

Organic Osmolyte Permeabilities of the Malaria-induced Anion Conductances in Human Erythrocytes

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ABSTRACT Infection of human erythrocytes with the malaria parasite *Plasmodium falciparum* induces new permeability pathways (NPPs) in the host cell membrane. Isotopic flux measurements demonstrated that the NPP are permeable to a wide variety of molecules, thus allowing uptake of nutrients and release of waste products. Recent patch-clamp recordings demonstrated the infection-induced up-regulation of an inwardly and an outwardly rectifying Cl⁻ conductance. The present experiments have been performed to explore the sensitivity to cell volume and the organic osmolyte permeability of the two conductances. It is shown that the outward rectifier has a high relative lactate permeability ($P_{\text{lactate}}/P_{\text{Cl}} = 0.4$). Sucrose inhibited the outward-rectifier and abolished the infection-induced hemolysis in isosmotic sorbitol solution but had no or little effect on the inward-rectifier. Furosemide and NPPB blocked the outward-rectifying lactate current and the sorbitol hemolysis with IC₅₀s in the range of 0.1 and 1 μM, respectively. In contrast, the IC₅₀s of NPPB and furosemide for the inward-rectifying current were >10 μM. Osmotic cell-shrinkage inhibited the inwardly but not the outwardly rectifying conductance. In conclusion, the parasite-induced outwardly-rectifying anion conductance allows permeation of lactate and neutral carbohydrates, whereas the inward rectifier seems largely impermeable to organic solutes. All together, these data should help to resolve ongoing controversy regarding the number of unique channels that exist in *P. falciparum*-infected erythrocytes.

KEY WORDS: plasmodium falciparum • new permeability pathways • osmolyte channel • red blood cells • patch-clamp

INTRODUCTION

The intraerythrocytic development of *P. falciparum* induces the so-called new permeability pathways (NPPs) that appear in the membrane of the parasitized erythrocyte. Via the NPP the host cell provides nutrients and vitamins (Saliba et al., 1998) and allows disposal of waste products such as lactic acid (Poole and Halestrap, 1993) and hemoglobin-derived amino acids (Lew et al., 2003). The NPP is thought to reflect ion channels with a high permeability for inorganic anions (Cl⁻, I⁻, SCN⁻), and also for a wide variety of anionic, cationic, and electro-neutral organic molecules (Kirk et al., 1994; Ginsburg and Kirk, 1998) such as amino acids, nucleosides, sugars, and polyols (Kirk, 2001). As a consequence, infected erythrocytes swell and hemolyze in isosmotic sorbitol solution (Lambros and Vanderberg, 1979; Ginsburg et al., 1983). The NPP is inhibited by classical anion channel/transport inhibitors (Breuer et al., 1987; Kirk and Horner, 1995a). Most recent studies disclosed the activation of anion conductances in the host cell membrane visible

by patch-clamp recording during the trophozoite stage of the blood cycle. On the basis of rectification behavior and difference in inhibitor sensitivity, two distinct anion conductances could be discriminated, i.e., an inwardly rectifying (Desai et al., 2000; Egee et al., 2002; Huber et al., 2002) and an outwardly rectifying conductance (Huber et al., 2002). The activation of these two conductances in infected human erythrocytes has recently been confirmed (Staines et al., 2003), but their functional significance for the malaria infection has not been defined. Since it is still on debate whether one or more channels/permeabilities contribute to the NPP, this study has been designed to elucidate whether the inwardly and outwardly rectifying channels display identical or different properties in permeability, drug sensitivity, regulation, and functional significance. A preliminary account of this work has been published in abstract form (Duranton et al., 2003a).

MATERIALS AND METHODS

Parasite Culture

The *P. falciparum* strain FCR-3 was permanently maintained in culture using a modified protocol of Trager and Jensen (Trager

Abbreviation used in this paper: NPP, new permeability pathway.

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and Jensen, 1976; Cranmer et al., 1997). In brief, RPMI medium 1640 (Biochrom) was supplemented with 25 mM HEPES/NaOH pH 7.4, 20 µg/ml gentamicin sulfate, 2 mM glutamine, 200 mM hypoxanthine, 0.5% Albumax II (GIBCO-BRL), and 2% of human serum. Washed human erythrocytes of blood group O⁺ (banked) were added to a hematocrit of 5%. Parasites were maintained at a parasitaemia of 2–5% in an atmosphere of 90% N₂/5% O₂/5% CO₂ at 37°C. For patch-clamp experiments parasites were taken directly from the asynchronous culture. For hemolysis and tracer flux experiments, trophozoite- and schizont-infected erythrocytes were enriched magnetically using MACS technology (Staalsoe et al., 1999). In brief, parasite cultures were centrifuged, resuspended in PBS/2% BSA/5 mM glucose and applied to a CS column in a SuperMACS (Miltenyi Biotech). The flow-through contained uninfected red blood cells and ring stages whereas the eluted fraction had a parasitaemia of up to 90% trophozoites/shizonts. All reagents used for culture and patch-clamp experiments were purchased from Sigma-Aldrich if not stated otherwise.

Light Microscopy

Analysis of parasite developmental stage: *P. falciparum*-infected erythrocytes were incubated 30 min in PBS buffer containing the fluorescent RNA/DNA dye SYTO 16 (50 µM, Molecular Probes). Fluorescence images were taken at 480/40 nm excitation and 535/50 nm emission wavelength (beamsplitter Q505LP, AHF-analysentechnik AG) and images were compared with the transmission light micrographs.

Erythrocyte cell volume changes during whole-cell recordings: Erythrocyte volumes were compared for different bath osmolarities. The experiments were performed with a 140 mM NMDGCl pipette solution (see below) combined with a NaCl bath solution (see below) in which the osmolarity was increased and decreased by adding sorbitol (100 mM) and removing NaCl (50 mM), respectively.

Patch-clamp Recordings

For control whole-cell experiments, erythrocytes from healthy donors (donors gave informed consent) were diluted (blood dilution ~1/1,000) in NaCl bath solution used for the patch-clamp experiments (see below).

P. falciparum-infected erythrocytes are chosen optically by the presence of a prominent (typically moving) light-dense intra-erythrocytic structure (hemozoin-containing food vacuole of the mid/late trophozoite-stage; Fig. 1). Infected erythrocytes were recorded at room temperature (patch-clamp data acquired in the authors' laboratory in infected erythrocytes at 37°C or at room temperature did not statistically differ in whole-cell current amplitude or phenotype; unpublished data). Continuous superfusion (~1 ml/min) was applied through a flow system inserted into the dish (bath volume ~200 µl).

