The Outward Rectifying Anions and Organic Osmolytes Conductance in Malaria-Infected Erythocytes: Myth or Reality?

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Abstract: Malaria-infected erythrocytes acquired New Permeability Pathways (NPPs) to meet the needs in nutrients and disposal of waste products of the intraerythocytic parasite development. The NPPs have been intensively studied for their putative interest as therapeutic targets for malaria treatment. Over the past 10 years, many electrophysiological studies have identified novel ion conductances (reflecting a part of the NPPs activities) in the host plasma membrane of *Plasmodium falciparum*-infected erythrocytes. In this article, we review the electrophysiological/biophysical properties of the malaria-induced outwardly rectifying anion conductance and compare this conductance to the other anion conductances and permeabilities already described in the literature.

Keywords: Patch-clamp, red blood cells, membrane permeability, plasmodium, malaria parasite.

1. ANION TRANSPORT AND PERMEABILITY IN NON-STIMULATED NON-INFECTED HUMAN ERYTHOCYTES

The control of the RBC ion homeostasis, volume, pH and membrane potential results from the interaction of many passive and active transport systems, cytoplasmic buffers, and from the charge and osmotic properties of haemoglobin and other major impermeant solutes. The human erythrocytes possess a wide range of transporters and channels which contribute to the maintenance of the cell volume, membrane deformability and/or stability in response to the shear stress generated by the blood circulation.

Among the variety of transporters, Band 3 (or Anion Exchanger-1) is the major erythrocyte membrane protein (approximately 1 million of copies per RBC). This Cl^-/bicarbonate anion exchanger-1 enhances the blood CO_2-carrying capacity, modulates the acid-base homeostasis and assists in the red cell dehydration [1]. This anion exchanger is considered as a potent therapeutic target in the treatment of sickle cell disease, and other hemoglobinopathies through the control of red cell dehydration [2,3]. Finally, it is generally admitted that the resting conductance of the human erythrocyte membrane is dominated by the activity of the non-electrogenic AE-1 exchanger without participation of anion channels. Patch-clamp experiments performed on intact mature unstimulated, non-stressed, non-infected human erythrocytes [4-9] indicated that the electrogenic RBC whole-cell conductance is in the sub-picoS range supporting the idea that endogenous anion channels are inactive when recorded under "physiological conditions". Nevertheless, 20 years ago, single patch-clamp recordings reported a 6 pS conductance channel with classical Cl^- selectivity spontaneously active at negative potentials and exhibiting rare openings events at positive potentials [10]. Isotopic flux measurements and differential laser-light scattering experiments revealed the presence of DIDS-insensitive Cl^- permeability in normal human RBCs [11]. Despite a low electrogenic Cl^- conductance, various studies have established that the membrane of RBCs contained a set of different Cl^- channel type: ClC-2 [12], CFTR [13,14], OR [6,9,15-17], PSAC [4,18], IR [6] and that these channels that are mostly silent in non-stressed, non-infected, non-stimulated RBCs might become active and functional when the right stimulus is applied. Interestingly, the activation of most of these channels is correlated to altered erythrocyte functions and is related to genetic or pathogenic diseases. Beyond the variety of stimuli known to activate ionic channels in red blood cells, the infection of the host erythrocytes by the malaria parasite was probably the most intensively studied.

2. THE MALARIA INFECTION: ACTIVATION OF VARIOUS ANION CONDUCTANCES IN PLASMODIUM-INFECTED ERYTHROCYTES

The intraerythrocytic development of *P. falciparum* depends on ample supply of nutrients and disposal of waste products. However, the low permeability of a normal RBC plasma membrane is not sufficient to satisfy the highly active metabolism of the parasite. To meet the requirement of the parasite development, new transport systems are activated or up-regulated in the host cell membrane which achieve various functions such as parasite nutrition, elimination of metabolism products and host volume regulation. For more than 2 decades, conventional isotopic flux measurements and hemolysis experiments have characterized the pathways responsible for the parasite-induced new transport systems. Commonly called New Permeability Pathways, these NPPs become apparent about 12-15h after the erythrocyte invasion and increase markedly up to a plateau at about 36 h post-invasion [19]. These NPPs have been postulated to

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be mostly anion selective but allow also the transport of a wide variety of charged and uncharged solutes. The NPPs have been intensively studied for their putative interest as therapeutic targets for malaria treatment. The NPPs are thought to reflect anion channels with a high permeability for inorganic anions (Cl^-, I^-, SCN^-, Br^-) and a lower but measureable permeability for organic molecules such as amino acids, nucleosides, sugars, polyols and even peptides.

