Plasmodium Induces Swelling-activated ClC-2 Anion Channels in the Host Erythrocyte*

Received for publication, July 7, 2004, and in revised form, July 21, 2004
Published, JBC Papers in Press, July 21, 2004, DOI 10.1074/jbc.M407618200

Stephan M. Huber‡‡, Christophe Duranton‡‡, Guido Henke‡, Claudia van de Sand‡, Volker Heussler‡, Ekaterina Shumilin‡, Ciprian D. Sandu‡, Valerie Tanneur‡, Verena Brand‡, Ravi S. Kasinathan‡, Karl S. Lang‡, Peter G. Kremsner, Christian A. Hübner**, Marco B. Rust**, Karin Dedek***‡‡, Thomas J. Jentsch**, and Florian Lang‡

From the Departments of *Physiology and **Parasitology, Institute of Tropical Medicine, University of Tübingen, 72076 Germany, the †Bernhard Nocht Institute and the **Center for Molecular Neurobiology, University of Hamburg, 20359 and 20251 Germany, and the ‡Department of Neurobiology, University of Oldenburg, 26111 Germany

Intraerythrocytic growth of the human malaria parasite Plasmodium falciparum depends on delivery of nutrients. Moreover, infection challenges cell volume constancy of the host erythrocyte requiring enhanced activity of cell volume regulatory mechanisms. Patch clamp recording demonstrated inwardly and outwardly rectifying anion channels in infected but not in control erythrocytes. The molecular identity of those channels remains elusive. We show here for one channel type that voltage dependence, cell volume sensitivity, and activation by oxidation are identical to ClC-2. Moreover, Western blots and FACS analysis showed protein and functional ClC-2 expression in human erythrocytes and erythrocytes from wild type (Clcn2+/+) but not from Clcn2−/− mice. Finally, patch clamp recording revealed activation of volume-sensitive inwardly rectifying channels in Plasmodium berghei-infected Clcn2+/+ but not Clcn2−/− erythrocytes. Erythrocytes from infected mice of both genotypes differed in cell volume and inhibition of ClC-2 by ZnCl2 (1 mM) induced an increase of cell volume only in parasitized Clcn2+/+ erythrocytes. Lack of ClC-2 did not inhibit P. berghei development in vivo nor substantially affect the mortality of infected mice. In conclusion, activation of host ClC-2 channels participates in the altered permeability of Plasmodium-infected erythrocytes but is not required for intraerythrocytic parasite survival.

Plasmodium falciparum is metabolically highly active and thus depends on ample supply of nutrients (1). In addition, the intraerythrocytic proliferation of the pathogen and the generation of waste products impose a severe challenge to volume constancy of the host red blood cell (RBC) (1). Moreover, the parasite impairs the pump leak balance of the host (2–7), which maintains a high cytosolic K⁺ and a low Na⁺ concentration in non-infected RBCs (8). From 15 and 36 h postinvasion, the K⁺ and Na⁺ leakage through the RBC membrane increases and the Na⁺/K⁺ pump activity decreases, respectively. Both processes result in a replacement of cytosolic K⁺ ions by Na⁺ in the late trophozoite/schizont-infected RBC (6). The parasite requires high Na⁺ and low K⁺ concentrations in the host cytosol (9) most probably to build up indirectly directed Na⁺ and outward directed K⁺ gradients across its plasma membrane. However, high cytosolic Na⁺ concentrations lead to cell swelling and eventually to colloid-osmotic hemolysis of the host RBC. Premature hemolysis is prevented by the concerted action of the parasite and the host RBC. The former lowers the colloid concentration by excess hemoglobin digestion and the latter exports the hemoglobin-derived amino acids out of the cell (7). To meet the requirements of the intraerythrocytic parasite development new transport systems are up-regulated in the host membrane which accomplish parasite nutrition, cation leakage, and maintenance of host volume constancy (1, 10).

