A High Specificity and Affinity Interaction with Serum Albumin Stimulates an Anion Conductance in Malaria-Infected Erythrocytes

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Key Words

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Abstract

The intraerythrocytic development of *P. falciparum* induces New Permeability Pathways (NPP) in the membrane of the parasitized erythrocyte which provide the parasite with nutrients, adjust the erythrocyte electrolyte composition to the needs of the parasite, and dispose of metabolic waste products and osmolytes. Patch-clamp recordings identified inwardly and outwardly rectifying (OR) anion conductances in the host erythrocyte membrane as electrophysiological correlate of the NPP. The OR conductance is regulated by serum. Here we show that serum albumin (SA) stimulated OR-generated Cl⁻ and lactate outward currents with an EC₅₀ of approximately 100 nM while other proteins such as ovalbumin or casein did not. The stimulatory efficacy did not differ between fatty acid free bovine SA and recombinant human SA and disruption of the SA tertiary structure abolished the

effect suggesting that intact SA protein and not other bound factors interact with the erythrocyte membrane. Taken together, the data indicate a high affinity and specificity interaction of native SA with the parasitized erythrocytes which might underlie the observed dependence of *P. falciparum* growth *in vitro* on SA.

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Introduction

Human erythrocytes infected with the malaria parasite *Plasmodium falciparum* acquire New Permeability Pathways (NPP) [1]. The NPP provide the intraerythrocytic parasite with essential nutrients such as pantothenate and dispose of metabolic waste products such as lactic acid [1]. In addition, the NPP-generated cation leak decreases the K⁺- and increases the Na⁺ concentration of the erythrocyte cytosol [2]. By this way, the NPP is building up an electrolyte environment for the intraerythrocytic parasite which resembles that of the blood and which is required for the parasite development

[3]. Short-circuiting the chemical gradients for K^+ and Na^+ across the erythrocyte membrane, however, results in colloid osmotic swelling and eventually hemolysis of the host erythrocyte. To avoid premature hemolysis, the parasitized erythrocyte and the parasite in concert lower the colloid osmotic pressure of the erythrocyte cytosol. The parasite digests hemoglobin in excess, i.e., much more than needed for its protein biosynthesis, and the host erythrocyte exports the hemoglobin-derived amino acids through the NPP [4]. Thus, the NPP serve multiple functions for the intraerythrocytic parasite development and, therefore, is considered as a potential target for future antimalarial therapy.

The permeabilities and the pharmacology of the NPP have been characterized by the use of tracer flux measurements and isosmotic hemolysis experiments. The latter method utilizes the new permeabilities to haemolyse the infected cells osmotically in isosmotic solutions of newly permeable solutes in which uninfected erythrocytes stay intact. These studies indicate that the NPP are anion-selective channels in the erythrocyte membrane which are also permeable for organic osmolytes and to a lesser extent for cations [5]. Previous studies suggest that the NPP is not generated by a unique broad specificity pathway but rather by at least two types of pathways [6, 7]. Accordingly, patch-clamp single channel and whole-cell studies performed on infected erythrocytes have identified up-regulation of at least four different types of channels: i) very low conductance inwardly rectifying ClC-2 anion channels [8] (which probably are identical [9] with supposed *Plasmodium*-encoded PSAC channels [10]), ii) *Plasmodium*-stimulated low conductance inwardly rectifying anion channels (IR) [11, 12], iii) outwardly rectifying anion channels (OR) [13], and iv) non-selective cation channels [14, 15] as putative electrophysiological correlates of the NPP. These channels differ in permselectivity, voltage dependence, and inhibitor sensitivities suggesting that they are generated by different proteins [16].

The channel type(s) generating the organic osmolyte permeability of parasitized erythrocytes is still under debate. A previous meta-study comparing data from tracer flux and isosmotic hemolysis experiments concluded that anions and nucleosides permeate through highly abundant anion-selective channels while other neutral osmolytes permeate through less abundant slightly cation-selective channels [6]. Upregulation of non-selective cation channels in *Plasmodium falciparum*-infected erythrocytes became evident in whole-cell patch-clamp record-