Historically, the first study using non-conventional techniques to measure NPPs was performed by Desai and coworkers [4]. In that study using on-cell and whole-cell patch-clamp techniques, the authors concluded that uninfected human RBCs had low linear conductance of about 100 pS while trophozoide-infected RBCs exhibited a dramatic increase of the chloride conductance (150 fold). Interestingly, despite using pipette and bath solution with symmetrical Cl^- concentration, the measured conductance exhibited a typical inward rectification with a plateau at positive potentials and a relative permeability for Cl^- over Na^+ of 10^5. This trophozoide-induced conductance is inhibited by various non-specific chloride transport inhibitors such as NPPB, niflumic acid, furosemide, glibenclamide and high doses of phloridzin. In the same study, using on-cell patch-clamp techniques the authors described an anion permeable channel of low unitary conductance (20 pS in symmetrical 1100 mM salt solution) with a typical fast gating activity and a significant voltage dependency (the open probability increases at positive potentials). This Cl^- permeable channel correlates well with the recorded *plasmodium*-induced inwardly rectifying whole-cell currents.

However, a large number of studies performed with patch-clamp technique since the past 10 years have identified at least four different types of channels as putative electrophysiological correlates of the new permeability pathways : 1/ small *c*onductance anion channels (SCC, 4-5pS) which most probably are identical to ClC-2 anion channels [12] and to the plasmodial surface anion channel (PSAC) [4,14,20], 2/ a CFTR-dependent *i*nwardly *r*ectifying anion *c*hannel (IRC, [13,20,21], 3/ a 80 pS *o*utwardly *r*ectifying anion *c*hannels (ORC, [22]), and finally 4/ a non-selective cation channel [23]. The exact physiological contribution of all these conductances in the intraerythrocytic parasite development remained elusive but the outwardly rectifying anion conductance was shown to be permeable to different organic osmolytes such as lactate, polyols, and sugars [9] while the ClC-2 channel [12] was demonstrated to be responsible for a volume-sensitive fraction of the inwardly rectifying current. Most of the already described ionic conductances observed on parasite infected erythtocytes might be used as target for future antimalaria therapy. However, the organic osmolyte and anion conductance is of high interest in two ways: 1/ as possible drug targets for antimalaria chemotherapy and 2/ according to specific biophysical properties as a route to deliver drugs from the blood to the intraerythrocytic parasite.

3. THE ANION AND ORGANIC OSMOLYTE PERMEABILITIES OF THE PLASMODIUM-INDUCED OUTWARDLY RECTIFYING (OR) CL^- CONDUCTANCE

The existence of an outwardly rectifying Cl^- conductance (OR) in *P. falciparum*-infected erythrocytes was primarily demonstrated by Huber and colleagues [6]. In this original study, the different sensitivity of the inward Cl^- current and the outward Cl^- current to increasing concentrations of well-known Cl^- inhibitors (NPPB, DIDS, glybenclamide, furosemide) allowed the authors to consider the existence of a second type of anionic conductance (different from the inward PSAC conductance previously described by Desai and co-workers [4]). The outward current was almost completely inhibited by ~1μM of NPPB (see Fig. **1**) and ~10μM of DIDS or glybenclamide. In constrast, the inward current remained unaffected by these modest concentrations of inhibitors (see Fig. **1**). The fraction of the total current inhibited by low doses of inhibitors exhibited a typical outwardly rectifying profile when recorded under symmetrical Cl^- solutions. Interestingly, this OR conductance is not dependent on glucose or ATP in the pipette solution, is active at room temperature and is not modulated by osmotic cell swelling or shrinkage [6,9]. Moreover, the OR Cl^- current exhibited a halide permselectivity of $Cl^->Br^-\sim I^->SCN^-$ [6,16] that strongly differs from that observed for the inwardly rectifying current (PSAC, $SCN^->I^->Br^->Cl^-$) or that reported for IR/ClC-2 ($Cl^->Br^->I^-$).