Tracer flux and isosmotic hemolysis experiments characterize the transport systems activated by the parasite as organic osmolyte and anion channels (with additional low but significant cation permeability) (1, 10) similar to those mediating regulatory volume decrease in many nucleated cells (11). Comparison of the available data on the parasite-induced transport suggests that infection of erythrocytes activates two classes of channels, anion-selective channels and organic osmolytes (and cation) channels (12). Recent whole cell patch clamp recordings revealed inwardly rectifying (13–16) and outwardly rectifying anion channels (14, 17, 18) as well as nonselective cation channels (19) in the cell membrane of infected erythrocytes confirming that more than one channel type contributes to the enhanced erythrocyte permeability. The outward rectifier is additionally permeable for organic osmolytes whereas a cell swelling-activated fraction of the inward rectifying anion channels is not (20).

Virtually identical inwardly and outwardly rectifying anion channels are observed in non-infected RBCs following oxidation (14). As P. falciparum is known to confer oxidative stress to the host cell (21–25), we hypothesized that P. falciparum generates the anion channels by oxidation of endogenous host cell membrane proteins (14). The nature and physiological significance of channel types identified by patch clamp recording, however, have remained a matter of discussion (26, 27). The aim of the present study was to elucidate the molecular identity of one of the infection-induced anion channels and to test for its functional significance for the cell volume maintenance of the infected host RBC.
**EXPERIMENTAL PROCEDURES**

**Parasites—**P. berghei ANKA-parasitized mouse RBCs (2 × 10^6) were injected intraperitoneally into sex- and age-matched wild type (C57BL/6J) knock-out mice (Clcn2^-/-) (28) and parasitemia was determined daily by Syto-16 staining in FACS analysis (see below). For patch clamp experiments, P. berghei-infected mouse RBCs were stored in RPMI 1640 medium. The human pathogen P. falciparum strains BNY (29) and FCR-3 (30) were grown in vitro in banked human RBCs (blood group O+). Parasites were cultured as described earlier (14) at a hematocrit of 5% and a parasitemia of 2–10% in RPMI 1640 medium supplemented with Albumin (tissue) (0.5%; Invitrogen) in an atmosphere of 35% N_2, 5% CO_2, 60% O_2.

**Patch Clamp—**Whole-cell currents were recorded at room temperature in late trophozoite stage-infected and non-infected human and mouse RBCs according to Huber et al. (14) with solutions buffered to pH 7.4. RBCs were bathed in (in mM): 115 NaCl, 10 MgCl_2, 5 CaCl_2, 20 Hepes/NaOH, a solution which was originally designed by Desai et al. (15) and which improves the sealing of the cells. Upon achievement of whole cell recording mode, cells were superfused with the recording solutions. After each measurement, cells and Petri dishes were replaced. In whole cell experiments, cytosolic and extracellular ion concentrations are defined by the bath and pipette solution. Electrophysiological driving forces result exclusively from the concentration difference of a permeable ion between both bath and pipette solutions and the applied voltage. An osmotic gradient across the recorded membrane to induce cell swelling was generated by either decreasing bath osmolality (Fig. 1 A–E) or increasing pipette osmolality (Figs. 1, F–I, 4, 5). Human RBCs were recorded with pipette solutions containing (in mM) 140 Na-X, 10 HEPES/NaOH, 5 MgCl_2, 1 Mg-ATP, 0.5 EGTA (with X as chloride or gluconate) combined with a bath solution of 100 sorbitol, 90 NaCl, 10 HEPES/NaOH, 1 MgCl_2. Cell swelling and shrinkage in Fig. 1, A–E was induced iso-ionically by decreasing and increasing the sorbitol to 0 and 200 mM, respectively. Further human RBCs (Fig. 1, F–I) as well as non-infected and parasitized mouse RBCs (Figs. 4 and 5) were recorded in a bath solution containing (in mM) 140 Na-MGD-Cl, 10 HEPES/MGD-Cl, 1 MgCl_2, in combination with an iso-osmotic (140 mM) bath solution (additionally containing in mM: 10 HEPES/NMDG, 0.5 EGTA, 1 M gramicidin, 1 Mg-ATP) and a pipette solution containing 400 mM NMDG-Cl, 100 mM Na-X, 10 HEPES/NaOH, 5 MgCl_2, 1 M gramicidin. Cell swelling and shrinkage were recorded in the presence of ZnCl_2 (1 mM) or (for control) of additional MgCl_2 (1 mM) as well as non-infected and parasitized mouse RBCs (Fig. 4, F, G–I) or a hypertonic (170 mM NMDG-Cl; Fig. 4, H, I) or a hypotonic sorbitol (200 mM) solution (Fig. 5, A–C) and further cell swelling and shrinkage were recorded in the presence of ZnCl_2. As illustrated in Fig. 1B (middle and right) osmotic cell shrinkage inhibited and osmotic cell swelling activated a fraction of the cell inward current. The volume-sensitive current fraction amounts to almost 50% of the total inward current in swollen infected cells (Fig. 1D). In sharp contrast to the infected RBC, swelling of non-infected human RBCs did not activate any appreciable current (Fig. 1, A and D, left two columns). This indicates that infection is a prerequisite for the activation of the current fraction by cell swelling.

