J Membrane Biol (2012) 245:797–805
DOI 10.1007/s00232-012-9482-y

Decreased Redox-Sensitive Erythrocyte Cation Channel Activity in Aquaporin 9-Deficient Mice

Yuliya V. Kucherenko · Stephan M. Huber · Søren Nielsen · Florian Lang

Received: 5 March 2012 / Accepted: 30 June 2012 / Published online: 27 July 2012

Abstract Survival of the malaria pathogen *Plasmodium falciparum* in host erythrocytes requires the opening of new permeability pathways (NPPs) in the host cell membrane, accomplishing entry of nutrients, exit of metabolic waste products such as lactate and movement of inorganic ions such as Cl^-, Na^+ and Ca^{2+}. The molecular identity of NPPs has remained largely elusive but presumably involves several channels, which partially can be activated by oxidative stress in uninfected erythrocytes. One NPP candidate is aquaporin 9 (AQP9), a glycerol-permeable water channel expressed in erythrocytes. Gene-targeted mice lacking functional AQP9 ($aqp^{-/-}$) survive infection with the malaria pathogen *Plasmodium berghei* better than their wild-type littermates ($aqp9^{+/+}$). In the present study whole-cell patch-clamp recordings were performed to explore whether ion channel activity is different in erythrocytes from $aqp^{-/-}$ and $aqp9^{+/+}$ mice. As a result, the cation conductance ($K^+ > Na^+ > Ca^{2+} \gg NMDG^+$) was significantly lower in erythrocytes from $aqp^{-/-}$ than in erythrocytes from $aqp9^{+/+}$ mice. Oxidative stress by exposure for 15–30 min to 1 mM H_2O_2 or 1 mM *tert*-butyl-hydroperoxide enhanced the cation conductance and increased cytosolic Ca^{2+} concentration, effects significantly less pronounced in erythrocytes from $aqp^{-/-}$ than in erythrocytes from $aqp9^{+/+}$ mice. In conclusion, lack of AQP9 decreases the cation conductance of erythrocytes, an effect that possibly participates in the altered susceptibility of AQP9-deficient mice to infection with *P. berghei*.

Keywords Red blood cell · New permeability pathway · Plasmodium · Malaria · Ca^{2+} · Oxidative stress

Y. V. Kucherenko · F. Lang (✉)
Department of Physiology, University of Tübingen, Gmelinstr. 5, 72076 Tübingen, Germany
e-mail: florian.lang@uni-tuebingen.de

Y. V. Kucherenko
Institute for Problems of Cryobiology and Cryomedicine, Ukrainian National Academy of Sciences, Kharkov, Ukraine

S. M. Huber
Department of Radiation Oncology, University of Tübingen, Tübingen, Germany

S. Nielsen
Water and Salt Research Center, Department of Biomedicine, Health, University of Aarhus, Aarhus, Denmark

Introduction

Infection with the malaria pathogen *Plasmodium* causes 300–500 million clinical cases and 1–3 million deaths per year (Kristoff 2007). *Plasmodium* spp. invade erythrocytes and depend for intraerythrocytic survival on a dramatic increase of transport across the cell membrane matching the extensive nutrient requirements of the pathogen (Kirk 2001). As a result, an infected erythrocyte takes up 40–100 times more glucose than a noninfected erythrocyte (Kirk 2001). The pathogen accomplishes the required transmembrane transport by induction of the so-called new permeability pathways (NPPs) (Ginsburg and Kirk 1998). NPPs transport nutrients, waste products and electrolytes such as Cl^-, Na^+ and Ca^{2+} (Kirk 2001). Electrolyte transport is required for maintenance of osmotic equilibrium and cell volume as well as for adjustment of the electrolyte composition of the host cytosol to the requirements of the parasite (Foller et al. 2009). Accordingly, intraerythrocyte survival of the pathogen is disrupted by inhibition of NPPs (Breuer et al. 1987; Ginsburg 1994; Kirk et al. 1993; Kirk and Horner 1995; Kutner et al. 1987). The percentage of infected erythrocytes is further

decreased by removal of extracellular Ca^{2+} (Brand et al. 2003; Wasserman et al. 1982; Wasserman 1990).

NPP is composed of inwardly rectifying anion channels (Desai et al. 2000; Egee et al. 2002; Huber et al. 2002; Staines et al. 2003), outwardly rectifying anion channels (Huber et al. 2002; Staines et al. 2003) and nonselective cation channels (Desai et al. 1996; Duranton et al. 2003; Staines et al. 2001; Thomas et al. 2001).

The pathogen imposes oxidative stress on the host cell (Atamna et al. 1994; Atamna and Ginsburg 1993, 1997; Becker et al. 1994; Ginsburg and Atamna 1994), which contributes to activation of the channels (Huber et al. 2002).

The molecular identity of the ion channels underlying NPP have remained elusive (Staines et al. 2007). Presumably, NPP is composed of several channels activated or inserted in *Plasmodium*-infected erythrocytes (Huber et al. 2005). One of those channel proteins may be aquaporin 9 (AQP9), which is expressed in erythrocytes and allows the passage of glycerol (Liu et al. 2007). The channel is considered to be a broadly selective neutral solute channel, being permeable to polyols, carbamides, purines and pyrimidines and inhibited by phloretin and mercurials (Rojek et al. 2007; Tsukaguchi et al. 1998, 1999). The channel is further thought to be permeable to lactate (Dibas et al. 2007; Tsukaguchi et al. 1999). In liver cells AQP9 expression is regulated by insulin via PI3 K/Akt signaling (Liu et al. 2010). AQP1 can be activated by cGMP, turning it into a cation-conductive channel permeable to Na^+, K^+, TEA^+ and Cs^+ (Boassa et al. 2006). Similar to AQP1, AQP9 has a tetrameric pore structure and acts as a nucleotide-regulated channel since it has a cAMP-activated protein kinase phosphorylation consensus site in the NH_3-terminal domain (Kuriyama et al. 1997). AQP9 is activated by cAMP, an effect fostered by interaction between AQP9, NHERF1 and CFTR (Pietrement et al. 2008).

Gene-targeted mice lacking AQP9 ($aqp^{-/-}$) have morphologically normal erythrocytes with normal H_2O and urea permeability but decreased glycerol permeability (Liu et al. 2007). Following infection with *Plasmodium berghei*, $aqp^{-/-}$ mice survive longer during the initial phase of infection than wild-type mice ($aqp9^{+/+}$) (Liu et al. 2007). The electrophysiological properties of the erythrocytes of those mice have, however, not been elucidated.

In the present study whole-cell patch-clamp experiments were performed to gain insight into the significance of AQP9 for cell membrane conductance in erythrocytes.

Materials and Methods

Mice

Experiments were performed in 9- to 16-week-old male and female gene-targeted mice lacking functional AQP9 ($aqp^{-/-}$) and their wild-type littermates ($aqp9^{+/+}$). The $aqp^{-/-}$ mice have been described previously (Liu et al. 2007). All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and approved by local authorities.