In addition to its specific halide permselectivity, the OR Cl^- conductance exhibited also a significant permeability to various organic osmolytes [9]. Whole-cell experiments performed by replacing equimolar concentration of Cl^- ions by L-lactate- ions in the bath solution confirmed a relatively high lactate permeability ($P_{lactate}$-/P_{Cl}-= 0.3-0.4). In sharp contrast, the permeability of the OR Cl^- conductance for gluconate ions is relatively low ($P_{gluconate}$-/P_{Cl}-= 0.04). Cl^--driven or lactate$^-$-driven OR currents exhibited the same profiles of inhibition for increasing concentrations of inhibitors (see for example, the NPPB sensitivity depicted in Fig. **1C**) suggesting that Cl^- and lactate ions share the same permeable pathway. Based on the demonstration that the OR Cl^- conductance is not sensitive to the extracellular osmolarity variations, patch-clamp experiments were performed by adding various polyols to test the putative permeability of the OR conductance to these osmolytes. Interestingly, replacement of sorbitol in the bath solution by equimolar amounts of mannitol or sucrose inhibited the Cl^- whole-cell currents of the *P. falciparum*-infected erythrocytes. The inhibition of the OR conductance occured instantaneously upon carbohydrate application, was reversible and concentration dependent. The inhibition efficacy of sorbitol, mannitol and sucrose differed significantly, suggesting that the level of inhibition of the OR-generated current is dependent on the nature of the osmolytes. The inhibition occurred according to the specific sequence sucrose>mannitol> sorbitol. Finally, the inhibitory effect of various carbohydrate species was dependent on the nature of the charge carrier : same concentration of mannitol (200mM) inhibits by ~50 % the OR-generated lactate current but by only ~25 % the OR-carried Cl^- current [9]. This interaction between charge carrier and neutral carbohydrates suggests a competition between the neutral and the charged solutes within the pore channel rather than an allosteric regulation of the carbohydrates on the channel/protein. The exact origin of the OR conductance is still under debate, but convincing studies demonstrated that the *P. falciparum*-induced OR conductance is most probably generated by endogenous RBC anionic channels which show low spontaneous activity