Contradictory data on whole-cell currents of *P. falciparum*-infected human erythrocytes have been published. Our previous study demonstrated inwardly and outwardly rectifying anion conductances (Huber et al., 2002), whereas three other groups, in sharp contrast, observed only an inwardly rectifying conductance in infected erythrocytes (Desai et al., 2000; Egee et al., 2002). To solve this conflict the groups of Roscoff and Oxford and ourselves worked together, comparing experimental protocols and culture conditions. As a result, we were able to demonstrate that serum (or albumax) of the culture medium induces in infected (but not in noninfected) cells the outwardly rectifying whole-cell current fraction (Staines et al., 2003). In our hands, superfusion of the recorded cell with serum (or albumax) -free solutions did not result in a run-down of the induced outward rectifying whole-cell current. Instead, inactivation of the outwardly rectifying cur-

rent component required an extensive washing of the erythrocytes. Since the present study worked on nonwashed infected cells, the outwardly rectifying component was present in the whole-cell experiments.

The bath was grounded via a bridge filled with NaCl bath solution (see below). Borosilicate glass pipettes (8–14 MΩ pipette resistance; GC150 TF-10, Clark Medical Instruments) manufactured by a microprocessor-driven DMZ puller (Zeitz) were used in combination with a STM electrical micromanipulator (Lang GmbH and Co. KG). Currents were recorded in fast whole-cell, voltage-clamp mode, and 3 kHz low-pass filtered by an EPC-9 amplifier (Heka) using Pulse software (Heka) and an ITC-16 Interface (Instrutech). After giga-Ohm seal formation, the membrane was ruptured by additional suction and/or brief electrical pulses (~700 mV during 100–200 µs). Rupture of the membrane and entry in whole-cell recording configuration was indicated by a minute increase in capacitance and a simultaneous bleaching of the erythrocyte due to dialysis of hemoglobin by the pipette solution.

The liquid junction potentials ΔE between the pipette and the bath solutions and between the salt bridge and the bath solutions were estimated as described earlier (Barry and Lynch, 1991). Data were corrected for the estimated ΔE values. Whole-cell currents were evoked by 10 to 11 voltage pulses (400 ms each) from –30-mV holding potential to voltages between –100 and +80–100 mV. Original whole-cell current traces are depicted after 500 Hz low-pass filtering. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces. Current values were analyzed by averaging the whole-cell currents between 350 and 375 ms of each square pulse.

Most cells were recorded with a pipette solution containing (in mM) 140 NMDG-Cl (pH 7.4), 10 HEPES/NMDG (pH 7.4), 5 MgCl₂, 1 Mg-ATP, 0.5 EGTA (=isotonic NMDG-Cl pipette solution). To swell or shrink the cells during whole-cell recording an isotonic bath solution was combined with a hypertonic or hypotonic NMDG-Cl pipette solution, respectively, containing (in mM): 140 NMDG-Cl (pH 7.4), 40 mannitol, 10 HEPES/NMDG (pH 7.4), 5 MgCl₂, 1 Mg-ATP, 0.5 EGTA (=hypertonic NMDG-Cl pipette solution) and 90 NMDG-Cl (pH 7.4), 10 HEPES/NMDG (pH 7.4), 5 MgCl₂, 1 Mg-ATP, 0.5 EGTA (=hypotonic NMDG-Cl pipette solution). Further pipette solutions were used to study the permeability of the inwardly rectifying conductance for monocarboxylates (in mM): 140 Na-L-lactate, 10 HEPES/NaOH (pH 7.4), 5 MgCl₂, 1 Mg-ATP, 0.5 EGTA.

Cells were superfused with standard bath solution (in mM): 140 NaCl, 10 HEPES/NaOH (pH 7.4), 1 CaCl₂, 1 MgCl₂. To determine the monocarboxylate permeability of the outwardly rectifying conductance, NaCl of the bath was isosmotically replaced by Na-L-lactate or -D-lactate: A Cl⁻-free bath solution containing (in mM) 145 Na-L(D)-gluconate, 10 HEPES/NaOH (pH 7.4) was used in further experiments. To study the effects of neutral carbohydrates on the whole-cell current part of bath NaCl (50 mM) was substituted by sorbitol, mannitol, or sucrose (100 mM each). In further records different concentrations of sucrose or mannitol were added to the NaCl or Na-L-lactate bath solution. To pre-shrink the cells in some experiments NaCl (25 mM) was added to the standard NaCl bath solution.

Hemolysis of Infected Cells

Enriched trophozoite-infected erythrocytes (parasitaemia >90%; hematocrit 5%) were spun down and 10 µl of the pellet were resuspended in 200 µl of isosmotic sorbitol solution (290 mM sorbitol/5 mM HEPES/NaOH pH 7.4), and hemolyzed for 10 min at 21°C in the presence and absence of NPPB or furosemide (from

0.01 to 100 μM). Further hemolysis experiments were performed using isosmotic solutions with isosmotic replacement of increasing concentrations of sorbitol by either NaCl or sucrose. After centrifugation the hemoglobin color of the supernatants (150 μl) was recorded qualitatively by image scanning. In addition, the hemoglobin concentration was determined quantitatively by photometry (absorbance at 546 nm after oxidation to cyanomet-hemoglobin).

Uptake of [¹⁴C] Sorbitol in Isosmotic Sorbitol Medium

Unidirectional flux of the polyol [¹⁴C] sorbitol (Biotrend) into enriched trophozoite/shizont-infected erythrocytes was measured according to the method described for rapid substrate uptakes (Kanaani and Ginsburg, 1991; Kirk and Horner, 1995b). Briefly, 150 μl of [¹⁴C] sorbitol (3.7×10^4 Bq/ml; 1.4×10^4 Bq/nmol specific activity) containing incubation solutions were layered over 200 μl of dibutylphthalate oil. The incubation solution additionally contained 290 mM sorbitol and 5 mM HEPES/NaOH pH 7.4. In further solutions, 20 and 60 mM of sorbitol was iso-osmotically replaced by either 20 and 60 mM sucrose or 10 and 30 mM NaCl, respectively. The uptake (21°C) was started by adding the trophozoite-infected erythrocytes (5 μl , >90% parasitaemia; 80% hematocrit) to the [¹⁴C] sorbitol containing solutions and terminated at the appropriate time by rapid centrifugation (10,000 g, 20 s) of the cells through the oil. The [¹⁴C] sorbitol solution which remained on top of the oil was removed and the tubes were washed five times by submerging the cell- and oil-containing tubes in distilled water and subsequently discarding the aqueous phase. After removal of the oil, the cell pellet was lysed and deproteinised by successive addition of Triton X-100 (0.1%; 0.5 ml) and trichloroacetic acid (6%, 0.5 ml). After 10 min of incubation (21°C) precipitated protein was separated by centrifugation (10 min, 10,000 g) and the radioactivity of the supernatant was measured using a β -scintillation counter (Wallac 1406).