Whole-cell patch-clamp experiments were performed in erythrocytes drawn from three $aqp^{-/-}$ and eight $aqp9^{+/+}$ mice, and intracellular free Ca^{2+} measurements were made utilizing FACS analysis in erythrocytes drawn from three $aqp^{-/-}$ and four $aqp9^{+/+}$ mice.

Electrophysiology

Whole-cell patch-clamp recordings were performed at room temperature. Patch electrodes were made of borosilicate glass capillaries (150 TF-10; Clark Medical Instruments, Lacey Green, UK) using a horizontal DMZ puller (Zeitz, Martinsried, Germany). Pipettes with high resistance from 12 to 17 MOhm were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (Heka, Lambrecht, Germany). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech, Great Neck, NY) and Pulse software (Heka, Lambrecht, Germany). For current measurements, erythrocytes were held at a holding potential (V_h) of −10 mV and 200-ms pulses from −100 to +100 mV were applied in increments of +20 mV. The original current traces are depicted without filtering (acquisition frequency of 3 kHz). Currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current–voltage relationship). *Applied voltage* refers to the cytoplasmic face of the cell membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solutions and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch (1991). Data were corrected for liquid junction potentials. Recordings were obtained in whole-cell mode after reaching >10 GΩ seals in on-cell configuration.

The pipette solution consisted of (in mM) 125 Na-gluconate, 10 NaCl, 1 $MgCl_2$, 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4). Where indicated, 1 mM H_2O_2 or 1 mM *tert*-butyl-hydroperoxide (t-BHP) were added acutely for 20 min to induce oxidative stress.

NaCl Ringer bath solution contained (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). In experiments where Cl^- was substituted with gluconate$^-$ the solution contained (in mM) 150 Na-gluconate, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). The KCl, NaCl and $CaCl_2$ bath solutions contained (in mM) 150 KCl, 150 NaCl and 100 $CaCl_2$, respectively,

further containing 10 HEPES titrated with Tris (KCl and NaCl solutions) or $CaOH_2$ ($CaCl_2$ solution) to pH 7.4. The NMDG-Cl bath solution contained (in mM) 180 NMDG (titrated with HCl to pH 7.4). Reagents were obtained from Sigma (Munich, Germany).

Intracellular Ca^{2+}

Intracellular free Ca^{2+} concentration in *aqp*$^{-/-}$ and *aqp9*$^{+/+}$ erythrocytes was measured after incubation for 30 min at +37 °C in the bath solution containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). Oxidative stress was induced in cells by incubation for 30 min at +37 °C in the bath solution containing 1 mM H_2O_2 (or 1 mM t-BHP).

Erythrocytes were washed in Ringer solution containing 5 mM $CaCl_2$ and loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. Cells were incubated at 37 °C for 20 min, washed once, resuspended in 5 mM Ca^{2+}-containing Ringer and subsequently analyzed in fluorescence channel FL-1 for FACS analysis.