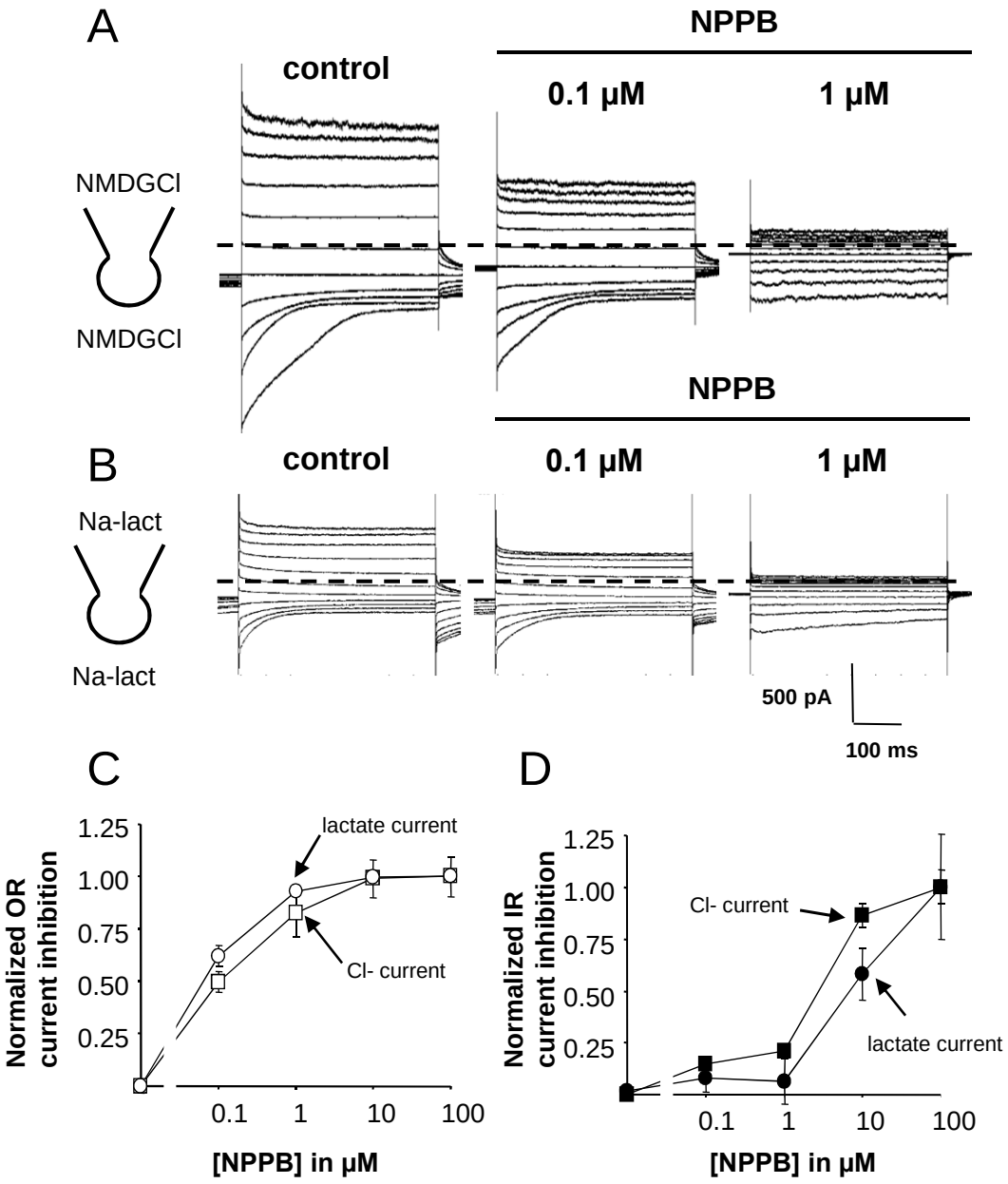

Fig. (1). NPPB-sensitivity of the *P. falciparum*-induced Cl^- and lactate permeabilities.

A-B: original whole-cell current traces of infected erythrocytes recorded in control condition and after addition of increasing concentrations of NPPB to the bath solution. Experiments were performed in (**A**) with symmetrical Cl^- concentrations (140 mM NMDGCl) in the bath and in the pipette solutions and in (**B**) with symmetrical concentrations of lactate (145 mM Na-L-lactate) in the bath and in the pipette solutions.

C-D: Dependence of the outward (**C**) and the inward currents (**D**) to increasing concentrations of NPPB. The curves represent the normalised current inhibition for both the Cl^-- (squares, n=7) and the lactate-driven currents (circles, n=5).

in non-infected erythrocytes but become active under oxidative stress as demonstrated by Huber and coworkers [6].

4. PH SENSITIVITY, BIOTINYLATION AND CHYMOTRYPSIN EFFECT ON THE *PLASMODIUM*-INDUCED ORGANIC OSMOLYTE AND ANION CHANNEL CONDUCTANCE

Another study [16] revealed another significant difference between the inwardly and the outwardly rectifying currents of the *P. falciparum*-infected RBCs. While the IR anion conductance was not sensitive to the alterations of extracellular pH (between pH 6.0 and 8.4), the OR anion conductance was strongly inhibited by external bath alkalinization. The OR current recorded under symmetrical Cl^- pipette and bath solutions was inhibited by ~70 % at pH 8.0. Isotopic lactate flux measurements performed in *P. falciparum*-infected RBCs confirmed the sensitivity of the lactate membrane permeability to alkaline pH. Additionally, *P. falciparum* growth assays performed at different pH showed a strong inhibition of the parasite development at pH

> 7.8 suggesting a causal link between parasite growth and the inhibition of the OR current.

