PfCRT, in $X$. laevis oocytes. Expression was not possible until we recon-
constructed the coding sequence on the basis of the yeast codon usage. The high A/T content of the original \textit{P. falciparum} sequence most likely prevented efficient translation, a problem often encountered when working with DNA from this parasite. The reconstructed \textit{pfcrt} coding sequence was designed such that translation of the corresponding \textit{in vitro} generated cRNA would produce the original full-length protein in \textit{X. laevis} oocytes without generating any kind of fusion protein. Successful expression was confirmed by Western analysis, and immunofluorescence located the protein at the oocyte plasma membrane (Fig. 1).

\textit{PfCRT}-expressing oocytes revealed changes in two basic physiological parameters. They exhibited a low resting $V_m$ and an alkaline pH. In addition, a chloride-dependent inward rectification was observed, which we did not explore further since it was apparent only at an unphysiologically negative $V_m$. At first sight, the modifications seen in \textit{PfCRT}-expressing oocytes are in agreement with studies postulating a function for \textit{PfCRT} in ion transport mechanisms or pH regulation (21, 28, 30, 45). For example, it has been suggested that \textit{PfCRT} mediates active or passive transmembrane Cl$^-$ movement (28, 30, 45), and another study has correlated polymorphisms within \textit{PfCRT} associated with chloroquine resistance with a reduced food vacuolar pH in \textit{P. falciparum} (45).

However, upon investigating the mechanisms underpinning the alkaline pH, and the low $V_m$ observed in \textit{PfCRT}-expressing oocytes, we were drawn to a different conclusion. Concerning the alkaline pH, we consider it unlikely that \textit{PfCRT} itself mediates amiloride-sensitive H$^+$ extrusion. Instead, we favor the hypothesis that the alkaline pH observed in \textit{PfCRT}-expressing oocytes is related to the activation of an endogenous NHE. We first noted that, from a thermodynamic point of view, the transmembrane chemical Na$^+$ gradient is large enough to...
drives the pH to the values recorded in PfCRT-expressing cells. A more direct argument in favor of activated Na⁺-H⁺ exchange is that established NHE inhibitors (40), such as amiloride, EIPA, and Hoechst 694, acidify the pH in PfCRT-expressing oocytes to values similar to those measured in control oocytes under the same experimental conditions (Fig. 3a). Amiloride, EIPA, and Hoechst 694 are also known to inhibit the NHE of X. laevis oocytes (39, 41). Further supporting the activation of an NHE, the amiloride-sensitive pH recovery rate from an acid load was significantly higher in PfCRT-expressing oocytes than in control oocytes (Fig. 3b and Table I). PfCRT has no homologies to NHEs or H⁺-ATPases and lacks an amiloride-binding site (46).

Concerning the low Vₘ measured in PfCRT-expressing oocytes, several lines of evidence suggest that the underpinning mechanism is a Gₛₑₐₜ induced by PfCRT expression. First, under voltage clamp conditions, when PfCRT-expressing oocytes were superfused with an Na⁺-free (substituted with choline⁺) medium, the Gₛₑₐₜ decreased, and the Eₘₑ decreased to more negative values (Fig. 5c). Replacing Na⁺ with choline⁺ induced a huge membrane hyperpolarization, which was not observed when Li⁺ was used instead of choline⁺ (Fig. 6, b and c). These results are consistent with an ion conductance having a poor discrimination between Na⁺ and Li⁺. Second, DPC, an established inhibitor of the oocyte Gₛₑₐₜ (32), induced a strong Vₘ hyperpolarization (Fig. 6d). This finding is in accordance with the inhibition of a DPC-sensitive conductance. Third, as expected from a Gₛₑₐₜ discrimination between Na⁺ and K⁺ was poor. The depolarization induced by a high K⁺ solution was significantly lower in PfCRT-expressing oocytes than in control oocytes. Moreover, in PfCRT-expressing oocytes, but not in control cells, the K⁺-induced depolarization increased in the presence of DPC (Fig. 7). Finally, NH₄⁺ influx seems to be enhanced in PfCRT-expressing oocytes. This again points toward a Gₛₑₐₜ which, according to previous reports, is permeable for NH₄⁺ in X. laevis oocytes (31, 44).