RESULTS

To study the lactate permeabilities of the erythrocyte membrane from normal and mid/late trophozoite stage-parasitized cells (Fig. 1), whole-cell currents were recorded upon replacing Cl^- in the bath solution by equiosmolar amounts of L-lactate. In noninfected erythrocytes, with NMDG-Cl pipette solution, whole-cell currents measured in the presence of NaCl or Na-L-lactate bath solutions were extremely low (Fig. 2, A and B). In sharp contrast, whole-cell currents of *P. falciparum*-infected erythrocytes (Fig. 2 C, top line) recorded with NMDG-Cl pipette solution were 50–100-fold larger. In contrast to the noninfected cells these currents were anion selective, as suggested by the reversal potential (V_{rev})³ of the infection-induced currents which were close to E_{Cl} (Fig. 2 D). In infected cells, replacement of Cl^- in the bath by lactate induced only a moderate shift of the V_{rev} by about $+20.0 \pm 1.9$ mV (Fig. 2 D) with the change of E_{Cl} (from 0 to +90 mV) and only an $\sim 60\%$ inhibition of the outward current indicating a $P_{\text{lactate}}/P_{\text{Cl}} = 0.3\text{--}0.4$ for the overall current in infected erythrocytes at V_{rev} . The inward current (e.g., at -100 mV voltage), which was carried by the outflow of Cl^- from the pipette into the bath, did not change by the substi-

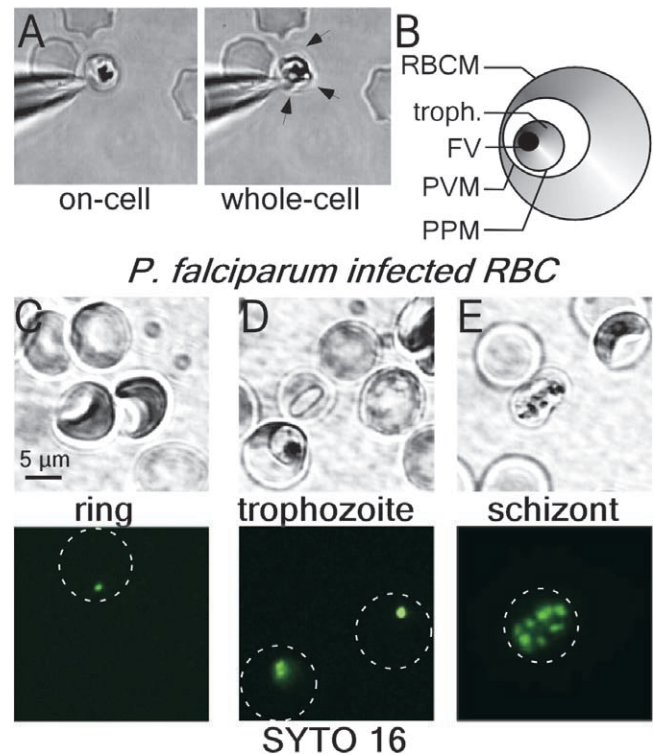


FIGURE 1. Whole-cell recording in mid/late trophozoite-infected red blood cells (RBCs). (A) Light micrograph of a parasitized erythrocyte recorded by patch-clamp techniques before (left, on-cell mode) and after rupture of the aspirated membrane (right, whole-cell mode). Note the bleaching of the erythrocyte cytosol by dialysis of the hemoglobin upon break-in into the whole-cell mode. The ghost membrane is indicated by arrows. (B) Scheme depicting the membrane organization of a trophozoite-parasitized erythrocyte (RBCM, red blood cell membrane; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; FV, food vacuole with hemozoin). (C–E) Transmission light (top line) and fluorescence images (bottom line) of (C) ring-stage-, (D) early and late trophozoite-, and (E) shizont-infected erythrocytes. Infected cells are indicated by the DNA/RNA-specific Syto16-fluorescence. Whole-cell records were obtained from noninfected and mid/late trophozoite-infected erythrocytes.

stitution of bath Cl^- with L-lactate, indicating that L-lactate did not block the conductance(s) (Fig. 2 D, circles and diamonds). In further experiments using Na-L-lactate in the pipette and NaCl in the bath (Fig. 2 C, bottom line) the whole-cell currents reversed at -22.8 ± 1.8 mV again between E_{Cl} (-66 mV) and E_{lactate} ($+\infty$). Substitution of bath Cl^- by L-lactate decreased the outward current by $\sim 60\%$ and shifted V_{rev} to -3.3 ± 1.7 mV, which was close to E_{lactate} (0 mV). With both pipette solutions, superfusion of the cells with Na-gluconate (a larger and less permeant anion than Cl^-) inhibited the outward currents almost completely and shifted the V_{rev} s toward high positive voltages (Fig. 2, D and E, squares), indicating a relatively low permeability for gluconate ($P_{\text{gluconate}}/P_{\text{Cl}} \sim 0.04$ and $P_{\text{gluconate}}/P_{\text{lactate}} \sim 0.1$).