A different approach has been used by two independent groups [7, 8] to characterize the organic osmolyte permeability of *P. falciparum*-infected RBCs. Both groups cross-linked the N-terminus of *P. falciparum*-infected RBC proteins with amine-reactive N-hydroxysulfosuccinimide (sulfo-NHS). As a result, sulfo-NHS produced covalent amide linkages between the amine group of protein N-terminus and the amino group of the lysine residues. Incubation of *P. falciparum*-infected RBCs with sulfo-NHS-LC-biotin led to 1/ biotinilation of intracellular proteins suggesting infection-induced biotin uptake, 2/ inhibition of infection-induced sorbitol hemolysis, 3/ inhibition of isotopic L-[^{35}S]-methionin and L-[^{14}C]-glutamic acid uptake and 4/ an irreversible block of the *P. falciparum*-induced OR conductance. Finally, these experiments demonstrated that the modification of OR conductance (as well as PSAC channel) by sulfo-NHS-LC-biotin exposure leads to a significant modification of *the plasmodium*-induced new permeability pathway. Nevertheless, the observed biotin effects are not final proofs of a direct interaction of biotin with PSAC or OR channels and these studies failed to characterize the molecular identity of PSAC or OR channels.

Since a fraction of the band 3 molecules is known to undergo covalent modifications following *P. falciparum*-infection [24,25], Baumeister and co-workers [26] hypothesise that post-infectious modifications of band-3 might be responsible for the NPPs. To verify this hypothesis, the integrity of band 3 proteins and the activity of the NPPs were quantified following protease treatment. Interestingly, α-chymotrypsin treatment of *P. falciparum*-infected cells resulted in the abolition of NPPs activities, and of the OR conductance. However, additional experiments finally excluded the possibility that the band-3 directly generated the OR conductance and the NPPs. The observed effects of chymotrypsin exposure were most probably due to a band-3 independent protein cleavage. Nevertheless, the close similarity between the effects of chymotrypsin on the NPPs and those on the OR anion current observed in infected cells is consistent with the hypothesis that the same permeability/ channels are responsible for these two phenomena.

5. REGULATION OF THE OUTWARDLY RECTIFYING (OR) CONDUCTANCE AND SORBITOL PATHWAY BY PURINOCEPTORS AND ATP PERMEABILITY OF THE OR CONDUCTANCE

Various stimuli (such as mechanical deformation, reduced pH, oxygen tension decrease) have been shown to stimulate ATP release from mature human RBCs. RBCs infected *by P. falciparum* or exposed to oxidation (t-BHP, 1mM) exhibited also a significant increase of ATP release as compared to non-stimulated non-infected RBCs (Fig. **2**). Moreover, extracellular addition of ATP stimulated the osmolyte permeability both in *P. falciparum*-infected RBCs and in oxidized RBCs leading to an increased sorbitol haemolysis as compared to non exposed cells (~30-40% [27]). However, ATP alone failed to activate directly the osmolyte permeability in non-infected non oxidized RBCs suggesting that ATP exerted only a positive feed back of the ATP-induced signalling cascade. Such ATP-induced ATP release has been demonstrated in human non-infected RBCs [28,29] and is classically depicted in nucleated cells via purinoceptor signalling. Involvement of purinoceptors in the induction of the OR conductance was strongly suspected since suramin as well as other purinoceptor antagonists inhibited the induction of the sorbitol permeability and the anion current of *P. falciparum*-infected RBCs [27]. MRS2179, a specific antagonist of P2Y1 inhibited the induction of the organic osmolyte permeability in malaria-infected RBCs as well as the oxidation-induced permeability of mature RBCs [27]. Finally, P2Y1$^{-/-}$ mice exhibited a delayed increase in parasitaemia when infected with *P. berghei* ANKA as compared to wild type mice while the RBCs from these P2Y1$^{-/-}$ mice exhibited a decreased OR permeability induced by both, oxidation- and *P.berghei*-infection.