ing upon removal of extracellular Cl^- [14, 15]. To which extend these channels contribute to the osmolyte permeability has not yet been defined. The ClC-2(PSAC) anion channels are not organic osmolyte channels and *P. berghei* grows well in ClC-2-deficient mice [8]. The IR is activated in uninfected erythrocyte by stimulation of the protein kinase A signalling [11] and depends on CFTR [12, 17]. *P. falciparum* develops well in cystic fibrosis (CF) erythrocytes and parasitized CF erythrocytes (albeit lower IR activity) have an organic osmolyte permeability identical to parasitized healthy human erythrocytes. Moreover, IR-generated currents and the osmolyte permeability as determined by non-electrophysiological means have opposite voltage dependence [7]. Taken together these observations suggest that the IR is not the only organic osmolyte channel in parasitized human erythrocytes. This conclusion is further supported by the fact that stimulation of uninfected erythrocytes with membrane-permeable cAMP derivatives does not induce an osmolyte permeability (unpublished own observations).

The OR, however, is permeable for organic osmolytes as deduced from the following observations: i) OR has a high lactate permeability [18]; ii) electroneutral carbohydrates modify the Cl^- and lactate currents through OR suggesting competition of organic osmolytes and anions in the channel pore [18]; iii) OR and the organic osmolyte permeability - as assessed by hemolysis in isosmotic sorbitol solution - develop with similar time course during the *P. falciparum* blood cycle [19]; iv) the up-regulation of both, OR and sorbitol permeability, depends on ATP-release and autocrine signalling through P2Y1 purinoceptors [19]; v) OR and the sorbitol permeability - as assessed by single cell hemolysis - are both stimulated by serum albumin [7, 20]. In summary, these observations indicate an involvement of OR in nutrient supply, disposal of metabolic waste, and reduction of colloid osmotic pressure and, therefore, a high functional significance of OR for the intraerythrocytic parasite development.

The stimulating effect of serum albumin on the activity of OR can be mimicked by erythrocyte-erythrocyte contact or by hemolysate [7] suggesting a more unspecific interaction between proteins in the serum on the one hand and the infected erythrocyte on the other. The present study aimed to characterize this interaction by the use of patch-clamp whole-cell recording in terms of serum albumin specificity and affinity of its binding to the infected erythrocyte.

Materials and Methods

Parasite culture

The *P. falciparum* strain FCR-3 or BINH were permanently maintained in culture using a modified protocol of Trager and Jensen [21] as described [19]. In brief, RPMI medium 1640 (Biochrom) was supplemented with 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH pH 7.4, 20 µg/ml gentamicin sulphate, 2 mM glutamine, 200 µM hypoxanthine, 0.5% Albumax II™ (Gibco-BRL). Washed human erythrocytes of blood group O⁺ (banked) were added to a hematocrit of 5%. Parasites (FCR3 and BinH) 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 infected erythrocytes were taken directly from the asynchronous culture. For control experiments, erythrocytes from healthy donors (donors gave informed consent) were used. The experiments have been approved by the local ethic commission.

Patch-clamp recordings

Mid/late trophozoite stages of *P. falciparum* infected erythrocytes were chosen optically by the presence of the light dense hemozoin-containing food vacuole. Most patch-clamp experiments were performed at room temperature. To control for a temperature effect, few experiments were performed at 37°C. Continuous superfusion (~1 ml/min) was applied through a flow system inserted into the dish (bath volume approx. 200 µl). 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, Harvard Apparatus, March-Hugstetten, Germany) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a STM electrical micromanipulator (Lang GmbH and Co KG, Germany). Currents were recorded in fast whole-cell, voltage-clamp mode (10 kHz sampling rate) and 3-kHz low-pass-filtered by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY, USA). After giga-Ohm seal formation (averaged seal resistance > 10 gigaohms), the membrane was ruptured by additional suction and/or brief electrical pulses (~700 mV during 100 to 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 between the pipette and the bath solutions and between the salt bridge and the bath solutions were estimated according to [22]. Data were corrected for the estimated liquid junction potentials. Whole-cell currents were evoked by 10-11 voltage pulses (400 or 700 ms each) from 0 mV or -30 mV holding potential to voltages between -100 mV 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 (650 and 675) ms of each square pulse. Paired experiments were performed in which several bath solutions were applied during continuous whole-cell recording. Since recording time until loss of seal was limited, short interpulse intervals were chosen in order to shorten the recording times between the solution exchanges. As a result of the short interpulse intervals, the holding current after a test pulse did not exactly reach the basal value before execution of the next test pulse (see Fig. 1A, 4th recordings) and, therefore, varied slightly bit within a pulse series.