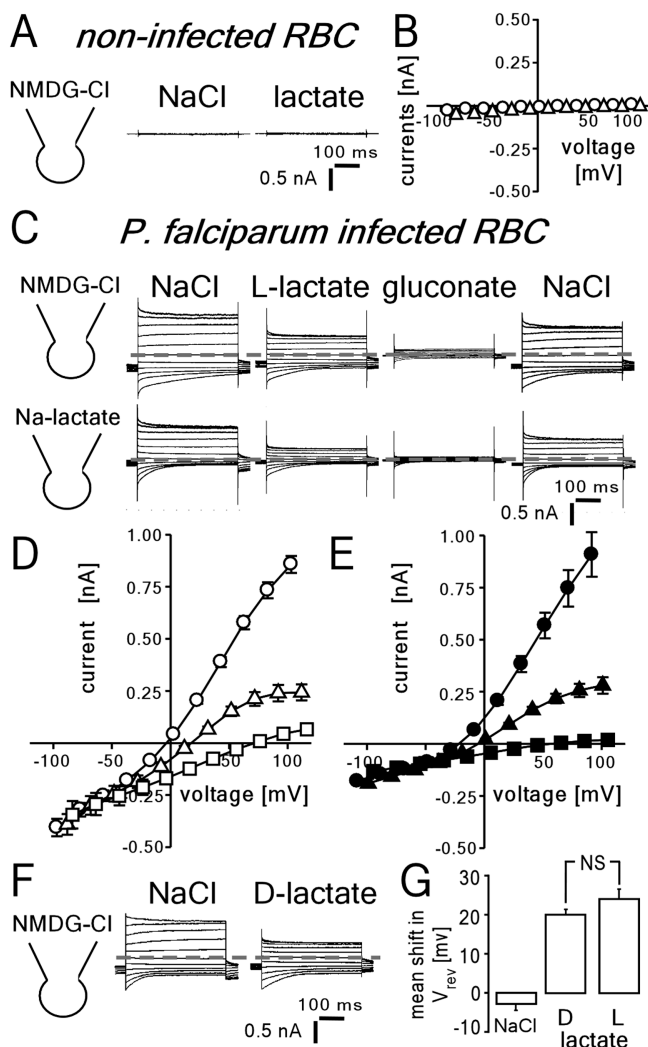


FIGURE 2. Lactate permeability of the plasma membrane of normal and *P. falciparum*-infected red blood cells. (A) Current traces of a noninfected erythrocyte recorded with NMDG-Cl pipette solution and NaCl or Na-L-lactate bath solution. Currents were obtained in fast whole-cell voltage-clamp mode. The membrane potential was held at -30 mV and currents were elicited by 400-ms square pulses to test potentials between -100 and $+100$ mV; zero current is indicated by dashed line. (B) Mean current-voltage (I-V) relations (\pm SE, $n = 4-8$) of noninfected erythrocytes recorded as in A with NaCl- (circles) or Na-L-lactate bath solution (triangles). Current values were analyzed between 350 and 375 ms of each square pulse. (C) Current traces of two infected cells recorded with NMDG-Cl (top line) or Na-L-lactate pipette solution (bottom line). Currents were measured in NaCl bath solution, after substitution of bath NaCl with Na-L-lactate and Na-gluconate, and after wash-out of Na-gluconate by NaCl. (D and E) Mean I-V relations (\pm SE) recorded as in C with NMDG-Cl (D, $n = 6-8$) and Na-lactate pipette solution (E, $n = 5-8$) combined with NaCl- (circles), Na-L-lactate (triangles), or Na-gluconate bath solution (squares). (F) Current traces recorded with NMDG-Cl pipette solution and NaCl or Na-D-lactate bath solution. (G) Mean shift in reversal potential (\pm SE) induced by replacement of NaCl by equimolar quantities of -L- or -D-lactate in the bath solution ($n = 4-8$).

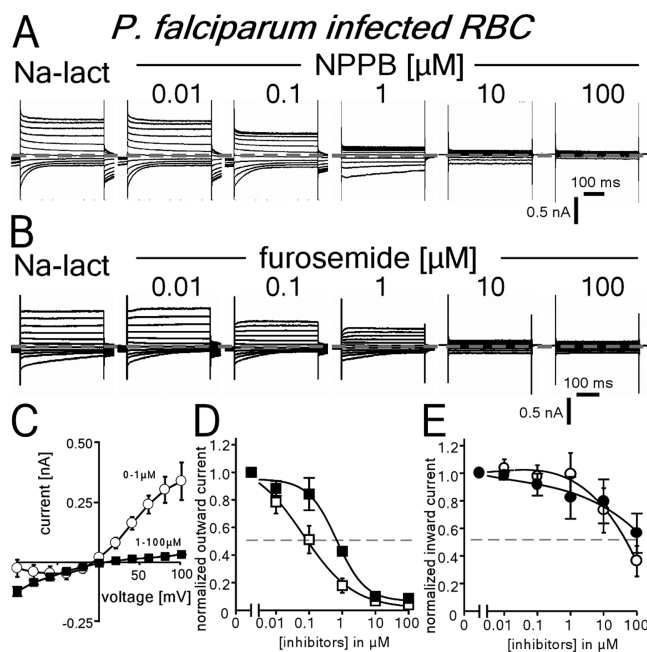


FIGURE 3. Blocker sensitivity of the *P. falciparum*-induced lactate permeability. (A and B) Current traces (Na-L-lactate was used in bath and in pipette solution) of an infected cell before (control) and during bath application of increasing concentrations (from 0.01 to 100 μ M) of NPPB (A) or furosemide (B). (C) Mean I-V relation of the current fractions (as recorded in F) sensitive to low ($\Delta I_{\text{low-NPPB}} = I_{\text{control}} - I_{\text{NPPB}} (1 \mu\text{M})$; circles) and high concentrations of NPPB ($\Delta I_{\text{high-NPPB}} = I_{\text{NPPB}} (1 \mu\text{M}) - I_{\text{NPPB}} (100 \mu\text{M})$; squares; means \pm SE; $n = 3$). (D and E) Dependence of the normalized outward (D) and inward current (E) on increasing concentrations of NPPB (open symbols) and furosemide (closed symbols). Currents were normalized by dividing the data by the value obtained at $+100$ mV (outward current) and -100 mV (inward current) under control conditions, respectively (data are means \pm SE; $n = 5$).

To test the stereo specificity of the lactate permeability, infected cells were recorded with NMDG-Cl pipette solution and NaCl or Na-D-lactate bath solution. Replacement of the NaCl bath solution by Na-D-lactate induced a decrease of the outward current and a shift of the V_{rev} to $+23.3 \pm 3$ mV (Fig. 2 F). Fig. 2 G shows that the mean reversal potential recorded either with Na-D- or Na-L-lactate bath solution did not differ (NMDG-Cl pipette solution), indicating that the lactate permeability did not discriminate between both lactate enantiomers.

To study the pharmacology of the lactate currents, infected cells were recorded with Na-L-lactate in the bath and pipette solutions at increasing concentrations of NPPB and furosemide, respectively. Both blockers inhibited first an outwardly rectifying current component and then at higher concentrations the remaining inwardly rectifying current fraction (Fig. 3, A and B). The IC_{50} s for outwardly and inwardly rectifying lactate currents were $\sim 0.1 \mu\text{M}$ and in the range of 30 μM for NPPB (Fig. 3, D and E, open symbols), and ~ 1 and 100

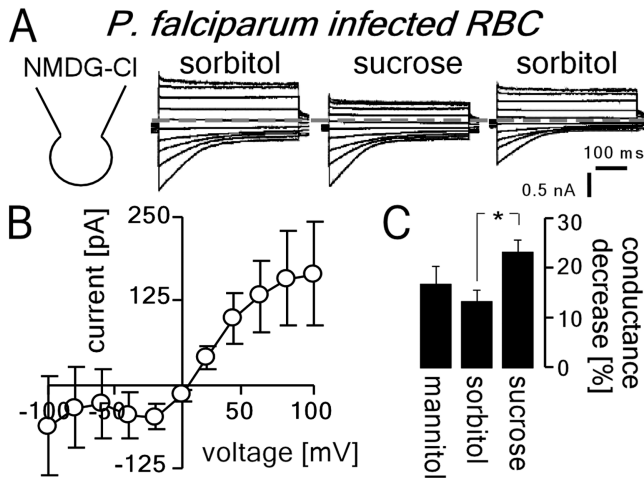


FIGURE 4. Influence of different carbohydrates on the *P. falciparum*-induced anion conductances. (A) Current traces recorded from an infected erythrocyte after partial replacement (50 mM NaCl) of the NaCl bath solution by isosmotic concentrations of sorbitol, sucrose, and again sorbitol (isotonic NMDG-Cl pipette solution). (B) I-V relation of the current fraction differentially inhibited by sorbitol and sucrose. Data are means \pm SE ($n = 6$) recorded as in A. (C) Mean decrease in outward conductance (\pm SE; $n = 6$) after partial bath replacement of NaCl (50 mM) by different carbohydrates (100 mM). Conductances were calculated from the individual I-V relations by linear regression of the outward current between +20 and +80 mV voltage.