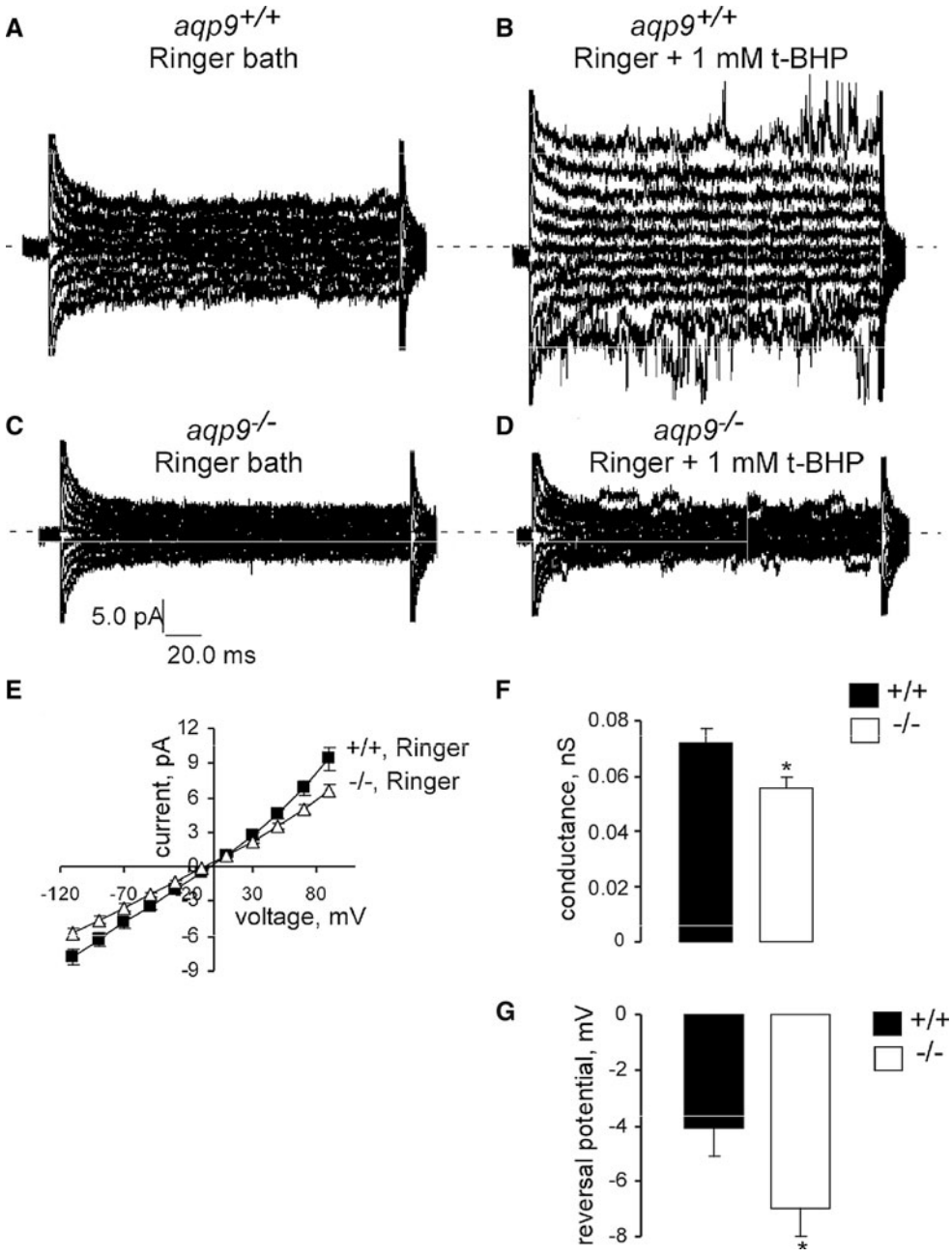

Fig. 1 Whole-cell patch-clamp currents and cation conductance in *aqp9*$^{+/+}$ and *aqp9*$^{-/-}$ erythrocytes. **a–d** Original whole-cell current tracings of control and oxidized (1 mM t-BHP) *aqp9*$^{+/+}$ (*upper tracings*) and *aqp*$^{-/-}$ (*lower tracings*) erythrocytes. Currents were recorded with Na-gluconate pipette and NaCl-Ringer bath solution. **e** Arithmetic means (±SEM) of current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in *aqp*$^{-/-}$ (*open triangles*, $n = 37$) and *aqp9*$^{+/+}$ (*closed squares*, $n = 38$) erythrocytes in NaCl-containing Ringer bath solution. **f** Arithmetic means (±SEM) of the conductance (as calculated for the inward currents by linear regression) of *aqp*$^{-/-}$ (*closed bar*) and *aqp9*$^{+/+}$ (*open bar*) erythrocytes in NaCl-Ringer. *Significant difference from *aqp9*$^{+/+}$ ($p \leq 0.05$, two-tailed *t* test). **g** Arithmetic means (±SEM) of the reversal potentials of *aqp*$^{-/-}$ (*closed bar*) and *aqp9*$^{+/+}$ (*open bar*) erythrocytes in NaCl-Ringer. *Significant difference from *aqp9*$^{+/+}$ erythrocytes ($p \leq 0.05$, two-tailed *t* test)

Ca^{2+} uptake in $aqp^{-/-}$ and $aqp9^{+/+}$ erythrocytes was measured in the presence of 2 mM vanadate to inhibit Ca^{2+}-ATPase. Briefly, cells were loaded with 2 μM Fluo-3/AM in Ringer solution containing 2 mM vanadate, 1 mM $CaCl_2$ and 5 mM EGTA (approximately 12 nM free Ca^{2+}) for 20 min at 37 °C. To induce oxidative stress, cells were treated (15 min at 37 °C) with 0.3 mM t-BHP dissolved in Ringer (2 mM vanadate, 1 mM $CaCl_2$ and 5 mM EGTA) before Fluo-3 loading. After loading, cells were washed once with the same Ringer bath solution (1 mM $CaCl_2$ and 5 mM EGTA). Then, prewarmed (+37 °C) 5 mM $CaCl_2$ and 2 mM vanadate-containing Ringer bath solution was added to the cells at time zero. Cells were placed in the water bath (+37 °C), and the time dependence of Ca^{2+} influx was measured in FACS analysis. Fluo-3 fluorescence data are presented as normalized data (for normalization the Fluo-3 data of $aqp9^{+/+}$ cells at the beginning of the experiment [5-min incubation] were used).

Statistics

Data are expressed as arithmetic means ± SEM, and a two-tailed *t* test or, where appropriate, a two-tailed, Welch-corrected *t* test was employed, $p \leq 0.05$ being considered statistically significant.

Results

Whole-cell membrane patch-clamp tracings were obtained in erythrocytes from gene-targeted mice lacking functional AQP9 ($aqp^{-/-}$) and their wild-type littermates ($aqp9^{+/+}$) to gain insight into the impact of AQP9 expression on ion channel activity in the erythrocyte cell membrane.

Whole-cell patch-clamp experiments on erythrocytes, performed with Na-gluconate pipette solution and NaCl-containing Ringer bath solution, revealed a difference in currents between $aqp9^{+/+}$ and $aqp^{-/-}$ mice. Currents recorded from $aqp^{-/-}$ mice were slightly but significantly decreased in comparison with those from $aqp9^{+/+}$ mice (see Fig. 1a, c, e). As a result, the cation conductance (calculated for inward currents by linear regression) was significantly lower in $aqp^{-/-}$ erythrocytes (56 ± 4 pS, $n = 37$) than in $aqp9^{+/+}$ erythrocytes (73 ± 5 pS, $n = 38$) (Fig. 1f). The reversal potential according to the *I–V* curve was significantly ($p \leq 0.05$) (Fig. 1g) less negative in erythrocytes of $aqp^{-/-}$ mice (−4.08 ± 1.04 mV, $n = 37$) than in erythrocytes of $aqp9^{+/+}$ mice (−6.99 ± 1.01 mV, $n = 38$). Oxidative stress (acute application of 1 mM t-BHP) increased whole-cell currents, an effect more pronounced in $aqp9^{+/+}$ erythrocytes (Fig. 1a, b) than in $aqp^{-/-}$ erythrocytes (Fig. 1c, d).

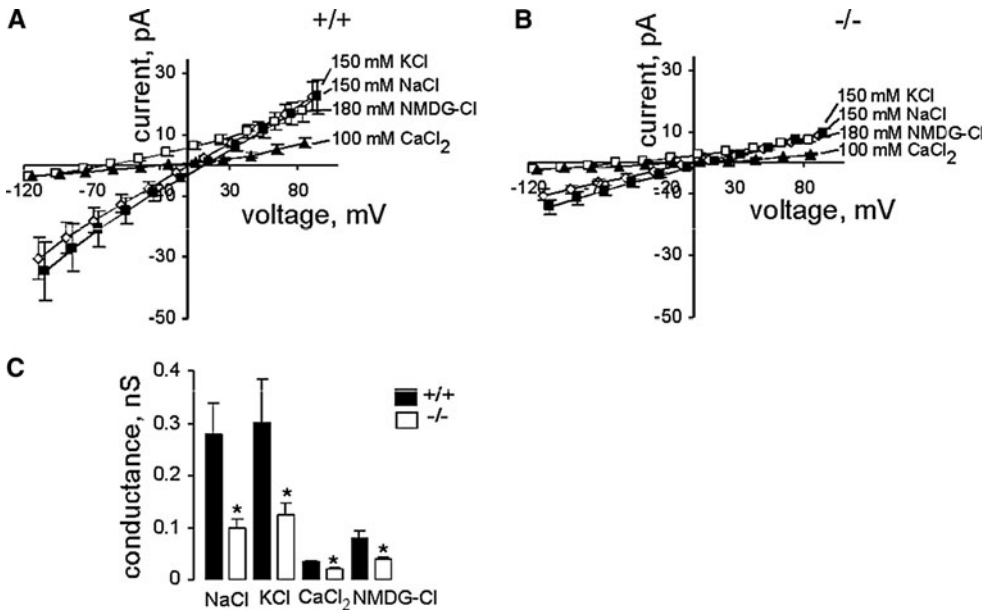

Fig. 2 Nonselective cation conductance in $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes. **a** Arithmetic means (±SEM) of the current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in $aqp9^{+/+}$ erythrocytes suspended in 150 mM KCl bath solution (*closed squares*, $n = 5$), 150 mM NaCl bath solution (*open diamonds*, $n = 5$), 100 mM $CaCl_2$ bath solution (*closed triangles*, $n = 5$) and 180 mM NMDG-Cl bath solution (*open squares*, $n = 5$). **b** Arithmetic means (±SEM) of the current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in $aqp^{-/-}$ mouse erythrocytes ($n = 5$). **c** Arithmetic means (± SEM, $n = 5$) of the conductance (as calculated for the inward currents by linear regression) of $aqp9^{+/+}$ (*closed bars*) and $aqp^{-/-}$ (*open bars*) erythrocytes as recorded with 150 mM KCl, 150 mM NaCl, 100 mM $CaCl_2$ or 180 mM NMDG bath solution. *Significant difference from $aqp9^{+/+}$ erythrocytes ($p \leq 0.05$, two-tailed, Welch-corrected *t* test)