Solutions and chemicals

Cells were recorded with a NMDG-Cl pipette solution containing (in mM): 140 N-methyl-D-glucamine-Cl (NMDG-Cl, pH 7.4), 10 HEPES/NMDG (pH 7.4), 1 MgCl₂, 1 Mg-ATP, 1 ethylene glycol-bis-(aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). In some recordings (Fig. 5A, B) a Na-gluconate pipette solution was used containing (in mM): 145 Na-D-gluconate, 10 HEPES/NaOH (pH 7.4), 5 MgCl₂, 1 Na₂-ATP, 1 EGTA. Cells were superfused with a NaCl bath solution which promotes frequent giga ohm formation containing (in mM): 115 NaCl, 10 MgCl₂, 5 CaCl₂, 20 HEPES/NaOH (pH 7.4) (Figs. 1A-D, 2, 3, 4, 5C, E) or a more physiological, glucose-containing extracellular solution (in mM): 125 NaCl, 32 HEPES/NaOH (pH 7.4), 5 KCl, 5 D-glucose, 1 MgCl₂, 1 CaCl₂ (Fig. 5A, B). The data did not differ between both solutions and were pooled for Figs. 1E, F, and 5D, F). In Fig. 6, the NaCl bath solution was replaced by Na-lactate solution containing (in mM): 145 Na-L-lactate, 10 HEPES/NaOH (pH 7.4), 1 CaCl₂.

Ultra-pure recombinant human serum albumin (rHSA, Recombum® albumin) was a kind gift from Novozymes Delta Limited (Nottingham, UK). All other chemicals were purchased from Sigma (Taufkirchen, Germany). For some experiments, rHSA (dilution of 0.1% in Ringer solution) was reduced by 10 mM dithiothreitol DTT (10 min) and the SH groups of the protein finally protected by iodoacetamide (50 mM for 30 min).

In further experiments the effect of rHSA fragments on the whole-cell currents of parasitized erythrocytes was studied. rHSA was diluted with phosphate buffered saline (PBS) to 0.2 % (w/v) and aliquots were incubated for 3 h at 37°C with trypsin (1 mg/ml) or formic acid (1:1; v:v). After treatment with trypsin the generated peptides were separated from uncleaved rHSA using centrifugal filter units (cutoff 5 kDa). The formic acid treated HSAr was separated into 3 fractions (filtration cutoff 50 kDa and 30 kDa) which subsequently were dialysed against 100 mM phosphate buffered saline for 8 hours.

Results

As demonstrated earlier [13], inwardly (IR) and outwardly rectifying (OR) anion-selective currents were observed in erythrocytes infected with *P. falciparum* in the trophozoite stage of the parasite's blood cycle. Cells directly taken from the culture medium have both

Fig. 1. Infection of human erythrocytes with *P. falciparum* induces inwardly and outwardly rectifying whole cell Cl^- currents.

A. Typical current tracings of an infected erythrocyte freshly withdrawn from the culture medium. Currents were elicited by 400-ms square pulses to test potentials between -100 and +80 mV (zero current is indicated by dashed line, region of data analysis by the vertical line shown for the 1st tracings) and recorded with NMDG-Cl pipette and constantly superfused with NaCl bath solution. Recordings were obtained first at 0 mV holding potential (first three tracings) and thereafter at -30 mV holding potential (last two tracings) in the absence (1st, 3rd and 4th tracings) and presence of NPPB (0.3 μM) in the bath solution (2nd and 5th tracings). **B-D.** Current-voltage (I/V) relationships of the total current (**B**) and the NPPB (0.3 μM)-sensitive (**C**) and NPPB (0.3 μM)-insensitive current fractions (**D**) recorded at 0 mV (open circles) and -30 mV holding potential (closed triangles) as indicated. Data were calculated from the experiment in (**A**) [the calculated NPPB-sensitive positive current at -100 mV voltage (closed triangles in Fig. 1C) results artificially from a spontaneous increase of the NPPB-insensitive IR current fraction during this particular recording (compare 4th and 5th tracings in Fig. 1A). The mean calculated NPPB-sensitive current at -100 mV, as depicted in Fig. 1E, is not different from 0 pA]. **E.** Mean current-voltage relationships ($\pm$ SE, $n = 3$) of the total current (open circles), the NPPB (0.3 μM)-sensitive current fraction (closed diamonds) and the NPPB (0.3 μM)-insensitive current fraction (closed triangles). Currents were recorded as in **A** (4th and 5th tracings) with -30 mV holding potential. Currents of the individual cells were normalized to the total current at +80 mV test voltage. **F.** Mean ratio ($\pm$ SE; $n = 3$) between the outward (G_o) and inward conductance (G_i) as calculated from the data in (**E**) by linear regression between 0 and +60 mV and between -60 and 0 mV, respectively. A ratio > 1 indicates an inward rectification while a ratio < 1 reveals an outward rectification (*: $p \leq 0.05$; **: $p \leq 0.01$, ANOVA).