μ M for furosemide, respectively (closed symbols). With the inhibition of outward current by low concentrations of NPPB (1 μ M), V_{rev} of the remaining (inwardly rectifying) current shifted significantly by $+25 \pm 1$ mV ($n = 3$) from $E_{lactate}$ toward E_{Cl} ($E_{Cl} = +23$ mV), suggesting no or a very low relative permeability for lactate ($P_{lactate}/P_{Cl} \ll 0.1$) of the inwardly rectifier as compared with the outward rectifier (Fig. 3 C, squares). The NPPB-1 μ M-sensitive outwardly rectifying current fraction reversed at +3 mV, indicating again a high relative lactate permeability ($P_{lactate}/P_{Cl} \sim 0.4$; Fig. 3 C, circles).

To test whether neutral carbohydrates influence the anion conductances, a fraction (50 mM) of the NaCl in the bath was iso-osmotically replaced by sorbitol, mannitol, or sucrose (100 mM each) during continuous whole-cell recording (NMDG-Cl pipette solution; Fig. 4). Partial isosmotic replacement of NaCl by sucrose, mannitol, or sorbitol each decreased the current (Fig. 4 C). Most importantly, sucrose inhibited the outwardly rectifying conductance significantly stronger than replacement with sorbitol (Fig. 4, A and C). The current fraction $\Delta I = I_{sorbitol} - I_{sucrose}$ showed an I/V relation typical for the outwardly rectifier (Fig. 4 B), indicating that the Cl^- current inhibited by neutral carbohydrates was outwardly rectifying.

To test for regulation of both *P. falciparum*-induced conductances by cell-volume changes, infected erythro-

cytes were swollen and shrunken by using osmolarity gradients between the pipette and the bath solutions (see MATERIALS AND METHODS). The osmolarity gradients induced changes in cell size of the whole-cell recorded cell as monitored by light microscopy (Fig. 5 A), indicating that osmolarity gradients indeed induced cell-volume changes of the recorded cell.

When infected cells were shrunken by hypotonic pipette solution, inward whole-cell currents inactivated time dependently within the first minutes and equilibrated within 4 min of recording (Fig. 5, C and E, triangles). This inactivation was not observed (within at least 10 min of recording), when cell-swelling was induced by a hypertonic pipette solution (Fig. 5, D and E, squares). In sharp contrast, the mean outward conductance recorded with hypotonic, isotonic, and hypertonic NMDG-Cl pipette solution remained virtually constant (Fig. 5 E). As a consequence, the cell volume-sensitive current fraction rectified inwardly (Fig. 5 F). Noninfected erythrocytes did not exhibit anion-selective whole-cell currents, even upon osmotic swelling by hypertonic NMDG-Cl pipette solution (isotonic NaCl bath solution; Fig. 5 B), indicating that the cell swelling-dependent inward current was induced by the infection.

In further experiments cells were first shrunken by addition of NaCl (25 mM) to the isotonic NaCl bath solution (isotonic NMDG-Cl pipette solution). Cell shrinkage induced a decrease of inward currents by $-45 \pm 12\%$ ($n = 6$) due to the cell volume dependence of the inwardly rectifying conductance without modifying the outward currents. Further addition of increasing concentrations of sucrose, in sharp contrast, reversibly decreased the whole-cell outward currents (Fig. 5 G). Fig. 5 H shows the I/V relations under control conditions (open circles) and after application of sucrose (400 mM, open triangles). The inhibited current fraction $\Delta I = I_{control} - I_{sucrose}$ (Fig. 5 H, closed diamonds) was outwardly rectifying, suggesting that the outward rectifier was inhibited by sucrose. The dose-dependent inhibition by sucrose of the relative outward conductance is shown in Fig. 5 I.

To compare the influence of neutral carbohydrates on lactate and Cl^- currents further experiments were performed using Na-lactate in pipette and bath solution or NMDG-Cl in the pipette and NaCl in the bath solution. Addition of mannitol (200 mM) to the bath reversibly decreased the whole-cell currents (Fig. 6 A). Similar to the action of sucrose on the Cl^- current (see Fig. 5 G), mannitol inhibited an outwardly rectifying fraction ($\Delta I = I_{control} - I_{mannitol}$) of the lactate current (Fig. 6 B). The relative inhibition exerted by mannitol on the lactate current was about two times higher than on the Cl^- current (Fig. 6 C), indicating that the inhibitory potency of the carbohydrate was dependent on the nature of the charge carrier.