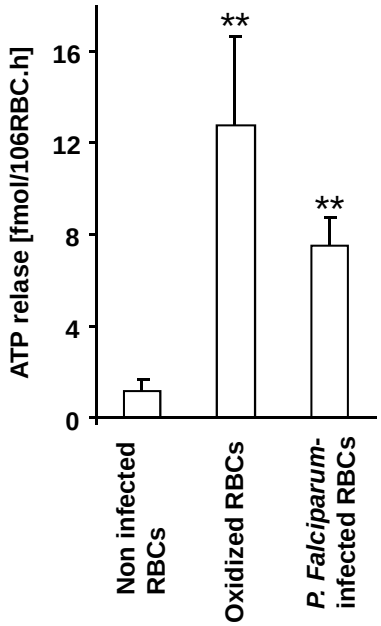

Fig. (2). Mean ATP release (±SE; n =11–12) of non-infected non-oxidized RBCs, of oxidized human RBCs and of *P. falciparum*-infected human RBCs (**$P \leq 0.01$ two-tailed t test) adapted from [27].

Since ATP stimulates both ATP release in mature RBCs and isoosmotic sorbitol hemolysis in *P. falciparum*-infected RBCs, it has been speculated that the ATP permeable conductance and the infection-induced new permeability pathway are generated by the same pathway. Interestingly, a recent study [30] demonstrated that ATP release by *P. falciparum*-infected RBCs was significantly reduced by low doses of NPPB. Moreover, patch-clamp experiments performed in whole cell-configuration confirmed also a low but significant permeability of the OR conductance to ATP ($P_{ATP}/P_{Cl^-} \sim 0.013$).

Altogether, human RBCs respond to malaria infection by releasing ATP that in turn may activate metabotropic purinoceptors (P2Y1) and induce organic osmolyte and OR anion conductance.

6. EFFECT OF SERUM ALBUMIN ON THE OUTWARDLY RECTIFYING (OR) CL^- CONDUCTANCE

Since the first patch-clamp study [4] that demonstrated the existence of a Cl^- channel (PSAC), an increasing number of studies have contributed to reveal the complexity of the currents that can be recorded in *P. falciparum*-infected RBCs. A number of studies performed with patch-clamp techniques reported additional findings and finally concluded the co-existence of 3 to 4 distinct anion conductances in the *P. falciparum*-infected RBCs. Nevertheless, conflicting results have led to controversy in this field. For example, the existence of an OR conductance remained controversial since many groups were unable to record an outwardly rectifying current in *P. falciparum*-infected RBC. The study of Staines and co-workers [15] finally demonstrated that addition of human serum (0.4% v/v) or serum albumin increased the outward current amplitude by ~4 fold suggesting that technical experimental situations altered the patch-clamp recordings (washing treatments resulted in a significant decrease of the overall OR current). Interestingly, the stimulating effect of serum albumin on the OR conductance was concentration dependent (EC_{50} in submicromolar range, with a maximal activation for 1% FCS corresponding to ~150 mM of albumin, Fig. **3**) reversible and other proteins such as ovalbumin or casein remained without effects [17]. Furthermore, sorbitol hemolysis experiments performed in *P. falciparum*-infected RBCs were shown to be sensitive to serum/BSA suggesting that the serum albumin-dependent OR current is involved in the sorbitol pathway [31].

A part of the discrepancies concerning the existence of an OR conductance were most probably due to the difficulties to record such outwardly rectifying anion channels (ORC)