current fractions. Reportedly, OR and IR differ in their sensitivity to the anion channel inhibitor NPPB [13] and in their dependence on the patch clamp holding potential [20]. In accordance to these previous studies [13] bath application of NPPB (0.3 μM) to infected erythrocytes inhibited a current fraction with OR phenotype while leaving an IR current fraction unchanged (Fig. 1). The latter was inhibited only at higher NPPB concentrations (data not shown and [13]). As reported earlier [20, 23], hyperpolarizing the holding potential from 0 mV to -30 mV changed the current phenotype. In particular, the NPPB-sensitive (Fig. 1A, 1st and 4th tracings and Fig. 1B, C) but not the NPPB-insensitive current fraction (Fig. 1A, 2nd and 5th tracings and Fig. 1D) was modified. The inward currents of the NPPB-sensitive fraction inactivated slowly at high negative test voltages when cells were held for some minutes at a holding potential of -30

mV. As a consequence of this time-dependent inactivation, the outward rectification of the OR current increased when analyzing the current values at the end of the test pulses (Fig. 1C, E-F). The comparison of the NPPB-insensitive current fractions recorded at 0 and -30 mV clearly excludes the possibility that the NPPB blockage itself was dependent on voltage or holding potential.

At -30 mV holding potential, tail inward currents were apparent upon repolarization from positive test voltages to the holding potential (Fig. 1A, 4th tracings). Because of the chosen short interpulse intervals (see Method section) these tail currents resulted in variations of the holding currents prior to the voltage pulses (Fig. 1A, 4th tracings). The tail currents were not due to instabilities of the electrical recordings but resulted from the slow deactivation kinetics of the OR current fraction, since NPPB (0.3 μM ; see Fig. 1A, 4th and 5th tracings) abo-

Fig. 2. Effect of membrane washing on whole-cell Cl^- currents of *P. falciparum*-infected human erythrocytes. A. Current tracings of infected erythrocytes (pRBC) freshly withdrawn from the culture medium (left) or after washing (right, 4 times for 20 min in serum albumin-free culture medium). Currents were recorded at -30 mV holding potential with NMDG-Cl in the pipette and NaCl in the bath. B. Mean I/V relationships ($\pm$ SE) recorded as in (A) in infected erythrocytes freshly taken from the culture (open circles, $n = 22$) or after washing (closed squares, $n = 32$).

lished both, the tail currents and the variation of the holding current prior to the test pulses. This is also evident from the observation that inactivation and activation of the OR current by wash-out and addition of serum albumin was accompanied by a de- and increase of holding current variations and tail currents, respectively (see Figs. 2 to 6). These experiments clearly demonstrate that only the slowly in-/deactivating current fraction, i.e., the OR current but not the holding potential- and time-independent and IR fraction is inhibited by $0.3\mu\text{M}$ NPPB. Both, the NPPB-sensitive OR and the less sensitive IR currents reversed at 0 mV voltage (Fig. 1B-D) when recorded with the chloride salt of the impermeant cation NMDG in the pipette and NaCl in the bath confirming the previously defined anion-selectivity of both current fractions.

Under the applied recording conditions (holding potential of -30 mV), the overall whole-cell current phenotype of trophozoite-infected erythrocytes directly derived from the culture was weakly outwardly rectifying (Fig. 1E, open circles) and most of the outward current was generated by the NPPB ($0.3\mu\text{M}$)-sensitive OR cur-