Before characterizing this oxidation-stimulated current fraction, we analyzed the cation selectivity of currents in nonoxidized $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes. The current–voltage (*I–V*) relations obtained from $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes superfused with isotonic KCl, NaCl, NMDG-Cl and $CaCl_2$ solutions are shown in Fig. 2a, b. In this series of experiments, the whole-cell currents in NaCl solution were higher (Fig. 2a, open diamonds) than in the previous series of experiments (Fig. 1e, closed squares). The difference cannot be explained by an increase in leak currents since superfusion of the impermeable cation $NMDG^+$ almost abolished inward currents and shifted the current reversal potential to highly negative voltages (Fig. 2a, open squares). As indicated below (see "Discussion" section), the difference may be due to the use of calcium-free solutions in the second series of experiments.

From the current reversal potentials in the different bath solutions a permselectivity rank order of $K^+ > Na^+ > Ca^{2+} \gg NMDG^+$ was deduced in $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes (Fig. 2a, b). The inward currents recorded with Na^+, K^+, Ca^{2+} as well as $NMDG^+$ in the bath were smaller in $aqp^{-/-}$ erythrocytes than in $aqp9^{+/+}$ erythrocytes. Accordingly, the conductances, as calculated for the inward currents by linear regression, differed significantly in all four solutions between $aqp^{-/-}$ and $aqp9^{+/+}$ erythrocytes (Fig. 2c).

Next, we analyzed the whole-cell currents in erythrocytes from $aqp9^{+/+}$ and $aqp^{-/-}$ mice following acute exposure to 1 mM H_2O_2 or 1 mM t-BHP (Fig. 1b, d). The mean increase of the current following acute application of 1 mM H_2O_2 (Fig. 3a) and 1 mM t-BHP (Fig. 3c) in NaCl-containing Ringer solution was significantly lower in $aqp^{-/-}$ erythrocytes (open triangles) compared to wild-type erythrocytes (closed squares). Accordingly, the fraction of the cation conductance induced by oxidative stress was significantly blunted in $aqp^{-/-}$ erythrocytes (Fig. 3b, d).

To determine the cation permselectivity of the oxidation-induced currents, H_2O_2- or t-BHP-exposed cells were recorded with isotonic solutions of NaCl, KCl, NaCl, $CaCl_2$ or NMDG-Cl. Similar to what was observed in untreated erythrocytes, the whole-cell currents of the

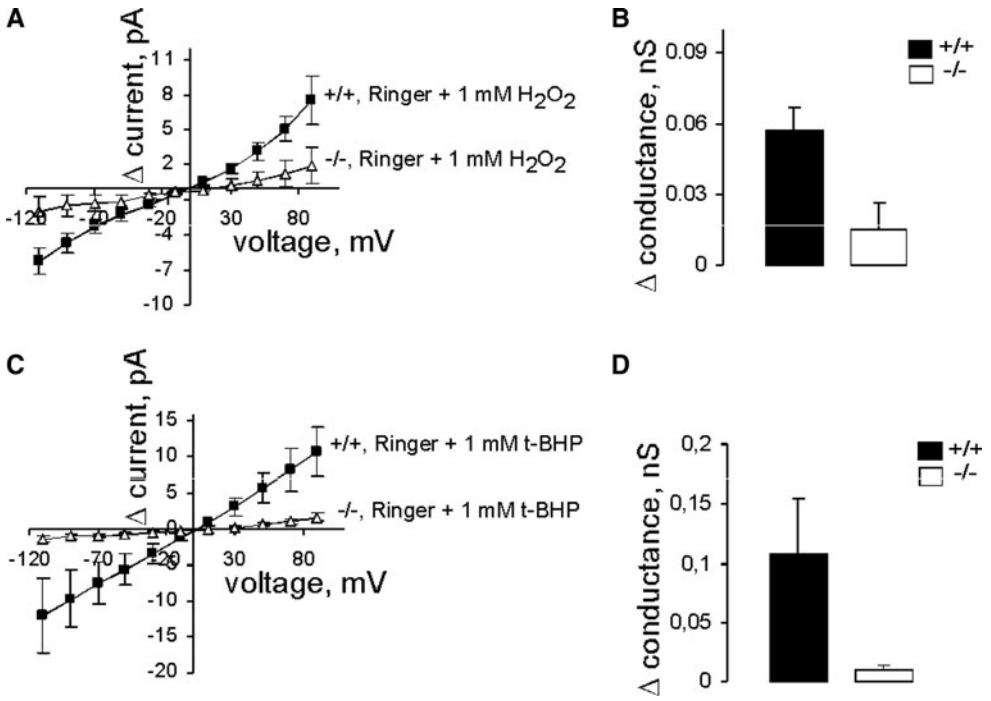

Fig. 3 Oxidation-induced cation conductance in $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes. **a** Arithmetic means (±SEM) of current increment (Δ current, calculated as difference between currents before and after acute oxidation for 20 min) as a function of voltage, recorded with Na-gluconate pipette solution, in $aqp^{-/-}$ (*open triangles*, $n = 7$) and $aqp9^{+/+}$ (*closed squares*, $n = 9$) erythrocytes in NaCl-containing Ringer bath solution in the presence of 1 mM H_2O_2. **b** Arithmetic means (±SEM) of the conductance (as calculated for the inward currents by linear regression) of $aqp^{-/-}$ (*closed bar*) and $aqp9^{+/+}$ (*open bar*) erythrocytes in NaCl-Ringer after acute 20-min application of 1 mM H_2O_2. *Significant difference from control ($p \leq 0.05$, two-tailed t test). **c** Arithmetic means (±SEM) of current increment (Δ current, calculated as difference between currents before and after acute [20-min] oxidation) as a function of voltage, recorded with Na-gluconate pipette solution, in $aqp^{-/-}$ (*open triangles*, $n = 14$) and $aqp9^{+/+}$ (*closed squares*, $n = 10$) erythrocytes in NaCl-containing Ringer bath solution in the presence of 1 mM t-BHP. **d** Arithmetic means (±SEM) of the conductance (as calculated for the inward currents by linear regression) of $aqp^{-/-}$ (*closed bar*) and $aqp9^{+/+}$ (*open bar*) erythrocytes in NaCl-Ringer after acute 20-min application of 1 mM t-BHP. *Significant difference from control ($p \leq 0.05$, two-tailed, Welch-corrected t test)