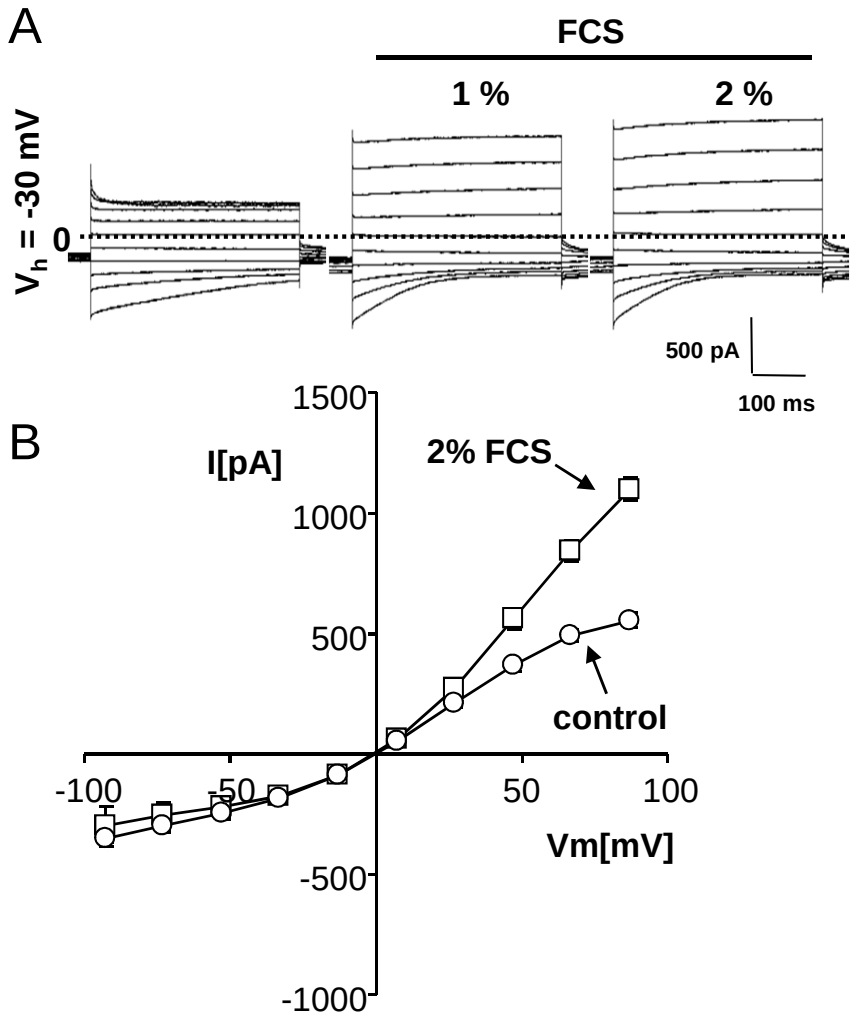

Fig. (3). Effects of foetal calf serum (FCS) on the whole-cell Cl^- currents of washed infected erythrocytes.

A. Whole-cell current tracings of a washed *P. falciparum*-infected RBC recorded at -30 mV holding potential with NMDG-Cl (145 mM) pipette and NaCl (125 mM) bath solution during addition of increasing concentrations of FCS to the bath solution. Whole-cell currents were evoked by 11 voltage pulses (400ms each) from –30 mV holding potential to voltages between -100 mV and +100 mV.

B. Corresponding I/V relationships (± SE, n = 19) of the whole-cell currents (obtained in paired experiments as depicted in (A) from washed infected cells before (circles) or after exposure to 2% of FCS (squares).

activity in single channel patch-clamp configuration. While inward rectifying channels (which are most probably the correlate of the PSAC/ClC-2 inward current recorded in whole-cell mode) were recorded in 80 % of all the experiments, outwardly rectifying intermediates conductance anion channels activity has been reported in only 3% of all the patches and only at the beginning of the recording [22]. With such a low occurrence ORC was considered to have only minor functional significance for the NPP and the parasite development. However, it is not easy to consider that single channel activity obtained in cell-attached mode configuration reflects the global electrical activity of an intact cell. Many technical problems have to be considered when using single channel patch-clamp configuration: the cell attached mode reflects, 1/ the electrical activity of a relatively small portion of the membrane, 2/ this restricted area is submitted to intense stretch (i.e. to obtain a tight seal) and finally, 3/ the exact composition of the intracellular compartment remained unknown. A hypothesis [32] has been established recently to explain the difficulties to record OR channels in cell attached mode: putative large endogenous substrates that slowly penetrate the channel and thereby inhibit the Cl^- current are brutally dialysing upon rupture of the membrane when reaching the whole-cell configuration. This rapid dialysis would result in the dis-inhibition of the OR channel.

CONCLUSION

In conclusion, the OR conductance might be the correlate (for a part) of the malaria-induced NPPs. This OR conductance exhibited a significant permeability for organic osmolytes (charged or uncharged) and might be of high interest in two ways: 1/ as possible drugs targets for anti-malaria chemotherapy and 2/ as a route to deliver drugs from the blood to the intraerythrocytic parasite.

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