Electrophysiological characteristics, molecular identity, regulation and physiological role of membrane anionic channels of the human erythrocyte in health and disease

Edyta Glogowska

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EDYTA GLOGOWSKA

Détermination des caractéristiques électrophysiologiques, de l'identité moléculaire, de la régulation et du rôle physiologique/patho-physiologique des canaux anioniques de la membrane des érythrocytes humains.

Thèse dirigée par le **Dr Serge THOMAS**
Equipe d'accueil: **Physiologie Cellulaire des Erythrocytes, Mer et Santé UMR 7150, Station Biologique, Roscoff**

Thèse soutenue à Roscoff, France
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devant le jury composé de :

M. François LALLIER,
Professeur CNRS-UPMC (Station Biologique, Roscoff, France)/ *président, examinateur*

M. Ingolf BERNHARDT,
Professeur de l'Université des Saarlandes (Saarbrücken, Allemagne) / *rapporteur*

M. Gordon LANGSLEY,
Docteur, DR CNRS Institut Cochin (Paris, France) / *rapporteur*

M. Christian BERTHOU,
Professeur de l'Institut de Cancérologie et d'Hématologie (Brest, France) / *examinateur*

M. Serge BONNEFOY,
Docteur, CNRS Institut Pasteur (Paris, France) / *examinateur*

M. Stéphane EGÉE,
Docteur, MCF, UPMC (Station Biologique, Roscoff, France) / *examinateur*

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* chapter IV. 2. was partly realized in Departments of Pediatrics and Genetics, Yale University School of Medicine, New Haven, USA, under Dr P. G. Gallagher supervision.

LIST OF ABBREVIATIONS.

4.1; 4.2; 4.9	cytoskeleton proteins
9-AC	9-aminocamptothecin
A	apicoplast
AE1	anion exchanger, band 3 protein (also SLC4A1)
AlbuMax	lipid-rich bovine serum albumin
ANT	adenine nucleotide transporter
ATP	adenosine triphosphate
ATPase	adenosine triphosphate catalyze enzyme
APS	ammonium persulfate
βR16	repeat 16 of β-spectrin
BSA	bovine serum albumin
C-A	cell-attached configuration of patch-clamp technique
cAMP	cyclic adenosine 3',5'-cyclophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
Cl^-	chloride anions
CLAG3	member of the *clag* multi-gene family
ClC-2	chloride channel type 2
Dg	dense granule
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonate
DMSO	methyl sulfoxide
DnaJ	molecular chaperone protein, Hsp40 (heat shock protein 40 kD)
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylene diamine tetraacetic acid
EIPA	ethylisopropylamiloride
E_m	resting membrane potential
EXP1	*P. falciparum* blood-stage antigen
FV	food vacuole
GABA	gamma-aminobutyric acid
GLUT1	glucose transporter
Hct	hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	hours post invasion
hRBC	human red blood cell
HRP1	histidine-rich protein 1
hSK4	Ca^{2+}-sensitive K^+ channel, Gardos channel
I	current amplitude
IBP	isoquinoline binding protein
IK1	Ca^{2+}-sensitive K^+ channel, Gardos channel
I-O	inside-out configuration of patch-clamp technique
IRC	inwardly rectifying channel

KCa 3.1	Ca^{2+}-sensitive K^+ channel, Gardos channel
kD	kiloDalton
K	knob
KAHRP	knob-associated histidine-rich protein
KO	knock out, mutant
KI	revertant
MACS	magnetic assisted cell sorting
MAHRP	membrane-associated histidine-rich protein
Mc	Maurer's cleft
MESA	mature-infected erythrocyte surface antigen
Mi	mitochondrion
Mn	microneme
N	nucleus
NaPi	sodium dihydrogen phosphate, $NaH_2PO_4 \cdot 2H_2O$
nA	nanoAmpere
NO	nitric oxide
NPPB	5-nitro-2-(phenylpropylamino)-benzoate
NPPs	new permeability pathways
nS	nanoSiemens
NSC	non selective cation channel
NS(VD)C	non selective (voltage dependent) cationic channel
NT1	nucleoside transporter
O-O	outside-out configuration of patch-clamp technique
ORC	outwardly rectifying channel
ORCC	outwardly rectifying chloride channel
pA	picoAmpere
PBR	peripheral-type benzodiazepine receptor
PBS	phosphate-buffered saline
pCa	$-\log[Ca^{2+}]$
P_{cat}	cation permeability channel
PEXEL	*Plasmodium* export element (translocon of exported proteins)
P. f.	Plasmodium falciparum (*P. falciparum*)
Pf155	*P. falciparum* 155kDa protein, RESA
PFA0110w	*resa1* gene
PF11_0509	*resa3* gene
PF11_0512	*resa2* gene
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfEMP3	*P. falciparum* erythrocyte membrane protein 3
pH	$-\log[H^+]$
PK11195	1-(2-chlorophenyl)-N-(1-methyl-propyl)-3-isoquinolinecarboxamide
PKA	protein kinase A
PMSF	phenylmethanesulfonyl fluoride, protease inhibitor
P_o	open probability of the channel
pS	picoSiemens
PSAC	plasmodial surface anionic channel
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
RBC	red blood cell
RESA	ring-infected erythrocyte surface antigen

REX	ring exported protein
Rh	rhoptry
Rifin	repetitive interspersed protein
Ro5-4864	4'- chlordiazepam
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
RT-PCR	real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qRT-PCR) or kinetic polymerase chain reaction
SBP1	skeleton binding protein 1
SCN^-	thiocyanate (also known as rhodanide)
SCC	small conductance channel
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STEVOR	subtelomeric variable open reading frame protein
TBS	tris buffered saline
TBST	tris buffered saline tween20
TEMED	tetramethylethylenediamine
TM	transmembrane domain
TSPO	translocator protein
TVN	tubulovesicular network
VDAC	voltage dependent anion channel
V_H	holding potential
V_m	membrane potential
V_p	holding potential applied to the pipette
VTS	vacuole transport signal
W-C	whole-cell configuration of patch-clamp technique
WT	wild type

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I. IN BRIEF.