The cell volume sensitive current fraction of infected RBCs activated time-dependently upon hyperpolarization (Fig. 1C) and exhibited an inwardly rectifying current voltage (IV) re-
P. falciparum induces cell volume-sensitive anion channels in human RBCs. A and B, whole cell current traces recorded (A) in a non-infected and (B) in a late trophozoite-infected human RBC in isotonic (control, left), hypertonic (shrinkage, middle), and hypotonic (swelling, right) NaCl bath solution combined with NaCl pipette solution. Currents were evoked by 10 voltage pulses (400 ms each) from −10 mV holding potential to voltages between −100 mV and +80 mV. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as entry of positive charge from the extracellular space into the cytosol, are negative currents and depicted as downward deflections of the original current traces. Zero current is indicated by gray line.

C, volume sensitive current fraction as calculated by subtracting the current traces in B obtained during cell shrinkage from those during cell swelling. D, mean conductance of inward current (as calculated by linear regression between −60 and 0 mV voltage) of non-infected and late trophozoite-infected human RBCs recorded as in (A and B) with hypertonic (closed bars) and hypotonic bath solution (open bars).

E, current-voltage (I/V) relationships of the volume-sensitive current fraction in infected human RBCs recorded as in B with NaCl (open circles) and sodium D-gluconate pipette solution (closed triangles). Data in D and E were analyzed by averaging the whole cell currents between 350 and 375 ms of each square pulse and depicted as means ± S.E. (n = 5–11; *, p < 0.05; two-tailed Student’s t test).

F and G, inhibition of cell swelling-induced whole cell currents by ZnCl₂ in infected human RBCs. Current traces (F) and I/V curves (G) recorded in the absence (left and right traces in F, open circles in G) and presence of ZnCl₂ (1 mM; middle traces in F, closed triangles in G). Continuous cell swelling was induced by combining a hypertonic pipette solution (170 mM NMDG-Cl) with an isotonic bath solution (140 mM NMDG-Cl). The current traces in the inset of F show the Zn²⁺-sensitive current fraction as calculated by subtracting the currents in F obtained during Zn²⁺ application from those obtained upon washout. H, I/V curve of the ZnCl₂-sensitive current fraction as calculated from the data in G, I, ZnCl₂ dose response curve of the whole cell inward current of infected human RBCs during cell swelling (data are means ± S.E.; n = 3–7).