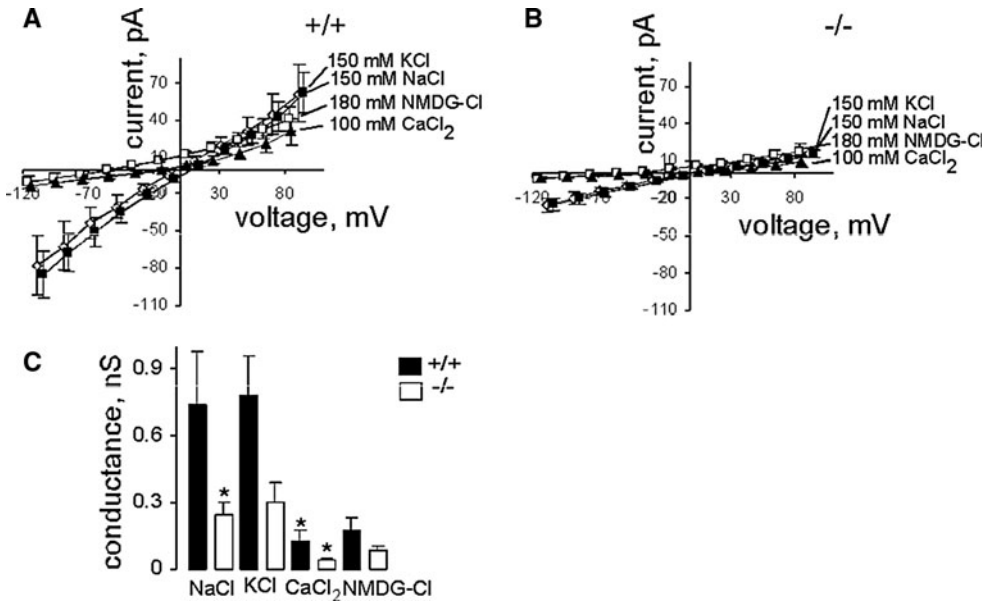

Fig. 4 Nonselective cation conductance in acutely oxidized $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes. **a, b** Arithmetic means (±SEM) of the current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in $aqp9^{+/+}$ erythrocytes (**a**, $n = 7–12$) and $aqp^{-/-}$ erythrocytes (**b**, $n = 9–12$) resuspended in 150 mM KCl bath solution (*closed squares*), 150 mM NaCl bath solution (*open diamonds*), 100 mM $CaCl_2$ bath solution (*closed triangles*) and 180 mM NMDG-Cl bath solution (*open squares*) after acute (20-min) oxidation with 1 mM t-BHP. **c** Arithmetic means (±SEM, $n = 7–12$) of the conductance (as calculated for the inward currents by linear regression) of oxidized (1 mM t-BHP for 20 min) $aqp9^{+/+}$ (*closed bars*) and $aqp^{-/-}$ erythrocytes (*open bars*) as recorded with 150 mM KCl, 150 mM NaCl, 100 mM $CaCl_2$ or 180 mM NMDG bath solution. *Significant difference from $aqp9^{+/+}$ erythrocytes ($p \leq 0.05$, two-tailed, Welch-corrected *t* test)

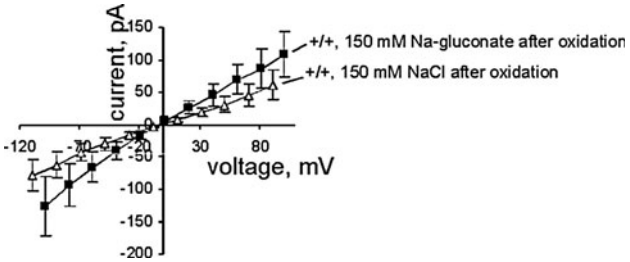

Fig. 5 Lack of anion conductance in acutely oxidized $aqp9^{+/+}$ erythrocytes. Arithmetic means (±SEM) of current as a function of voltage (*I–V* relationship), recorded with Na-gluconate pipette solution, in acutely oxidized (20 min with 1 mM t-BHP) $aqp9^{+/+}$ erythrocytes resuspended in 150 mM NaCl (*open triangles*, $n = 10$) and in 150 mM Na-gluconate (*closed squares*, $n = 6$) bath solutions

oxidized cells in NaCl solution were higher than those in NaCl-containing Ringer solution and the currents were smaller in oxidized $aqp^{-/-}$ erythrocytes than in oxidized $aqp9^{+/+}$ erythrocytes (Fig. 4a, b). As a result, following oxidation, the cation conductances recorded in KCl, NaCl or $CaCl_2$ were significantly lower in $aqp^{-/-}$ erythrocytes than in $aqp9^{+/+}$ erythrocytes. The cation permselectivity was not different between oxidized $aqp9^{+/+}$ and oxidized $aqp^{-/-}$ erythrocytes (compare Figs. 2a, b and 4a, b).

Acute oxidation (20 min with 1 mM H_2O_2 or 1 mM t-BHP) did not modify the anion conductance of erythrocytes from both genotypes. As shown in Fig. 5, substitution of Cl^- in isotonic 150 mM NaCl solution with gluconate did not shift the reversal potential when recorded with Na-gluconate pipette solution. An increase in inward and outward currents (Na^+ currents) induced by acute oxidation was due to activation of cation channels in Cl^--free medium. Furthermore, we did not observe a shift in the reversal potential and current increase when Cl^- in NaCl-containing Ringer bath solution was substituted with L-lactate$^-$ (data not shown).

Since the whole-cell patch-clamp experiments revealed an increase in Ca^{2+} conductance in both untreated and oxidized erythrocytes from either $aqp9^{+/+}$ and $aqp^{-/-}$ mice (Figs. 2c, 4c), a further series of experiments was performed utilizing FACS analysis to estimate intracellular free Ca^{2+} by means of Fluo-3 fluorescence.