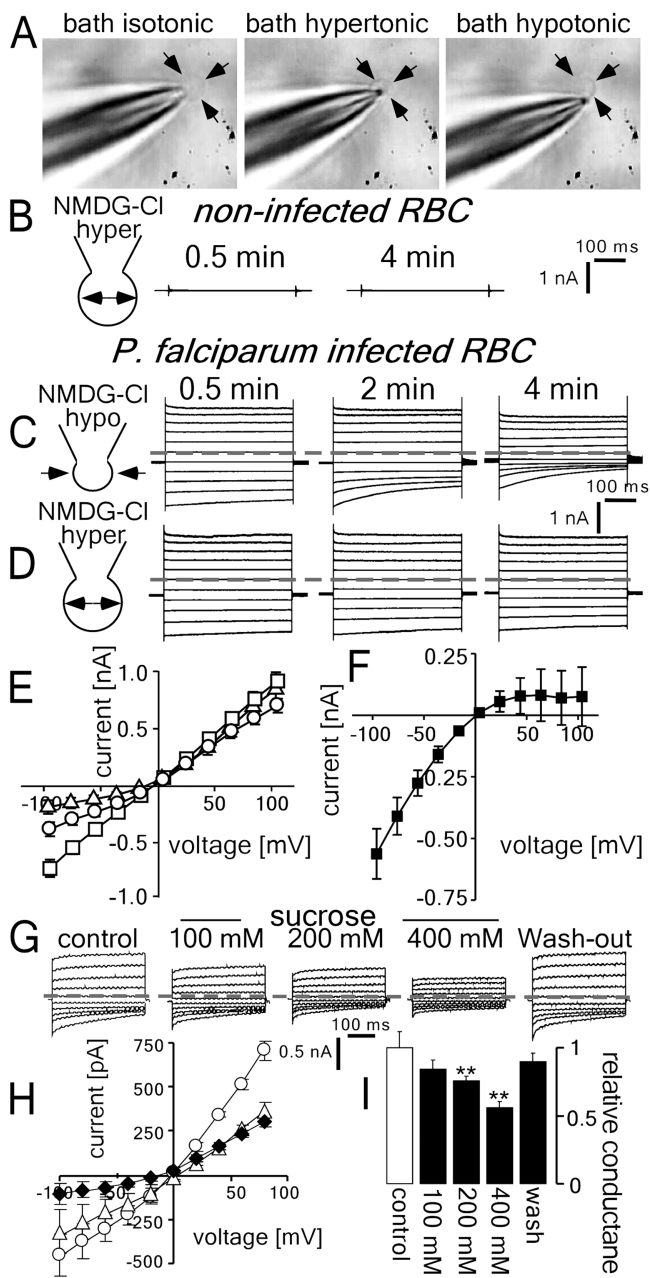


FIGURE 5. Effect of pipette and bath osmolarity on Cl^- whole-cell currents of *P. falciparum*-infected human erythrocytes. Inhibition by hyperosmotic addition of sucrose to the bath. (A) Cell volume change of a noninfected erythrocyte during continuous whole-cell recording in isosmotic NaCl bath solution (left), upon hypertonic cell shrinkage (+100 mM sorbitol; middle), and hypotonic reswelling (-50 mM NaCl; right). Ghost membrane is indicated by arrows. (B–D) Current traces recorded at different time points (as indicated) upon achievement of whole-cell recording mode. Records were obtained in noninfected (B) and infected erythrocytes (C and D) with hypertonic (B and D, ~330 mosM) and hypotonic (C, ~190 mosM) NMDG-Cl pipette solution. In these experiments, the noninfected and infected erythrocytes were permanently superfused with isosmotic NaCl bath solution. (E) Mean I-V relations (\pm SE) recorded as in C and D, 4 min after achievement of whole-cell configuration either with hypertonic (squares, $n = 17$), hypotonic (triangles, $n = 5$), or isotonic NMDG-Cl pipette

Finally, the NPPB and furosemide inhibition of the hemolysis in isosmotic sorbitol solution was compared with those of both conductances (Fig. 7, A and B). NPPB and furosemide inhibited the sorbitol hemolysis of infected erythrocytes in a dose-dependent manner (Fig. 7 A) with IC_{50} s ($\sim 1 \mu\text{M}$ for NPPB and $\sim 10 \mu\text{M}$ for furosemide; Fig. 7 A) similar to those for the outward rectifying lactate current (Fig. 3 D). In addition, the effect of sucrose on the infection-induced hemolysis was determined by isosmotic substitution of sorbitol with increasing concentrations of sucrose and—for control—of NaCl (Fig. 7 B). Replacement by sucrose but not by NaCl inhibited hemolysis of trophozoite-infected cells with an IC_{50} of $\sim 10 \text{ mM}$, indicating (a) competition between sorbitol and sucrose within the permeability pore and (b) low permeability of sucrose.

To further test for the inhibitory effect of sucrose on the sorbitol pathway of infected erythrocytes, isotopic flux measurements of ^{14}C sorbitol were performed under the experimental conditions used for the hemolysis experiments. Fig. 7 C shows the time course of the ^{14}C sorbitol uptake in isosmotic sorbitol solution. Within 2 min of incubation the uptake apparently saturated. Incubation (30 s) of the infected cells in the presence of 20 or 60 mM of sucrose (replacing the same amount of sorbitol) significantly decreased the uptake of ^{14}C sorbitol as illustrated in Fig. 7 D. In contrast, substitution of 20 and 60 mM of sorbitol by 10 and 30 mM of NaCl did not significantly decrease ^{14}C sorbitol uptake, respectively (Fig. 7 D).

DISCUSSION

The malaria parasite *Plasmodium falciparum* has been demonstrated to lower the concentration of imperme-

solute (circles; $n = 9$). (F) I-V relations of the volume-sensitive current fraction $I_{\text{swell}}-I_{\text{shrink}}$ as calculated from E by subtracting the currents recorded with hypotonic ($\sim 190 \text{ mosM}$, $n = 5$) from those obtained with hypertonic ($\sim 330 \text{ mosM}$, $n = 17$) NMDGCl pipette solutions. Currents were recorded with isotonic NaCl bath solution 4 min after achievement of whole-cell configuration. (G) Concentration-dependent inhibition of the outwardly rectifying Cl^- current by sucrose. Current traces recorded from an infected cell before and immediately after addition of increasing concentrations of sucrose to the NaCl bath solution, and after wash-out of sucrose (NMDG-Cl pipette solution). Prior to the application of sucrose, cells were preshrunk by addition of NaCl (+25 mM) to the bath solution. (H) Mean I-V relations (\pm SE; $n = 6$) of the outwardly rectifying conductance recorded as in E under control conditions (NaCl bath; circles) and after addition of sucrose (400 mM) to the bath (triangles). In addition, the calculated sucrose-sensitive current fraction is shown (diamonds). (I) Mean relative outward conductances (\pm SE; $n = 6$) in the absence (control) and presence of increasing concentrations of sucrose in the bath solution (as indicated), and after replacement of sucrose by NaCl solution (wash-out). Conductances were calculated from (E and F) by linear regression of the outward current between +20 and +80 mV voltage.

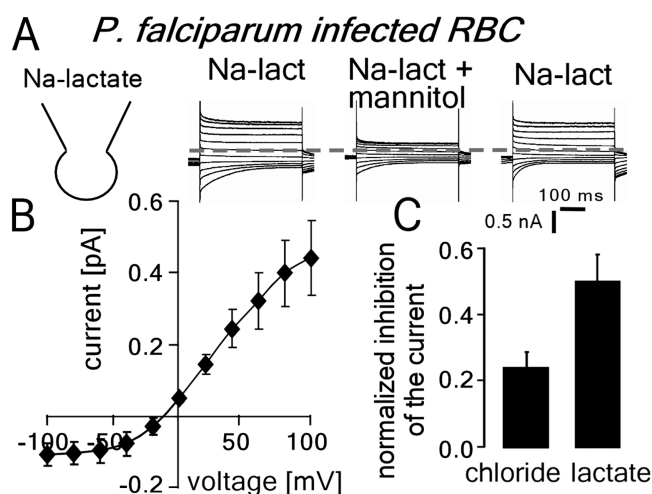


FIGURE 6. Comparison of the mannitol inhibition on the *P. falciparum*-induced lactate or Cl^- current. (A) Original current traces recorded from an infected cell (from -100 to $+80$ mV) before, and immediately after hyperosmotic addition of mannitol (200 mM) to the Na-L-lactate bath solution and after wash-out (Na-L-lactate pipette solution). (B) Mean I-V relation (\pm SE; $n = 4$) of the normalized current fraction which was sensitive to mannitol as recorded in A. The mannitol sensitive current fraction ($\Delta I = I_{\text{control}} - I_{\text{mannitol}}$) of the individual experiments was normalized to the control current at $+100$ mV voltage. (C) Inhibition of outward conductance by addition of neutral carbohydrates to the bath. Shown is the normalized inhibition of the outward conductance ($\Delta G = (G_{\text{control}} - G_{\text{mannitol}})/G_{\text{control}}$ as calculated between $+20$ and $+80$ mV voltage; \pm SE, $n = 4$) with lactate (data from A and B) or with chloride (NMDG-Cl pipette and NaCl bath solution) as principal anion.

able osmolytes in the host cytosol by excess hemoglobin digestion and discharge of the amino acids via the NPP (Lew et al., 2003). In addition, the NPP has been proposed to dispose of lactate since the endogenous erythrocytes transport pathways are not sufficient to cope with the rate of lactic acid production by the parasite (Poole and Halestrap, 1993). Thus, by the release of organic osmolytes the NPP counteracts infection-induced swelling and avoids premature lysis of the host cell (Kirk, 2001; Staines et al., 2001, 2003; Lew et al., 2003).