ClC-2 in Parasitized Erythrocytes

Fig. 1. P. falciparum induces cell volume-sensitive anion channels in human RBCs. A and B, whole cell current traces recorded (A) in a non-infected and (B) in a late trophozoite-infected human RBC in isotonic (control, left), hypertonic (shrinkage, middle), and hypotonic (swelling, right) NaCl bath solution combined with NaCl pipette solution. Currents were evoked by 10 voltage pulses (400 ms each) from −10 mV holding potential to voltages between −100 mV and +80 mV. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as entry of positive charge from the extracellular space into the cytosol, are negative currents and depicted as downward deflections of the original current traces. Zero current is indicated by gray line. C, volume sensitive current fraction as calculated by subtracting the current traces in B obtained during cell shrinkage from those during cell swelling. D, mean conductance of inward current (as calculated by linear regression between −60 and 0 mV voltage) of non-infected and late trophozoite-infected human RBCs recorded as in (A and B) with hypertonic (closed bars) and hypotonic bath solution (open bars). E, current-voltage (IV) relationships of the volume-sensitive current fraction in infected human RBCs recorded as in B with NaCl (open circles) and sodium D-gluconate pipette solution (closed triangles). Data in D and E were analyzed by averaging the whole cell currents between 350 and 375 ms of each square pulse and depicted as means ± S.E. (n = 5–11; *, p < 0.05; two-tailed Student’s t test). F and G, inhibition of cell swelling-induced whole cell currents by ZnCl₂ in infected human RBCs. Current traces (F) and I/V curves (G) recorded in the absence (left and right traces in F, open circles in G) and presence of ZnCl₂ (1 mM; middle traces in F, closed triangles in G). Continuous cell swelling was induced by combining a hypertonic pipette solution (170 mM NMDG-Cl) with an isotonic bath solution (140 mM NMDG-Cl). The current traces in the inset of F show the Zn²⁺-sensitive current fraction as calculated by subtracting the currents in F obtained during Zn²⁺ application from those obtained upon washout. H, I/V curve of the ZnCl₂-sensitive current fraction as calculated from the data in G, I, ZnCl₂ dose response curve of the whole cell inward current of infected human RBCs during cell swelling (data are means ± S.E.; n = 3–7).
the swelling-induced inwardly rectifying anion current fraction in *P. falciparum*-infected human RBCs resembled that of the ubiquitously expressed, swelling-activated Cl− channel CIC-2 (28, 31, 35–37). If the inwardly rectifying infection-induced anion conductance was indeed generated by CIC-2 then heterologously expressed CIC-2 channels should be activated by oxidative stress. In order to test for sensitivity of CIC-2 to oxidation, we injected mRNA encoding CIC-2 into *Xenopus laevis* oocytes as described previously (35). Two electrode voltage clamp demonstrated volume sensitive and inwardly rectifying anion currents in *Xenopus* oocytes expressing CIC-2 but not in water-injected oocytes (Fig. 2A). Exposure of the CIC-2 expressing oocytes to the oxidant tert-butylhydroperoxide (t-BHP, 1 mM) resulted in a strong activation of inwardly rectifying anion currents (Fig. 2, B and C), which was reversed upon reduction by dithiothreitol (5 mM; Fig. 2, B and C). No similar current was induced by oxidation in water-injected oocytes (Fig. 2D) indicating that the current was indeed due to activation of CIC-2. The heterologously expressed CIC-2 current was reversibly inhibited by ZnCl2 added to the bath solution with an apparent IC50 in the range of about 30 μM (Fig. 2D). Those experiments allow us to suggest that, if CIC-2 is expressed in RBCs, it will be activated by oxidative stress imposed by either infection with *P. falciparum* or exposure to oxidants.

Western blot analysis of RBC membrane preparations shows that CIC-2 protein is indeed expressed in erythrocytes. As illustrated in Fig. 3A, staining with a CIC-2-specific antibody yielded a single band in human RBCs and two bands in mouse RBCs at the expected molecular size in the range of 100 kDa. The bands were absent in RBCs from *Cln2−/−* mice (Fig. 3A) confirming the specificity of the antibody.

To test for functional significance of CIC-2 in non-treated mouse RBCs, erythrocyte count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration; and percentage of reticulocytes were measured in non-treated and non-infected mouse RBCs, it will be activated by oxidative stress imposed by either infection with *P. falciparum* or exposure to oxidants.

Western blot analysis of RBC membrane preparations shows that CIC-2 protein is indeed expressed in erythrocytes. As illustrated in Fig. 3A, staining with a CIC-2-specific antibody yielded a single band in human RBCs and two bands in mouse RBCs at the expected molecular size in the range of 100 kDa. The bands were absent in RBCs from *Cln2−/−* mice (Fig. 3A) confirming the specificity of the antibody.