To test for Ca^{2+} uptake, Fluo-3-loaded and Ca^{2+}-depleted erythrocytes were resuspended in Ca^{2+}-containing medium and the increase in Fluo-3 fluorescence intensity was measured in the presence of orthovanadate, an inhibitor of the erythrocyte Ca^{2+} pump. As shown in Fig. 6a, b, the normalized Fluo-3 fluorescence increased more steeply in control and preoxidized (0.3 mM t-BHP for 15 min) $aqp9^{+/+}$ erythrocytes than in control and preoxidized $aqp^{-/-}$ erythrocytes, respectively. Accordingly, both basal and oxidation-induced Ca^{2+} permeability were apparently higher in $aqp9^{+/+}$ than in $aqp^{-/-}$ erythrocytes. Along these

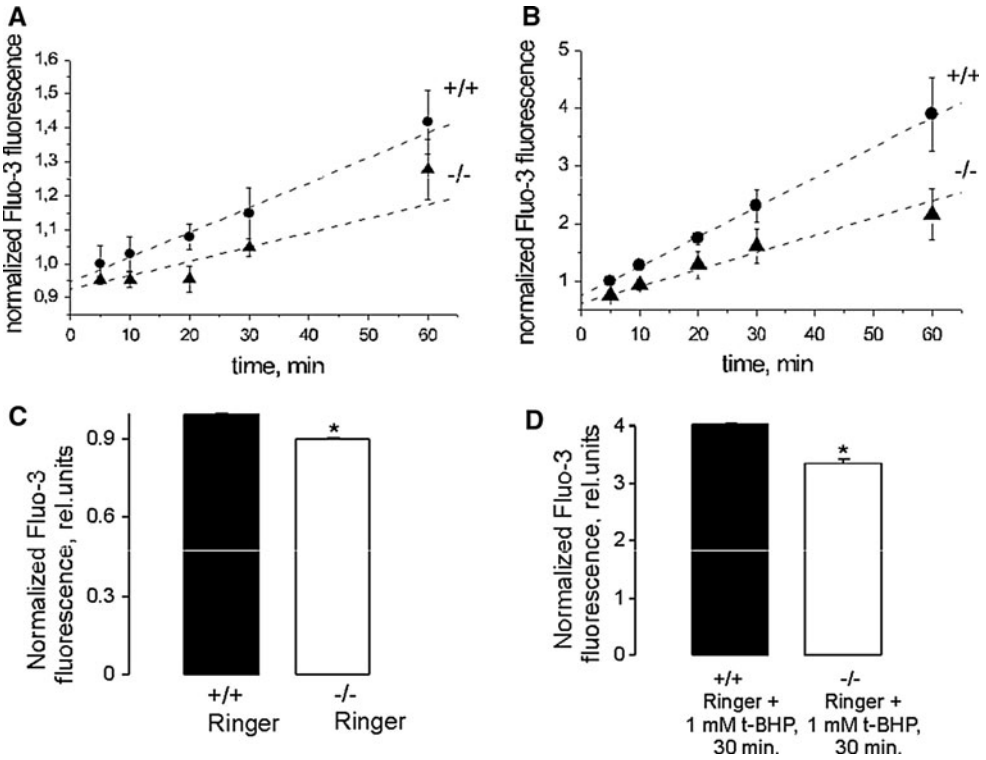

Fig. 6 Cytosolic free Ca^{2+} concentration and Ca^{2+} uptake in control and oxidized $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes. **a**, **b** Time-dependent increase of normalized Fluo-3 fluorescence (arithmetic means ± SEM, $n = 3$) in vanadate (2 mM)-treated control (**a**) and oxidized (0.3 mM t-BHP for 15 min) (**b**) $aqp9^{+/+}$ (*closed circles*) and $aqp^{-/-}$ (*closed triangles*) erythrocytes. **c**, **d** Arithmetic means (±SEM, $n = 12$–16) of normalized Fluo-3 fluorescence of control (**c**) and oxidized (1 mM t-BHP for 30 min) (**d**) $aqp9^{+/+}$ (*closed bars*) and $aqp^{-/-}$ (*open bars*) erythrocytes in NaCl-containing Ringer bath solution. *Significant difference from $aqp9^{+/+}$ erythrocytes ($p \leq 0.05$, two-tailed t test)

lines, in the absence of Ca^{2+} ATPase inhibition, the steady-state Fluo-3 fluorescence was in the absence or presence of t-BHP (1 mM for 30 min) slightly but significantly higher in $aqp9^{+/+}$ than in $aqp^{-/-}$ erythrocytes (Fig. 6c, d).

In conclusion, our data suggest that lack of AQP9 decreases the Ca^{2+}-permeable cation conductance of mouse erythrocytes, an effect that possibly contributes to the altered susceptibility of AQP9-deficient mice to infection with *P. berghei*.

Discussion

The present observations reveal a subtle difference in cation channel conductance between erythrocytes from gene-targeted mice lacking functional $aqp^{-/-}$ and erythrocytes from their wild-type littermates ($aqp9^{+/+}$).

In theory, the differences in cation conductance between $aqp^{-/-}$ and $aqp9^{+/+}$ erythrocytes could result from a cation permeability of AQP9. The channel is considered to be permeable for a wide variety of neutral solutes, such as polyols, carbamides, purines and pyrimidines, and inhibited by phloretin and mercurials (Rojek et al. 2007; Tsukaguchi et al. 1998, 1999). It is noteworthy, though, that the related AQP1 with a similar tetrameric pore structure can be turned into a cation-conductive channel, permeable to Na^+, K^+, TEA^+ and Cs^+, following activation by cGMP, which is generated by the endogenous receptor guanylate cyclase (Boassa et al. 2006).

Since AQP9 has been reported to be activated by cAMP (Kuriyama et al. 1997; Pietrement et al. 2008), it may participate in the regulation of cation channel activity in the cell membrane. When exposed to normal NaCl-containing Ringer bath solution, the currents were not large in $aqp9^{+/+}$ erythrocytes. The cation conductance was even lower, but not absent, in erythrocytes from $aqp^{-/-}$ mice. Notably, whole-cell currents were larger when recorded with calcium-free solutions. Binding of calcium within the channel pore may decrease in calcium-permeable cation channels the current carried by monovalent cations. Hence, the increased monovalent cation currents in the absence of calcium further suggest calcium permeability of the AQP9-dependent conductance.

The residual cation currents in control or oxidized AQP9-deficient erythrocytes suggest that AQP9 might contribute to, but does not fully account for, the cation conductance of the erythrocytes. Like AQP1, a small part of AQP9 molecules could be cation-conductive. Assuming a large number of AQP channel copies in the cell membrane, AQP9 proteins may be responsible for the observed 17-pS difference in cation conductance between $aqp9^{+/+}$ and $aqp^{-/-}$ erythrocytes.

In earlier experiments (Foller et al. 2008), evidence suggested a contribution of TRPC6 channels in the total cation current in erythrocytes. Again, the cation conductance was decreased, but not absent, in gene-targeted mice lacking TRPC6. It is possible that the residual conductance in TRPC6-deficient mice is in part due to AQP9.