In the present and two previous reports (Huber et al., 2002; Staines et al., 2003), inwardly and outwardly rectifying whole-cell anion conductances were identified in *P. falciparum*-infected human erythrocytes. Recent studies using similar whole-cell protocols (Desai et al., 2000; Durantón et al., 2002, 2003b; Egee et al., 2002; Huber et al., 2002; Lang et al., 2003) indicate that these conductances are not active spontaneously in the majority of noninfected cells, but activate upon oxidative stress, protein kinase A stimulation, or membrane stretch (Egee et al., 2002; Huber et al., 2002). Thus, these conductances at least in part are generated

by endogenous erythrocyte proteins. In this study, no spontaneous activity of the inwardly and outwardly rectifying anion conductance was observed in noninfected cells. In addition, no activation of these conductances was elicited in noninfected cells by cell swelling or by the use of nonphysiological pipette and bath solutions, strongly suggesting that the observed conductances in parasitized cells were indeed induced by the infection but not by the experimental setup. In theory, the inwardly and the outwardly rectifying current phenotype of the infected erythrocyte might be generated by two individual channel proteins, by a multichannel complex, or by one molecule with two functional states. In any case, inward and outward rectifiers seem to be regulated differentially (serum, voltage, cell volume), pointing to different functions of both conductances for the erythrocyte-parasite interaction.

The inwardly and outwardly rectifying anion conductances of infected erythrocytes differed in lactate permeability (see Fig. 2). The highly lactate-permeable current fraction shows (a) NPPB and furosemide sensitivities, (b) time-dependent inactivation at hyperpolarizing voltage, and (c) outwardly rectifying current voltage relationship identical to those reported previously for the outwardly rectifying Cl^- currents (Huber et al., 2002). As demonstrated for the infection-induced NPP by tracer flux (Cranmer et al., 1995), this outwardly rectifying conductance did not differentiate between D- and L-lactate enantiomers thus exhibiting no stereo specificity.

As a consequence of its rectification behavior, the outward rectifier mediates a much higher lactate influx into the cell at positive membrane potentials than lactate efflux at negative potentials. This voltage dependence, therefore, does not seem to be optimally suited for the disposal of lactate out of the cells. Nevertheless, the outward rectifier activated at high negative voltages and generated lactate inward currents in the range of -40 pA at -40 mV voltage (see the $1 \mu\text{M}$ NPPB-sensitive current fraction in Fig. 3 C, open circles). This current corresponds to the permeation of ~ 50 fmol lactate per cell and second. This value is huge compared with the calculated lactate disposal of infected cells (0.003 fmol per cell and second; Poole and Halestrap, 1993), suggesting that the outwardly rectifying conductance can indeed transport physiologically relevant amounts of lactate.

The inwardly rectifying conductance, in sharp contrast to the outward rectifier, was much less lactate permeable. These data seem to be at variance to a previous study (Desai et al., 2000) reporting a high relative lactate permeability for an infection-induced inwardly rectifying anion conductance, but in this report the authors did not observe outwardly rectifying currents. The inward rectifier in this previous study (Desai et al.,