Although our data demonstrate an enhanced amiloride-sen-

![Figure 6](https://www.jbc.org/doi/10.1074/jbc.M112.369444)

**Representative recordings of the resting Vₘ and pH in control and PfCRT-expressing oocytes determined under different extracellular bath conditions.**

- **a**, effect of Cl⁻ removal from the bath on the Vₘ and pH. Arrows indicate the time point at which the superfusate was changed from Ringer’s solution to a Cl⁻-free solution.
- **b**, effect of Na⁺ removal from the bath on the Vₘ and pH. Arrows indicate the time point at which Na⁺ was removed (downward arrows) or reintroduced (upward arrows).
- **c**, effect of Na⁺ substitution with Li⁺ on the Vₘ. The downward arrow indicates the time point at which the superfusate was changed from Ringer’s solution to an Na⁺-free solution. The upward arrow indicates the time point at which the superfusate was changed from an Na⁺-free solution to an Na⁺-free, Li⁺-containing solution. The dashed arrow indicates the change to Ringer’s solution. **d**, effect of DPC on the Vₘ and pH. Arrows indicate the time point at which 1 mM DPC was added to (downward arrows) or removed from (upward arrows) Ringer’s solution. Solid lines, PfCRT-expressing oocytes; dashed lines, control oocytes. Bar = 2 min. Vₘ determinations were independent of whether a conventional microelectrode or a pH-sensitive double-barreled electrode was used. The statistical analysis of the data are compiled in Table II.

**Table II**

|                | Ringer’s Na⁺-free | Cl⁻-free | Na⁺-free/Li⁺ | DPC |
|----------------|-------------------|----------|--------------|-----|
| Vₘ (mV)        |                   |          |              |     |
| Control        | −45.00 ± 1.07 (11) | −49.14 ± 1.32 (11)⁺ | −52.00 ± 1.16 (11)⁺ | −54.00 ± 2.08 (3; NS) |
| PfCRT          | −23.18 ± 0.83 (11) | −40.04 ± 0.84 (11)⁺ | −24.36 ± 0.80 (7)⁺ | −19.67 ± 1.45 (3; NS) |
| Control        | −48.68 ± 1.23 (11) | −52.00 ± 1.16 (11)⁺ | −54.00 ± 2.08 (3; NS) | −57.50 ± 0.43 (10)⁺ |
| PfCRT          | −22.86 ± 0.76 (7) | −24.36 ± 0.80 (7)⁺ | −19.67 ± 1.45 (3; NS) | −36.64 ± 1.02 (14)⁺ |

⁺ Significant differences (p < 0.001) between cells bathed in Ringer’s solution and modified solutions.
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Fig. 7. Changes in \( \Delta V_m \) induced by increasing the [K\(^{+}\)] in control and PfCRT-expressing oocytes. The extracellular K\(^{+}\) concentration was increased 5-fold, and \( \Delta V_m \) values were monitored. The experiment was performed in the presence (shaded bars) and absence (open bars) of DPC. The bath contained ouabain (0.1 mM) and was free. The data represent the means ± S.E. of four or more independent determinations. *significant differences in \( \Delta V_m \) (\( p < 0.05 \)) in control oocytes and PfCRT-expressing oocytes.