The decreased cation conductance of erythrocytes from $aqp^{-/-}$ mice may contribute to the less severe clinical course of malaria in those mice. As shown earlier (Brand et al. 2003), the growth of the malaria parasite *Plasmodium falciparum* in human erythrocytes was highly dependent on the entry of both extracellular Na^+ and extracellular Ca^{2+} into parasitized erythrocytes. Accordingly, the intraerythrocytic growth of the parasite was disrupted by inhibition of the Na^+- and Ca^{2+}-permeable, nonselective cation conductance in the host cell membrane by ethylisopropylamiloride ($IC_{50} < 10$ μM). Moreover, the percentage of *Plasmodium*-infected erythrocytes was significantly blunted by replacement of NaCl with KCl, NMDG-Cl or raffinose (Brand et al. 2003). Half-maximal growth was observed at about 25 mM Na^+. Finally, the parasitemia was significantly decreased by lowering the extracellular free Ca^{2+} concentration with EGTA. At excessive extracellular Ca^{2+} concentrations, EGTA was not effective, indicating that the lack of Ca^{2+}, not the presence of EGTA, disrupted parasite growth (Brand et al. 2003).

Stimulation of cation channels is a double-edged sword (Foller et al. 2009) as excessive Ca^{2+} entry leads to triggering of erythrocyte death (Bhavsar et al. 2010, 2011; Eberhard et al. 2010; Felder et al. 2011; Foller et al. 2009; Gatidis et al. 2011; Ghashghaeinia et al. 2011; Lang et al. 2005, 2007, 2009, 2010, 2011; Nguyen et al. 2011; Qadri et al. 2011a, 2011b), thus limiting the life span of the infected erythrocyte and of the intracellular pathogen. Accordingly, stimulating the suicide of infected erythrocytes may decrease parasitemia and thus favorably influence the clinical course of malaria (Bobbala et al. 2010; Foller et al. 2009; Siraskar et al. 2010).

In conclusion, the present observations provide evidence for an effect of AQP9 on the cation conductance of the erythrocyte cell membrane. Either by allowing the permeation of cations itself or by regulating the activity of cation channels, AQP9 contributes to the cation permeability of erythrocytes. As intraerythrocytic *Plasmodium* spp. depend on Na^+ and Ca^{2+} entry across the host cell membrane, the decreased cation conductance of AQP9-deficient erythrocytes could well contribute to the relative protection of those mice during malaria.

References

Atamna H, Ginsburg H (1993) Origin of reactive oxygen species in erythrocytes infected with *Plasmodium falciparum*. Mol Biochem Parasitol 61:231–241

Atamna H, Ginsburg H (1997) The malaria parasite supplies glutathione to its host cell—investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*. Eur J Biochem 250:670–679

Atamna H, Pascarmona G, Ginsburg H (1994) Hexose-monophosphate shunt activity in intact *Plasmodium falciparum*-infected erythrocytes and in free parasites. Mol Biochem Parasitol 67:79–89

Barry PH, Lynch JW (1991) Liquid junction potentials and small cell effects in patch-clamp analysis. J Membr Biol 121:101–117

Becker K, Gui M, Traxler A, Kirsten C, Schirmer RH (1994) Redox processes in malaria and other parasitic diseases. Determination of intracellular glutathione. Histochemistry 102:389–395

Bhavsar SK, Bobbala D, Xuan NT, Foller M, Lang F (2010) Stimulation of suicidal erythrocyte death by alpha-lipoic acid. Cell Physiol Biochem 26:859–868

Bhavsar SK, Gu S, Bobbala D, Lang F (2011) Janus kinase 3 is expressed in erythrocytes, phosphorylated upon energy depletion and involved in the regulation of suicidal erythrocyte death. Cell Physiol Biochem 27:547–556

Boassa D, Stamer WD, Yool AJ (2006) Ion channel function of aquaporin-1 natively expressed in choroid plexus. J Neurosci 26:7811–7819

Bobbala D, Alesutan I, Foller M, Huber SM, Lang F (2010) Effect of anandamide in *Plasmodium berghei*-infected mice. Cell Physiol Biochem 26:355–362

Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F (2003) Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte. Cell Physiol Biochem 13:347–356

Breuer WV, Kutner S, Sylphen J, Ginsburg H, Cabantchik ZI (1987) Covalent modification of the permeability pathways induced in the human erythrocyte membrane by the malarial parasite *Plasmodium falciparum*. J Cell Physiol 133:55–63

Desai SA, McCleskey EW, Schlesinger PH, Krogstad DJ (1996) A novel pathway for Ca^{++} entry into *Plasmodium falciparum*-infected blood cells. Am J Trop Med Hyg 54:464–470

Desai SA, Bezrukov SM, Zimmerberg J (2000) A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. Nature 406:1001–1005

Dibas A, Yang MH, Bobich J, Yorio T (2007) Stress-induced changes in neuronal aquaporin-9 (AQP9) in a retinal ganglion cell-line. Pharmacol Res 55:378–384

Duranton C, Huber S, Tanneur V, Lang K, Brand V, Sandu C, Lang F (2003) Electrophysiological properties of the *Plasmodium falciparum*-induced cation conductance of human erythrocytes. Cell Physiol Biochem 13:189–198

Eberhard M, Ferlinz K, Alizzi K, Cacciato PM, Faggio C, Foller M, Lang F (2010) FTY720-induced suicidal erythrocyte death. Cell Physiol Biochem 26:761–766

Egee S, Lapaix F, Decherf G, Staines HM, Ellory JC, Doerig C, Thomas SL (2002) A stretch-activated anion channel is up-regulated by the malaria parasite *Plasmodium falciparum*. J Physiol 542:795–801

Felder KM, Hoelzle K, Ritzmann M, Kilchling T, Schiele D, Heinritzi K, Groebel K, Hoelzle LE (2011) Hemotrophic mycoplasmas induce programmed cell death in red blood cells. Cell Physiol Biochem 27:557–564

Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L, Lang F, Huber SM (2008) TRPC6 contributes to the Ca^{2+} leak of human erythrocytes. Cell Physiol Biochem 21:183–192

Foller M, Bobbala D, Koka S, Huber SM, Gulbins E, Lang F (2009) Suicide for survival—death of infected erythrocytes as a host mechanism to survive malaria. Cell Physiol Biochem 24: 133–140

Gatidis S, Zelenak C, Fajol A, Lang E, Jilani K, Michael D, Qadri SM, Lang F (2011) p38 MAPK activation and function following osmotic shock of erythrocytes. Cell Physiol Biochem 28:1279–1286

Ghashghaeinia M, Toulany M, Saki M, Bobbala D, Fehrenbacher B, Rupec R, Rodemann HP, Ghoreschi K, Rocken M, Schaller M, Lang F, Wieder T (2011) The NFkB pathway inhibitors Bay 11-7082 and parthenolide induce programmed cell death in anucleated erythrocytes. Cell Physiol Biochem 27:45–54

Ginsburg H (1994) Transport pathways in the malaria-infected erythrocyte. Their characterization and their use as potential targets for chemotherapy. Biochem Pharmacol 48:1847–1856

Ginsburg H, Atamna H (1994) The redox status of malaria-infected erythrocytes: an overview with an emphasis on unresolved problems. Parasite 1:5–13