Fig. 3. Effect of recombinant human serum albumin (rHSA) on the whole-cell Cl^- currents of washed uninfected and infected erythrocytes. A. Current tracings of an uninfected erythrocyte recorded at -30 mV holding potential with NMDG-Cl pipette and NaCl bath solution before and after addition of recombinant human serum albumin (rHSA; 0.1%) to the bath solution. B. Current tracings of a washed *P. falciparum*-infected RBC recorded as in (A) during addition of increasing concentrations of rHSA to the bath solution. C,D. Corresponding I/V relationships ($\pm$ SE, $n = 7$) of the whole-cell currents (C) and of the rHSA-activated current fractions (D) obtained in paired experiments as depicted in (B) from washed cells before (closed circles) or after exposure to 0.0001% (open diamonds), 0.001% (open squares), 0.01% (open triangles) or 0.1% rHSA (open circles).

rent fraction (Fig. 1E, closed diamonds). Fig. 1F compares the rectification behaviour of the total, the NPPB ($0.3\mu\text{M}$)-sensitive and the NPPB-insensitive current fractions.

On average, the mean total outward currents ($\pm$ SE; $n = 22$, holding potential of -30 mV) of trophozoite-parasitized cells amounted to 1124 ± 57 pA at 80 mV test voltage. Washing the cells (4 times for 20 min in serum- and Albumax-free culture medium) prior to whole-cell recording led to a change of the current phenotype. The mean outward currents ($\pm$ SE) declined significantly ($p < 0.001$; two-tailed Welch-corrected t-test) to 506 ± 31

Fig. 4. Effects of bovine serum albumin (BSA) on the whole-cell Cl^- currents of washed infected erythrocytes. **A.** Current tracings of a washed *P. falciparum*-infected RBC recorded at -30 mV holding potential with NMDG-Cl pipette and NaCl bath solution during addition of increasing concentration of BSA to the bath solution. **B, C.** Corresponding I/V relationships ($\pm$ SE, $n = 7$) of the whole-cell currents (**C**) and of the rHSA-activated current fractions (**D**) obtained in paired experiments as depicted in (**A**) from washed cells before (closed circles) or after exposure to 0.0001% (open diamonds), 0.001% (open squares), 0.01% (open triangles) or 0.1% BSA (open circles). **D.** Dependence of mean whole-cell outward currents ($\pm$ SE; $n = 7$) of washed infected erythrocytes on rHSA (closed circles) and BSA concentrations (open circles). Outward currents in Fig. 3B (rHSA) and 4B (BSA) were analyzed at $+80$ mV voltage.

pA ($n = 32$;) at $+80$ mV voltage while the inward currents did not change (Fig. 2A). As a result, the current-voltage relationship changed from an outwardly rectifying to a sigmoid curve (Fig. 2B) indicating partial inactivation of the OR current during the washing procedure. The outward currents in washed cells (Fig. 2A 2nd tracings and Fig. 2B, closed squares), however, were still higher than those of the NPPB-treated un-washed cells (Fig. 1A, 5th tracings and Fig. 1D). In addition, washed cells in contrast to NPPB-treated cells still exhibited slowly inactivating inward currents which indicated a residual OR current activity. The comparison of NPPB-treated and washed infected erythrocytes suggested inactivation of more than 50% of the OR currents during washing.

For the further experiments washed erythrocytes were used to define the factors in serum (Albumax, bovine serum albumin) that stimulate the reported re-activation of the whole-cell currents in trophozoite-parasitized human erythrocytes [20, 23]. As a result, neither ATP (100 μM , $n = 5$), nor IGF-1 (50 ng/ml, $n = 7$), IGF-2 (0.1 ng/ml, $n = 3$), erythropoietin (1 U/ml, $n = 3$), insulin (10 $\mu\text{g}/\text{ml}$, $n = 4$), PGE_2 (5-10 μM , $n = 3$), or thromboxane (10 μM , $n = 2$) induced any significant increase of the whole-cell currents when applied acutely during continuous whole cell recording (data not shown). In sharp contrast, ultrapure recombinant human serum albumin (rHSA, recombuninTM), while having no effect on whole-cell currents of uninfected human erythrocytes (Fig. 3A), activated the OR currents of parasitized erythrocytes (Fig. 3B). rHSA stimulated the currents of trophozoite-parasitized erythrocyte during continuous whole-cell