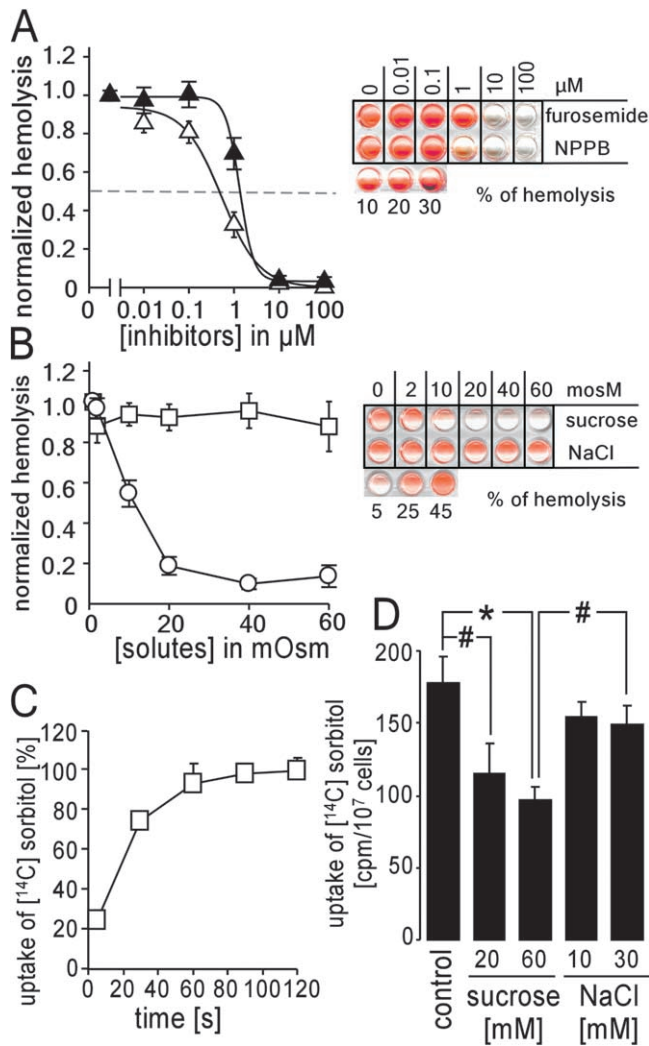


FIGURE 7. Inhibition by NPPB, furosemide, and sucrose of the sorbitol-induced hemolysis. (A) Enriched trophozoide-infected erythrocytes were incubated (10 min/21°C) in isosmotic sorbitol solution in the presence of increasing concentrations (0.01–100 μM) of NPPB (open triangles; $\pm\text{SE}$, $n = 12$) or furosemide (closed triangles, $\pm\text{SE}$; $n = 9$). Incubation was stopped by centrifugation and hemolysis was indicated by hemoglobin in the supernatant. In the insert on the right are the scanned images of supernatants from an individual experiment. The hemoglobin concentration of the supernatant for each condition (NPPB and furosemide) was determined photometrically (546 nm). Data are expressed as the percentage of the 100 μM NPPB inhibited fraction (maximal inhibition = $86 \pm 2\%$ of total hemolysis, left). (B) Substrate competition of the infection-induced sorbitol hemolysis. Hemolysis (10 min/21°C) was assessed in solutions where different quantities of sorbitol (0–120 mosM) have been isosmotically replaced by sucrose (circles) or NaCl (squares). Mean normalized hemolysis ($\pm\text{SE}$; $n = 8$; left) and imaged supernatants of an individual experiment (right) are shown as in A. (C) Uptake of [^{14}C] sorbitol into *P. falciparum*-infected human erythrocytes incubated in isosmotic sorbitol solution. The uptake of radiolabeled sorbitol was measured after 5, 30, 60, 90 and 120 s of incubation. Individual data of three experimental series were expressed as a percentage of the respective 2 min values and averaged (means $\pm\text{SE}$; $n = 4$ –12). (D) Inhibition of [^{14}C] sorbitol uptake in isosmotic sorbi-

2000) resembled the outward rectifier of the present study by the relative lactate permeability (0.4 and 0.43, respectively) and the high sensitivity to furosemide and NPPB (1 μM furosemide and 0.8 μM NPPB inhibited 30% and 41% of the inward rectifier in the previous study while 1 μM furosemide and 1 μM NPPB inhibited $\sim 55\%$ and 80% of the outward rectifier in the present work). This might suggest that the inward rectifier of the previous study (Desai et al., 2000) and the outward rectifiers of the present study possibly represent modifications of the same conductance type. The inward rectifier of this previous study, however, differs substantially from the inward rectifier of the present study (relative lactate permeability <0.1 ; IC_{50} for NPPB and furosemide $>10 \mu\text{M}$). The present study further discloses that the inwardly rectifying conductance is sensitive to cell volume: Cell shrinkage inactivates and cell swelling activates the inward rectifier. The outwardly rectifying conductance, in contrast, did not exhibit any apparent cell volume dependence.

In addition to cell volume regulation, the infection-induced NPP of the host cell membrane supplies the parasite with nutrients, molecules in general larger than lactate. For instance, the parasite needs pantothenate for coenzyme A synthesis (Saliba et al., 1998) and isoleucine for protein synthesis (which cannot be provided by hemoglobin digestion; Sherman, 1983). To test for the putative permeabilities of the two infection-induced anion conductances to large molecules, the present study analyzed the interference of neutral carbohydrates with the Cl^- and lactate currents of infected erythrocytes. As a result, neutral carbohydrates applied iso-osmotically or iso-ionically inhibited a current fraction with a current-voltage relation typical for the outwardly rectifier. This inhibition occurred instantaneously upon carbohydrate application, was concentration dependent, reversible, and not dependent on cell swelling or shrinkage. These data indicate that the effect of the carbohydrates is not the result of changes in ionic strength or tonicity of the bath solution or of increase in transmembrane osmotic pressure. The efficacy of sorbitol, mannitol, and sucrose was not identical, indicating that the inhibitory effect on the outwardly rectifying Cl^- current is dependent on the nature of the osmolyte. In addition, the inhibitory effect of any given carbohydrate species was dependent on the nature of the charge carrier: mannitol in the

tol by sucrose. Mean uptake ($\pm\text{SE}$; $n = 9$ –10) of [^{14}C] sorbitol (30 s values minus the 5 s blank value) in isosmotic sorbitol solution (control) or in sorbitol solutions where 20 and 60 mM of sorbitol were replaced by 20 and 60 mM of sucrose or 10 and 30 mM of NaCl, respectively (uptake conditions: $3.7 \times 10^4 \text{ Bq/ml}$ [^{14}C] sorbitol, 2.5% final hematocrit, $>90\%$ parasitemia). * and # correspond to $P \leq 0.01$ and 0.05, respectively, ANOVA.

bath (200 mM) inhibited the lactate current by some 50% but the Cl⁻ current only by some 25%. This interplay between charge carrier and neutral carbohydrate strongly suggests a competition between neutral and the charged solute within the permeation pore rather than an allosteric effect of the carbohydrates on the channel protein. The outwardly rectifying lactate current and the sorbitol permeability (as measured by isosmotic hemolysis) were similarly sensitive to NPPB and furosemide. Finally, disaccharides reportedly block isosmotic sorbitol hemolysis of infected cells (Ginsburg et al., 1983) but, in contrast, failed to inhibit [¹⁴C] sorbitol uptake (Ginsburg et al., 1985). In this study, sucrose blocked the hemolysis in isosmotic sorbitol solution and exerted a moderate but significant inhibitory effect on the outwardly rectifying Cl⁻ current (IC₅₀ in the range of 400 mM). In contrast [¹⁴C] sorbitol uptake in sorbitol solution was inhibited by 20 mM sucrose. Moreover, the [¹⁴C] sorbitol uptake in isosmotic sorbitol solution reached a plateau within 1–2 min, whereas uptake saturation in NaCl reportedly is not complete at 30 min of incubation (Ginsburg et al., 1985). This suggests that the sorbitol permeability itself and the competition between sorbitol and sucrose at the pore are strongly depending on the ionic environment. E.g., suspending the infected cells in isosmotic sorbitol strongly depolarizes (<0 mV in NaCl and >100 mV in sorbitol) the erythrocyte membrane potential, enhancing the conductance of the outwardly rectifying anion channel.

The sucrose inhibition of the sorbitol hemolysis and uptake points to a competition between both substrates with a permeability of sorbitol >> sucrose. Permeability for sugars/polyols in the rank order of sorbitol > mannitol >> sucrose have been demonstrated for *P. falciparum*-infected (Ginsburg et al., 1985; Huber et al., 2002) and -oxidized erythrocytes (Huber et al., 2002) by isosmotic hemolysis experiments. This rank order is inverse to the carbohydrate inhibition of the outwardly rectifying current which occurs in the sequence of sucrose (> mannitol) > sorbitol. This inverse correlation suggests that (a) channels that are occupied by carbohydrates are not permeable for charged molecules and (b) slowly (or non) permeating carbohydrates are occupying the channel pore for a longer period and are therefore more potent inhibitors of the current than fast permeating carbohydrates. Together, all those observations indicate that the outwardly rectifier participates in the sorbitol permeation pathway.

In conclusion, *P. falciparum*-infected erythrocytes express two types of anion conductances at the mid-late trophozoite stage of parasite development. The inwardly rectifying anion conductance is activated by cell swelling. In contrast, the outwardly rectifying current is insensitive to cell volume and might be involved in transport of lactate, amino acids, and further organic osmolytes.

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