Ginsburg H, Kirk K (1998) Membrane transport in malaria-infected erythrocyte. In: Sherman IW (ed) Malaria: parasite biology, pathogenesis, and protection. American Society for Microbiology, Washington DC, pp 219–232

Huber SM, Uhlemann AC, Gamper NL, Duranton C, Kremsner PG, Lang F (2002) *Plasmodium falciparum* activates endogenous Cl^- channels of human erythrocytes by membrane oxidation. EMBO J 21:22–30

Huber SM, Duranton C, Lang F (2005) Patch-clamp analysis of the "new permeability pathways" in malaria-infected erythrocytes. Int Rev Cytol 246:59–134

Kirk K (2001) Membrane transport in the malaria-infected erythrocyte. Physiol Rev 81:495–537

Kirk K, Horner HA (1995) Novel anion dependence of induced cation transport in malaria-infected erythrocytes. J Biol Chem 270: 24270–24275

Kirk K, Horner HA, Spillett DJ, Elford BC (1993) Glibenclamide and meglitinide block the transport of low molecular weight solutes into malaria-infected erythrocytes. FEBS Lett 323:123–128

Kristoff J (2007) Malaria stage-specific vaccine candidates. Curr Pharm Des 13:1989–1999

Kuriyama H, Kawamoto S, Ishida N, Ohno I, Mita S, Matsuzawa Y, Matsubara K, Okubo K (1997) Molecular cloning and expression of a novel human aquaporin from adipose tissue with glycerol permeability. Biochem Biophys Res Commun 241:53–58

Kutner S, Breuer WV, Ginsburg H, Cabantchik ZI (1987) On the mode of action of phlorizin as an antimalarial agent in in vitro cultures of *Plasmodium falciparum*. Biochem Pharmacol 36: 123–129

Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F (2005) Mechanisms of suicidal erythrocyte death. Cell Physiol Biochem 15:195–202

Lang F, Huber SM, Szabo I, Gulbins E (2007) Plasma membrane ion channels in suicidal cell death. Arch Biochem Biophys 462: 189–194

Lang PA, Kasinathan RS, Brand VB, Duranton C, Lang C, Koka S, Shumilina E, Kempe DS, Tanneur V, Akel A, Lang KS, Foller M, Kun JF, Kremsner PG, Wesselborg S, Laufer S, Clemen CS, Herr C, Noegel AA, Wieder T, Gulbins E, Lang F, Huber SM (2009) Accelerated clearance of *Plasmodium*-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. Cell Physiol Biochem 24:415–428

Lang F, Gulbins E, Lang PA, Zappulla D, Foller M (2010) Ceramide in suicidal death of erythrocytes. Cell Physiol Biochem 26:21–28

Lang E, Jilani K, Zelenak C, Pasham V, Bobbala D, Qadri SM, Lang F (2011) Stimulation of suicidal erythrocyte death by benzethonium. Cell Physiol Biochem 28:347–354

Liu Y, Promeneur D, Rojek A, Kumar N, Frokiaer J, Nielsen S, King LS, Agre P, Carbrey JM (2007) Aquaporin 9 is the major pathway for glycerol uptake by mouse erythrocytes, with implications for malarial virulence. Proc Natl Acad Sci USA 104:12560–12564

Liu H, Mei ZC, Xiao X (2010) Effects of insulin and LY294002 inhibitors of PI3 K on the regulations and expression of aquaporin 9 in normal liver cells [in Chinese]. Zhonghua Gan Zang Bing Za Zhi 18:455–458

Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, Bernhardt I (2011) Regulation of phosphatidylserine exposure in red blood cells. Cell Physiol Biochem 28:847–856

Pietrement C, Da Silva N, Silberstein C, James M, Marsolais M, Van Hoek A, Brown D, Pastor-Soler N, Ameen N, Laprade R, Ramesh V, Breton S (2008) Role of NHERF1, cystic fibrosis transmembrane conductance regulator, and cAMP in the regulation of aquaporin 9. J Biol Chem 283:2986–2996

Qadri SM, Bauer J, Zelenak C, Mahmud H, Kucherenko Y, Lee SH, Ferlinz K, Lang F (2011a) Sphingosine but not sphingosine-1-phosphate stimulates suicidal erythrocyte death. Cell Physiol Biochem 28:339–346

Qadri SM, Kucherenko Y, Zelenak C, Jilani K, Lang E, Lang F (2011b) Dicoumarol activates Ca^{2+}-permeable cation channels triggering erythrocyte cell membrane scrambling. Cell Physiol Biochem 28:857–864

Rojek AM, Skowronski MT, Fuchtbauer EM, Fuchtbauer AC, Fenton RA, Agre P, Frokiaer J, Nielsen S (2007) Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proc Natl Acad Sci USA 104:3609–3614

Siraskar B, Ballal A, Bobbala D, Foller M, Lang F (2010) Effect of amphotericin B on parasitemia and survival of *Plasmodium berghei*-infected mice. Cell Physiol Biochem 26:347–354

Staines HM, Ellory JC, Kirk K (2001) Perturbation of the pump-leak balance for Na^+ and K^+ in malaria-infected erythrocytes. Am J Physiol Cell Physiol 280:C1576–C1587

Staines HM, Powell T, Ellory JC, Egee S, Lapaix F, Decherf G, Thomas SL, Duranton C, Lang F, Huber SM (2003) Modulation of whole-cell currents in *Plasmodium falciparum*-infected human red blood cells by holding potential and serum. J Physiol 552:177–183

Staines HM, Alkhalil A, Allen RJ, De Jonge HR, Derbyshire E, Egee S, Ginsburg H, Hill DA, Huber SM, Kirk K, Lang F, Lisk G, Oteng E, Pillai AD, Rayavara K, Rouhani S, Saliba KJ, Shen C, Solomon T, Thomas SL, Verloo P, Desai SA (2007) Electrophysiological studies of malaria parasite-infected erythrocytes: current status. Int J Parasitol 37:475–482

Thomas SL, Egee S, Lapaix F, Kaestner L, Staines HM, Ellory JC (2001) Malaria parasite *Plasmodium gallinaceum* up-regulates host red blood cell channels. FEBS Lett 500:45–51

Tsukaguchi H, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, van Hoek AN, Hediger MA (1998) Molecular characterization of a broad selectivity neutral solute channel. J Biol Chem 273:24737–24743

Tsukaguchi H, Weremowicz S, Morton CC, Hediger MA (1999) Functional and molecular characterization of the human neutral solute channel aquaporin-9. Am J Physiol Renal Cell Physiol 277:F685–F696

Wasserman M (1990) The role of calcium ions in the invasion of *Plasmodium falciparum*. Blood Cells 16:450–451

Wasserman M, Alarcon C, Mendoza PM (1982) Effects of Ca^{++} depletion on the asexual cell cycle of *Plasmodium falciparum*. Am J Trop Med Hyg 31:711–717