recording within few minutes of superfusion in a dose-dependent and reversible manner (Fig. 3B,C). In particular, rHSA (0.1% w/v rHSA which is equivalent to ~ 15 μM) increased the outward currents (at $+80$ mV voltage) significantly ($p \leq 0.001$; two-tailed paired t-test) from 544 ± 36 pA to 1142 ± 58 pA ($n = 7$, Fig. 3C). The outward current of rHSA-stimulated cells did not differ from that of cells directly taken from the culture medium (see above). The rHSA-induced current ($\Delta I = I_{\text{BSA}} - I_{\text{control}}$) was strongly outwardly rectifying and exhibited a reversal potential close to 0 mV (i.e., at the Cl^- equilibrium potential defined by the NMDG-Cl pipette and the NaCl bath solution; Fig. 3D). The rHSA-induced current inactivated slowly (within 20 min) during continuous superfusion with albumin-free washing solution (data not shown). This indicates reversible activation of the OR current fraction by rHSA. Stimulation of washed infected erythrocytes with rHSA (0.1%) at 37°C incubation temperature also significantly ($p \leq 0.01$; two-tailed paired t-test) increased the outward currents (at $+80$ mV voltages) from 662 ± 154 pA to 1130 ± 160 pA ($n = 3$, data not shown) indicating that the stimulatory effects of rHSA did not differ between 37°C and room temperature.

Recombinant HSA is produced in yeast. To exclude the possibility that contaminations of rHSA by yeast-derived factors confer the effect on OR rather than rHSA itself, the experiments were repeated with fatty acids-free bovine serum albumin (BSA). As shown in Fig. 4A-C, BSA exerts a stimulatory and dose-dependent effect on OR identical to that observed with rHSA.

Fig. 5. Ovalbumin, casein and reduced rHSA fail to activate the whole-cell Cl⁻ currents in washed infected erythrocytes. rHSA has no re-inducing effect on the biotinylation-inactivated currents. A. Whole-cell current tracings of a washed infected erythrocyte before (1st tracings) and during consecutive exposure to ovalbumin (1%; 2nd tracings), casein (1%; 3rd tracings) and rHSA (0.1%, 4th tracings) and after wash-out of rHSA (5th tracings). B. Mean conductance increase ($\pm$ SE; $n = 3-4$) induced by casein (1%, left) or ovalbumin exposure. C. Current tracings of a washed infected erythrocyte before (left) and after stimulation with reduced rHSA (0.1%, middle) and non-reduced rHSA (0.1%, right). D. Mean conductance increase ($\pm$ SE; $n = 3$) stimulated by rHSA (open bar) or reduced rHSA (0.1% each). E. Effect of rHSA on the whole-cell current of a washed infected erythrocyte before (2nd trace) and after irreversible inactivation (5th trace) of the outwardly rectifying current by covalent modification with LC-sulfo-NHS-(+)-biotin (1 mM for 2 min). F. Mean conductance increase ($\pm$ SE; $n = 3$) stimulated by rHSA in washed untreated (open bar) or biotinylated infected erythrocytes (closed bar). Recordings were obtained at -30 mV holding potential with Na-gluconate (A, B) or NMDG-Cl pipette solution (C-F) combined with NaCl bath solution. The conductances in (B), (D) and (E) were calculated by linear regression of the current voltage relationship between 0 and +80 mV (*: $p \leq 0.05$; **: $p \leq 0.01$, two-tailed t-test).

valent modification with LC-sulfo-NHS-(+)-biotin (1 mM for 2 min). F. Mean conductance increase ($\pm$ SE; $n = 3$) stimulated by rHSA in washed untreated (open bar) or biotinylated infected erythrocytes (closed bar). Recordings were obtained at -30 mV holding potential with Na-gluconate (A, B) or NMDG-Cl pipette solution (C-F) combined with NaCl bath solution. The conductances in (B), (D) and (E) were calculated by linear regression of the current voltage relationship between 0 and +80 mV (*: $p \leq 0.05$; **: $p \leq 0.01$, two-tailed t-test).

The dose-response curves of BSA and rHSA indicate an EC_{50} in the submicromolar range (Fig. 4D).

To test whether sera of further animal species are also capable to activate OR in trophozoite-infected human erythrocytes, mouse serum was applied during continuous whole-cell recording. As a result mouse serum activated OR confirming the inter-species cross-activity (data not shown). To study the specificity of the serum albumin effect, other proteins such as ovalbumin or casein (1% w/v) were applied. Both proteins exerted no stimulatory effect on the OR currents (Fig. 5A, B). In further experiments serum albumin was enzymatically or chemically modified to define the conformation and/or subdomains of the protein required to interact with the infected erythrocyte. The three-dimensional structure of serum albumin is maintained by several disulfide bonds bridging the molecule [24]. Reduction of the disulfide bridges by dithiothreitol and covalent protection of the SH groups by iodoacetamide (1 mM) abolished the stimulatory effect of rHSA (0.1%; $n = 3$; Fig 5C, D)). Identical loss of stimulatory function was observed after

cleavage of rHSA by trypsin, or formic acid (applied concentrations of the rHSA-derived oligopeptides were $\sim 15 \mu\text{M}$; $n = 2-3$; data not shown).