ative H\(^{+}\) extrusion and the presence of a pathway permeable to cationic species in PfCRT-expressing oocytes, we do not suggest that PfCRT itself mediates these ion transport properties. A direct role of PfCRT in H\(^{+}\) extrusion and in nonselective cation transport is unlikely since these transmembrane pathways appear to be independent of each other both electrophysiologically and pharmacologically. For example, some external ion replacements induced rapid changes in the \( V_m \) without inducing concurrent changes in the pH (Fig. 6). Given that both the \( G_{cat} \) and NHE are present in the plasma membrane of \( X. laevis \) oocytes (39, 41, 47), the simplest explanation is that the low \( V_m \) and the high pH observed in PfCRT-expressing oocytes result from the activation of endogenous transport systems, viz. an NHE and a \( G_{cat} \). Despite their enhanced activities, no significant differences in \( \alpha_{\text{Na}} \) between PfCRT-expressing and control oocytes were measured (Table I). A plausible explanation is that the cells have adjusted Na\(^{+}\) efflux, mediated by the Na\(^{+}\)-K\(^{+}\)-ATPase, to maintain \( \alpha_{\text{Na}} \). It is well known that the Na\(^{+}\)-K\(^{+}\)-ATPase activity, in \( X. laevis \) oocytes as well as in other cells, is highly sensitive to changes in \( \alpha_{\text{Na}} \) (48, 49). The activation of an NHE and a \( G_{cat} \) appears to be a specific effect, as expressing and non-conducting heterologous proteins does not result per se in the activation of endogenous transporters in \( X. laevis \) oocytes (50–52).

A previous study has suggested that PfCRT mediates transmembrane Cl\(^{-}\) movement (30). This conclusion is based on the observation that inside-out vesicles from \( P. pastoris \) membranes containing PfCRT fused to a biotin acceptor domain acidified only in the presence of a symmetrical transmembrane Cl\(^{-}\) gradient. Acidification consumed ATP and was more pronounced in vesicles containing the mutant PfCRT protein associated with chloroquine resistance than in those containing the wild-type protein. As PfCRT itself has no homologies to the \( \text{Na}^{+}\)-\( \text{K}^{+}\)-ATPases, it was argued that PfCRT supports endogenous H\(^{+}\)-ATPase activity to produce larger \( \Delta pH \) values by facilitating transmembrane Cl\(^{-}\) movement, which would shunt changes in \( V_m \) resulting from the H\(^{+}\)-ATPase activity (30). Is it possible that, in \( X. laevis \) oocytes, PfCRT induces changes in Cl\(^{-}\) homeostasis that subsequently activate endogenous transport systems? Although this is an intriguing hypothesis, our data provide no supporting evidence. The \( V_m \) and \( \Delta pH \) did not respond to external Cl\(^{-}\) removal in PfCRT-expressing oocytes, and no significant differences in \( \alpha_{\text{Cl}} \) were observed between control and PfCRT-expressing oocytes.

Although we found no evidence for a direct or indirect function of PfCRT in Cl\(^{-}\) conductance, the similarities between the two studies are nevertheless striking. In both cases, the expression of PfCRT resulted in the activation of endogenous transport systems: the \( G_{cat} \) and NHE in \( X. laevis \) oocytes and an H\(^{-}\)-ATPase in \( P. pastoris \). One may wonder whether the Cl\(^{-}\) movement observed in the \( P. pastoris \) system is also due to the activation of an endogenous Cl\(^{-}\) transporter by PfCRT.

Thus, there appears to be a common theme of PfCRT activating or modulating transport systems. Currently, we have no explanation of the underlying mechanism. It may involve protein-protein interactions, as exemplified by the activation of an Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) cotransporter by the trout erythrocyte anion exchanger-1 (53) or of channels by single membrane-spanning proteins (54–59). It may also involve electric or thermodynamic coupling such as with the Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) cotransporter and KCl transporter (60). As reported for amino acid transporters, coupling of transport activities may serve to recycle ions or to buffer ionic or \( V_m \) changes induced by the transporter of interest (61–64). Alternatively, PfCRT may interfere with second messengers, such as Ca\(^{2+}\), which is known to regulate the activity of numerous membrane transporters, including the NHE (46, 65), \( G_{cat} \) (66), and H\(^{-}\)-ATPase (67).

A function for PfCRT as an activator or modulator of other transporters would be consistent with most models proposed to explain chloroquine resistance in \( P. falciparum \). Mutations within PfCRT that create chloroquine resistance may alter functional coupling of PfCRT to other transport systems, as seen in the \( P. pastoris \) system (30), which, in turn, may affect chloroquine transport or pH regulation of the food vacuole. Further studies will help distinguish between these possibilities.

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Evidence for Activation of Endogenous Transporters in *Xenopus laevis* Oocytes Expressing the *Plasmodium falciparum* Chloroquine Resistance Transporter, PfCRT

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