To test for functional expression of CIC-2, the cation permeability was increased in mouse RBCs by addition of the ionophores valinomycin (2 μM) and gramicidin A (4 μM), and the cells were bathed in a high KCl solution to maintain a high cytosolic K+ concentration. The subsequent dilution of the medium by KCl-free hypotonic sorbitol solution imposed a strong KCl gradient leading to cellular KCl loss and cell shrinkage. Due to the high cation permeability of the cell membrane the rate of cell shrinkage depended only on anion channel activity. Initial cell swelling in hypotonic sorbitol solution and hyperpolarization of the cation-selective cell membrane are expected to activate putative CIC-2 channels. Inwardly and outwardly rectifying anion channels of infected RBCs differ in NPPB sensitivity (14). Therefore, the experiments were performed in the presence of NPPB at a concentration (50 μM) which should be sufficient to block the outwardly rectifying anion conductance (see Fig. 5C) but which should not inhibit CIC-2-like currents in mouse erythrocytes (38). Under those experimental conditions, RBCs from wild-type and *Cln2−/−* mice swelled and started to re-adjust their volume.

![Fig. 2. Activation of rCIC-2 channels heterologously expressed in *X. laevis* oocytes by cell swelling and by oxidation. A, activation of rCIC-2 by exposure to hypotonic bath. Left, two electrode voltage clamp traces of a water-injected oocyte (upper panel) and a rCIC-2-expressing cells (lower panel) recorded under isotonic (control) and hypotonic conditions (swelling). Currents were evoked by applying square pulses (10 s each) from −60 mV holding potential to voltages between −120 mV and +40 mV as shown in B (inset). Right, resulting mean I/V relations (±S.E.) of water-injected (upper plot; n = 4) and rCIC-2-injected oocytes (lower plot; n = 24) bathed in isotonic (open squares) and hypotonic solution (closed circles). B, time course of current change of an rCIC-2-expressing oocyte subsequently submitted to oxidation (t-BHP; 1 mM) and reduction (dithiothreitol; 5 mM). Shown is a current trace at −60 mV holding potential and during applied voltage pulses (pulse protocol is depicted in the inset). C, activation of rCIC-2 by oxidation. Left, current traces and, right, corresponding mean I/V-curves (±S.E.) from water-injected (upper panel; n = 4) and rCIC-2-injected oocytes (lower panel; n = 24) recorded under control conditions (left traces; open squares), upon oxidation (right and middle traces, respectively; closed circles) and upon reduction (right traces; open triangles). Experimental conditions are as in B. D, ZnCl2 sensitivity of rCIC-2. Left, original traces recorded in a rCIC-2-injected oocyte in the presence of increasing concentrations of ZnCl2. Currents were obtained at −60 mV holding potential and during a voltage square pulse to −120 mV; right, dose response curve as calculated from the data in D (means ± S.E., n = 9–15).
within 5 min of incubation in hypotonic sorbitol solution (Fig. 3D, open symbols) as measured by changes of forward scatter in FACS analysis. This regulatory volume decrease was significantly (p < 0.05; two-tailed Welch-corrected t test) more pronounced in wild type than in Clcn2<sup>−/−</sup> RBCs (slope of forward scatter decrease: −20 ± 6 <i>versus</i> −6 ± 2 rel. units/min in wild type and Clcn2<sup>−/−</sup> RBCs, respectively; n = 4) suggesting low basal CIC-2 activity prior to oxidation. Following oxidative stress (addition of 1 mM 2-BHP for 15 min) the same maneuver led to rapid shrinkage of RBCs, which was significantly (p ≤ 0.01; two-tailed Welch-corrected t test) faster in RBCs from wild-type mice than from Clcn2<sup>−/−</sup> mice (slope of forward scatter decrease: −110 ± 15 <i>versus</i> −35 ± 8 rel. units/min in oxidized wild-type and oxidized Clcn2<sup>−/−</sup> RBCs, respectively; n = 4; Fig. 3D, closed symbols). Thus, CIC-2 is functionally expressed and activated by oxidative stress in mouse RBCs. The observed slow shrinkage of oxidized Clcn2<sup>−/−</sup> RBCs (Fig. 3D, closed triangles) may be due to residual activity of non-CIC-2 anion conductances similarly activated by oxidation but not fully blocked by NPPB (50 μM). Therefore a higher concentration of NPPB (100 μM) was used in the following experiments.