Serum albumin might increase the open probability of OR. It might also stimulate the formation of new channels, i.e., either the insertion of parasite proteins into the erythrocyte membrane or the recruitment of channels from a pool of silent erythrocyte proteins. This channel induction can be studied upon irreversible blockage of active channels by biotinylation [25]. Further incubation of biotinylated infected erythrocytes reportedly leads to slow re-induction of channel activity [19]. As shown in Fig. 5E, F exposure of biotin-treated infected erythrocytes to rHSA failed to re-induce the channel activity within the time needed to activate OR in non-biotinylated cells.

Finally, the effect of rHSA was tested on the infection-induced lactate currents (Fig. 6). The NaCl bath solution was replaced by Na-L-lactate solution leading to a decrease in outward currents which were now generated by lactate influx (compare Fig. 6B, closed circles with Fig. 3C, closed circles). The pipette was filled

Fig. 6. Effect of rHSA on whole-cell lactate currents of washed infected erythrocytes. A. Current tracings of a washed infected erythrocyte recorded at -30 mV holding potential with NMDG-Cl pipette and Na-L-lactate bath solution before (left) and after addition of rHSA (0.1%) to the bath solution (right). B. Mean I/V relationships ($\pm$ SE; $n = 4$) recorded as in (A) in paired experiments from washed infected erythrocytes before (closed circles) and after addition of rHSA (0.1%, open circles) to the bath solution.

with the standard NMDG-Cl pipette solution. This current decrease was accompanied by only a small shift of the reversal potential along the change of Cl^- equilibrium potential indicating high relative lactate permeability of OR (compare Fig. 6B, closed circles with Fig. 3C, closed circles). Addition of rHSA (0.1 %) to the bath solution induced an increase of lactate outward current (Fig. 6A-C) and the stimulated current fraction (difference between open squares and closed circles in Fig. 6B) exhibited typical outward rectification. This indicates stimulation of lactate and Cl^- whole-cell currents by serum albumin through activation of OR in trophozoite-infected erythrocytes.

Discussion

Alteration of the whole-cell currents in parasitized erythrocytes by serum albumin and holding potential might result from a change in the kinetics of already active channels as suggested recently [9]. Against such a transformation of e.g., an IR into an OR current phenotype argues the observations that IR and OR differ at whole-cell level in permselectivity ($\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$ vs. $\text{Cl}^- \geq \text{Br}^- \approx \text{I}^- > \text{SCN}^-$), pH-dependence [26], inhibitor sensitivities ([13] and present study), and voltage dependence (present study). Although not excluded, such profound modifications of the intrinsic properties of a channel protein are very unlikely. Rather, IR and OR are most likely generated by different proteins and only the latter is activated by serum albumin.

The present study demonstrates that the reported stimulation of whole-cell currents in *P. falciparum*-in-

fectured human erythrocytes by serum factors [7, 20] results from a high specificity and affinity interaction of serum albumin with the infected cells. This interaction requires an intact serum albumin molecule in its native three-dimensional configuration since reduction of the disulfide bonds or digestion into peptides abolished the effect of serum albumin. At least in theory, serum albumin might remove inhibitory lipophilic factors from the membrane and by that way dis-inhibit the *Plasmodium* induced outwardly rectifying anion conductance (OR). These lipid withdrawing and binding properties of serum albumin are used experimentally to, e.g., abrogate the inhibition of K^+ channels by oleyl-CoA [27]. The activation of the Cl^- and lactate currents by serum albumin, however, was reversible in the present study arguing against such activation mechanisms. Rather, the serum albumin-induced activation of OR resulted from binding to a specific binding site (receptor) in the erythrocyte membrane. Serum albumin receptors (cubulin, megalin) are expressed, e.g., in the brush border membrane of proximal tubular cells where they trigger receptor-mediated endocytosis of filtered serum albumin [28]. Remarkably, these receptors operate with K_D values [29] similar to the EC_{50} values found in the present study for the serum albumin effect on the OR current. However, we were not able to identify cubulin by immunoblotting in the erythrocyte membrane (data not shown). Moreover, erythrocytes lack the endocytosis machinery and megalin or cubulin are promiscuous receptors which bind a variety of proteins [30]. Taken together, it seems very unlikely that serum albumin confers its effect on human erythrocytes via megalin or cubulin. Rather, serum albumin interacts more directly with the channel protein and not via