Late trophozoite-infected RBCs have high cytosolic Na<sup>+</sup> and low cytosolic K<sup>+</sup> concentrations (2–7). To test for CIC-2 activation by <i>P. berghei</i>-infection Na<sup>+</sup>-permeabilized mRBCs from malaria-infected wild-type and Clcn2<sup>−/−</sup> mice (>70% parasitemia) were resuspended in isosmotic sorbitol solution, and cell shrinkage was assessed by FACS forward scatter in the presence of NPPB (100 μM). Fig. 3E shows that Clcn2<sup>−/−</sup> deficiency significantly (p < 0.001; two-tailed Welch-corrected t test) de-
increased the slope of forward scatter decline \((-87 \pm 12 \text{versus} -28 \pm 6 \text{rel. units/min in wild-type and Clcn2}^{-/-} \text{RBCs, respectively; } n = 3-6\) indicating infection-induced activation of CIC-2.

To directly test for activation of CIC-2 channels by Plasmodium infection, patch clamp whole cell recordings were performed in P. berghei-infected RBCs from wild-type and Clcn2^{-/-} mice. In vivo infected mouse RBCs exhibited whole cell currents which resembled those of infected human RBCs in anion selectivity, current amplitude, rectification, and time-dependent in-/activation at strong hyper-/depolarizing voltages (compare Fig. 4B, third trace with Fig. 1B, second trace). In analogy to human RBCs, cell swelling activated and cell shrinkage inactivated an anion current fraction in infected but not in non-infected RBCs from wild-type mice (Fig. 4, A and B). In sharp contrast, no cell volume-sensitive current fraction was measurable in infected RBCs from Clcn2^{-/-} mice (Fig. 4, C, E, and F). The current phenotype of infected Clcn2^{-/-} RBCs was identical to that of infected wild-type RBCs during cell shrinkage (compare Fig. 4, C with B, third traces or 5A, first traces) indicating similar expression of the infection-induced non-CIC-2 anion conductances by both genotypes. Comparison of the I/V curves recorded in both genotypes under control conditions (Fig. 4, D and E; closed triangles) and during cell swelling (Fig. 4, D and E; open circles) revealed a CIC-2-dependent inwardly rectifying current fraction which amounted to about 3 nS (Fig. 4G). It contributed about 50% of the inward current in swollen cells (Fig. 4D) similar to the volume sensitive inward current fraction of infected human RBCs (Fig. 1D). The phenotype of the CIC-2 current in infected mouse RBCs (Fig. 4G) did not differ from the infection-induced, volume sensitive, and inwardly rectifying anion conductance of human RBCs (Fig. 1, C and E) and CIC-2 channels heterologously expressed in Xenopus laevis oocytes (Fig. 2) in rectification behavior and slow activation at hyperpolarizing voltages. ZnCl2 (1 mM) and NPPB (100 \mu M) added to the bath inhibited the whole cell currents of swollen RBCs from wild-type mice additively (Fig. 5, A–C) suggesting that NPPB (100 \mu M) had only a minor effect on the...
ZnCl₂-sensitive current fraction but inhibited the ZnCl₂-insensitive outwardly rectifying current fraction almost completely. ZnCl₂ inhibited the total inward current of swollen RBCs from wild-type mice with an IC₅₀ in the range of 100 μM (Fig. 5D).

Comparison of the ZnCl₂ (1 mM)-sensitive current fraction between whole cell currents of swollen wild-type (Fig. 5E, open circles), swollen Clcn2⁻/⁻ RBC (Fig. 5E, open diamonds) and non-swollen wild-type RBCs (Fig. 5E, closed triangles) indicated Zn²⁺-sensitivity of the cell volume sensitive CIC-2-generated current fraction.

The swelling-induced activation of CIC-2 points to a possible role of this channel in regulatory volume decrease of swollen RBCs. Thus, CIC-2 deficiency may impair cell volume regulation of infected RBCs leading to an increase in host cell volume.
To test this possibility, RBCs freshly drawn from *P. berghei*-infected *Clen2*−/− and wild-type mice were stained with the DNA/RNA fluorescence dye Syto 16 and analyzed by FACS. (Fig. 6A). Because mature mouse and human RBCs are devoid of nuclei, mitochondria, and RNA, this staining allows to differentiate between non-infected (i.e. Syto 16-negative) and parasitized RBCs (i.e. Syto 16 positive). Both, non-infected and parasitized cells from infected *Clen2*−/− mice, exhibited a significantly (*p*<0.05, one-way analysis of variance) higher forward scatter (488 ± 7 and 515 ± 10 rel. units, *n* = 20, respectively) than the corresponding wild-type RBC groups (457 ± 6 and 481 ± 7 rel. units, *n* = 16, respectively). As *P. berghei* amplifies in an asynchronous manner in mice (39) all stages are encountered at a particular time point and differences in cell volume, therefore, cannot be attributed to difference in stages of parasite development (6). Thus, the observed differences in forward scatter suggest an enhanced RBC volume induced by *Clen2* deficiency. In further experiments, the effect of CIC-2 inhibition on forward scatter was determined in non-infected and parasitized RBCs from both genotypes. Incubation with ZnCl2 (1 mM for 4 h at 37 °C) induced a significant increase in forward scatter of parasitized wild-type RBCs but not of non-infected wild type or non-infected and parasitized *Clen2*−/− RBCs (Fig. 6B). Thus inhibition of CIC-2 increased cell volume only in parasitized RBCs.

To test for the functional significance of CIC-2 for the malaria infection *in vivo*, *Clen2*−/− mice and their wild-type littermates (20–29 mice each) were infected with *P. berghei* Anka (2 × 10⁶ parasitized RBCs intraperitoneally) and increase in parasitemia and animal survival monitored. Infection was followed by increasing parasitemia in both genotypes with similar time courses (albeit parasites initially developed somewhat faster in *Clen2*−/− mice; Fig. 6, C and D). Some of the wild-type littermates tended to die slightly earlier than *Clen2*−/− mice and their wild-type littermates eventually approached similar survival rates. Taken together, lack of CIC-2 did not prevent infection with *P. berghei* Anka and had no profound influence on the course of the disease in mice.

**DISCUSSION**

The present observations provide conclusive evidence for expression of CIC-2 channels in RBCs and show that the channels are activated by oxidation and by infection with *Plasmodium*. The present study thus confirms the participation of host cell membrane proteins in the altered permeability of infected erythrocytes. The data further disclose the functional significance of CIC-2 in cell volume maintenance of infected host cells. Inhibition of CIC-2 resulted in a cell volume increase in infected RBCs, suggesting that Cl− efflux via inorganic monovalent anion-selective channels contributes to regulatory volume decrease of the infected cells.

A prerequisite for anion channel-generated Cl− efflux during regulatory volume decrease is a membrane potential more negative than Cl− equilibrium potential. This occurs for instance in dying human RBCs when increased free cytosolic Ca²⁺ concentrations stimulate the activation of Gardos K⁺ channels (40). Consequently, human RBCs hyperpolarize toward K⁺ equilibrium potential. Hyperpolarization in turn imposes an outwardly directed driving force for Cl− leading to channel-mediated efflux of K⁺, Cl−, and osmotically obliged H₂O, and RBC shrinkage.