a complex signalling. This assumption is supported by the observation that repeated addition and removal of serum albumin in the bath reversibly and repeatedly activated and inactivated the outward currents during continuous (up to 60 min) whole-cell recording (data not shown). This argues against the dependence of the OR currents on signalling molecules (such as kinases, phosphatases, phospholipases, etc.) that are expected to be diluted by the pipette solution during long lasting recording which in turn would result in a run-down of the OR current.

In the present study, OR activity decreased slowly and incompletely during washing of the cells in serum albumin-free culture medium or during whole-cell recording when the cells were superfused with serum albumin-free bathing solution. This might hint either to very slow or ineffective signalling processes decreasing OR activity after removal of the stimulating factor or to a very tight (unspecific) binding of serum albumin to the erythrocyte surface. Then, during wash-out serum albumin might be recruited for the specific receptor ligation from this pool of unspecifically bound albumin. Serum albumin-specific bands in gel electrophoresis of white membranes prepared from tightly washed erythrocytes argue for such contamination by serum albumin. This surface contamination might also underlie the reported dependence of OR in infected erythrocytes on the hematocrit, i.e., the stimulation of the current by cell-cell contact [7]. Moreover, the intraerythrocytic parasite reportedly has access to serum albumin indicating that serum albumin must enter the infected erythrocyte [31]. The presence of serum albumin stored within the erythrocyte might explain the observed stimulation of OR by hemolysate of infected erythrocytes [7].

In the present study, washed parasitized erythrocytes still exhibited time-dependently inactivating inward currents at highly negative voltages and appreciable outward currents (Fig. 2), strongly suggesting that the wash-out of serum albumin was not complete. Consequently, serum albumin exposure was followed by only an about two-fold increase in outward current (Fig. 3). In a previous study utilizing washed parasitized erythrocytes, an about 4-fold increase of outward currents upon stimulation with human serum has been demonstrated [20]. Since the serum-stimulated outward currents at +80 and +100 mV voltage, respectively, of the parasitized erythrocytes were similarly high in this previous [20] and the present study, the difference in the serum stimulatory effect between both studies is most probably due to differences in the washing efficacy.

On the one hand, serum albumin might stimulate the

recruitment of channels from a pool of silent erythrocyte proteins or trafficked parasite-derived proteins. On the other hand, serum albumin might activate preformed/inserted channels or stimulate their open probability. Biotinylation of the erythrocyte membrane reportedly inhibits the OR current and the osmolyte permeability irreversibly [25]. Post-culturing of biotinylated infected cells, however, leads to the partial restoration of the osmolyte permeability [19]. In the present study, serum albumin did not stimulate the OR current of biotinylated cells, suggesting that serum albumin rather induces activation/modulation of pre-formed channels than channel recruitment.

Human serum albumin, the major protein component of blood plasma, builds up the principal fraction of the colloid osmotic pressure. In addition, it binds a number of relatively insoluble endogenous molecules, such as unesterified fatty acids, bilirubin, and bile acids and thus facilitates their transport in the circulation. In plasma human erythrocytes are facing serum albumin concentrations of about 500 - 650 μ M casting some doubt on the physiological significance of a signalling through a putative serum albumin receptor on the erythrocyte membrane. The functional significance of serum albumin for the intraerythrocytic *P. falciparum* development is evident from the fact that *in vitro* culture of *P. falciparum* requires serum albumin in the medium [32]. Serum albumin might deliver fatty acids to the parasitized erythrocytes needed for the parasite's membrane biosynthesis. In addition, serum albumin has been suggested to act as amino-acids source for the parasite development [31].

The present paper confirms a further function of serum albumin for the erythrocyte-parasite interaction. Serum albumin activates OR, which most probably is the electrophysiological correlate of the infection-stimulated organic osmolyte permeability [18, 19]. This activation is highly specific and exhibits high affinity, and thus, might be used as a target of future antimalarial therapy.

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