Parasitized RBCs, reportedly, do not activate Gardos K⁺ channels (41) due to only moderate Ca²⁺ leakage through the host membrane (42), unimpaired Ca²⁺ ATPase activity of the host RBC (43), and Ca²⁺ uptake by the parasite (44, 45), which together prevent an increase of the cytosolic-free Ca²⁺ concentration in the host cytosol. The infection-induced permeabilities, however, have themselves a low but significant permeability for monovalent inorganic cations, which probably is generated by a Ca²⁺-permeable cation channel type different from the anion channels (19). This cation permeability exhibits a K⁺-to-Na⁺ permeability ratio of about 2 (6, 19, 46). Therefore, the activity of the cation permeability should drive Cl− out of the cell (because the loss of K⁺ is expected to exceed the uptake of Na⁺) especially in the first phase of trophozoite development where the Na⁺ pump activity maintains high outwardly directed K⁺- and inwardly directed Na⁺ gradients across the host membrane (6, 19, 46). In this infection stage net loss of monovalent cations together with Cl− counteracts the expansion of the host volume by the parasite metabolisms. Accordingly, cell volume of infected RBCs was increased upon inhibition of CIC-2 channels.

Besides CIC-2 at least two further anion channel types have been demonstrated in *P. falciparum*-infected human erythrocytes. An outwardly rectifying channel type, which exhibits an additional permeability for organic osmolytes such as lactate, sorbitol, and mannitol (20) and an inwardly rectifying channel type (13, 15, 16). The latter has been reported to be dependent on CFTR (16) and to be activated in non-infected human RBCs by membrane stretch, by protein kinase A phosphorylation (47), and by hypertonic shrinkage (16) indicating that this channel type is also generated by host proteins. The pharmacology of this inwardly rectifying anion channel resemble those determined with tracer flux and isosmotic hemolysis in *P. falciparum*-infected human RBCs suggesting an organic osmolyte permeability also for this inwardly rectifying anion channel (13, 15). More recently, this function has been challenged by the observation that parasites develop well in RBCs from cystic fibrosis patients (mutations in both CFTR alleles), i.e. in the absence of these inwardly rectifying anion channels (16). Nevertheless, there might be considerable functional redundancy of inwardly and outwardly rectifying (putative) organic osmolyte and anion channels. Moreover, whether a principally inwardly or outwardly rectifying current phenotype is measurable in *P. falciparum*-infected human RBCs strongly depends on the applied experimental patch-clamp protocol suggesting that parasitized RBCs can recruit the (putative) organic osmolyte and anion channel types in dependence on extracellular signals (serum factors) or membrane potential (18). In contrast to these (putative) osmolyte channels, the swelling-induced inwardly rectifying anion conductance of *P. falciparum*-infected human RBCs is reportedly not permeable to organic osmolytes (20) indicating that channels others than CIC-2 account for the hemolysis of *Plasmodium*-infected RBCs in isoosmotic solutions of organic osmolytes.

A further result of the present study is that *P. berghei* Anka infection *in vivo* induced inwardly and outwardly rectifying anion channels in the mouse RBC membrane very similar to those observed in human RBC infected *in vitro* with *P. falciparum*. Reportedly, RBCs from mice infected with *P. vinckei* increase the rate of furosemide-sensitive choline, taurine, and Rb⁺ influx (48). As in *P. falciparum*-infected human RBCs (1), the infection-induced choline uptake is dependent on the counter anion in the medium and occurs via a nonsaturable pathway. Taken together these data strongly suggest that *Plasmodium* infection induces similar changes in erythrocyte membrane permeability in mouse and man.

Functionally, *P. falciparum*-infected human RBCs resemble many nucleated cell types which accomplish regulatory volume decrease by the concerted action of CIC-2 channels and organic osmolyte and anion channels (11, 49). The present study demonstrates that both, CIC-2 and the organic osmolyte and anion
channels, are contributing to cell volume constancy of the parasitized RBC. Swelling-dependent CIC-2 activity might sense changes in RBC volume and might fine tune cell volume of parasitized RBCs (especially during early infection) while the cell volume-independent activity (20) of the organic osmolyte parasitized RBCs (especially during early infection) while the changes in RBC volume and might fine tune cell volume of channels, are contributing to cell volume constancy of the parasite and Dr. Mark Waidmann, Department of Transfusion Medicine, University of Tuebingen for providing human RBCs.

REFERENCES