I. 1. Aim of the study.

Red blood cells (RBCs) are from a structural point of view the simplest of all eukaryotic cells. Devoid of intracellular organelles they are a classic model system for studying how ions, nutrients and other solutes cross the plasma membrane. Although RBCs have been intensively studied for several years (see review: Bernhardt and Ellory, 2003), still many questions concerning these cells are not fully answered. Erythrocytes accomplish their main function (the transport of O_2 from lungs or gills to tissues, and the reverse transport of CO_2) by two specialized molecular machines: haemoglobin and an anion exchange carrier (Cl^-/HCO_3^-). All the other transporters found in the RBC membrane (e.g. pumps, exchangers, cotransporters) are aimed at cell homeostasis. They maintain the constancy of volume and elastic properties that allow RBCs to bend and flow through the narrowest of capillaries. It was revealed during the last three decades that RBC membrane is endowed with a large variety of ionic channels (cationic and anionic). However, the standard proteomic protocols for detection of these channels were very limiting, taking into account how little protein represents a few hundred channels. Furthermore, for a long time most of the available data on the ion movements across the RBC membrane came from the isotopic fluxes experiments. The patch-clamp electrophysiological technique, allowing recording of unitary currents resulting from the flow of ions through the channels, was the best method to objectivize these conductive pathways. Although its successful application, due to the technical difficulties related to the small size of erythrocytes and their remarkable deformability a lot of informations about ionic channels in the RBCs membrane still remain unclear. For instance, their role is not fully understood, as well as their molecular identity and regulation. It is unlikely that they participate in red cell homeostasis which is rather based on cotransporters and on the almost total absence

of cationic permeability and minute anionic conductance (pump-leak concept, Tosteson and Hoffman, 1960). However, it was acknowledged that channels are involved in the physiological process of senescence of red blood cells as well as in pathological conditions such as sickle cell disease or malaria. Therefore, it is still unclear if ionic channels play a specific role in erythrocyte physiology or if they are a threat for cell homeostasis or just residues of a lost previous function during evolution or maturation process.

Since 1999, the research work of the group of "Comparative Physiology of Erythrocytes" from Station Biologique in Roscoff (France) was focused on description at the molecular level of ionic channels present in the RBCs membrane of nucleated (fish and birds) and non-nucleated (human) erythrocytes, their nature, regulation and role in physiological and pathological (malaria) conditions. Their initial observations have indicated that in human RBCs these channels seem to be inactive in the 'resting cell' and, when activated experimentally, can lead to a very high single cell conductance and potentially induce disorders, with the major risks of fast dehydration and dissipation of gradients. What does this mean therefore? How we can explain the existence of ionic channels in the human RBC membrane?

Because the previous projects realized in our laboratory gave already some characterizations of these conductive pathways in the human RBC membrane, both in health and disease, (Egee et al., 2002; Decherf et al., 2004; Bouyer et al., 2006; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009; Dyrda et al., 2010) the main purpose of my PhD thesis was to go further, with the specific intention to answer the still open questions: what is the nature, regulation and especially physiological role of ionic channels in the human red blood cell membrane?
The present work, done in Station Biologique in Roscoff under the leadership of Dr Serge Thomas gives us new insights into the nature and role played by ionic channels in the human RBC membrane in health and disease.

I. 2. Global context and objectives.

In a global view on research on ionic channels in the human RBC, up-to-now, it appears that their possible physiological role depends a lot from the molecular nature of the anionic conductance and its regulation. In contrast to the cationic channels already well characterized in erythrocytes, anion transport through the human RBC membrane attracted attention only recently and historically has been perceived to be mediated by a two-component system: a large electroneutral Cl^-/HCO_3^- exchanger (band 3, AE1) and a smaller electrogenic component corresponding to a few hundred channels. Several groups of electrophysiologists, including our team, have attempted to identify channels responsible for the anionic conductance in the RBC membrane. Using the *cell-attached* and *excised* configurations of patch-clamp technique the group of "Comparative Physiology of Erythrocytes" identified three types of anionic channels in human erythrocytes: a small conductance channel (SCC, ~5 pS), an inwardly rectifying channel (IRC, ~15 pS) and an outwardly rectifying channel (ORC, 75 - 85 pS).

The molecular characterization of these conductive pathways was still unknown, but the important is that this kind of ions transport was specifically active in *Plasmodium-falciparum* malaria infected human RBCs. It was demonstrated (Desai et al., 2000; Huber et al., 2002; Egee et al., 2002; Thomas and Lew, 2004; Bouyer et al., 2006; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009) that after parasite invasion there is an induction of new channel activity in the host plasma membrane, called New Permeability Pathways (NPPs), displaying anionic channel properties (Ginsburg et al., 1983; Ginsburg, 1994; Kirk, 2001). The question of whether this activity is host- (up-regulated and/or modified endogenous RBC proteins) or parasite-derived has not been resolved yet.

On the other hand, a recent study from this group on ionic channels in healthy erythrocytes (Dyrda et al., 2010) presents electrophysiological evidence that anionic channel activity is secondarily generated by Gardos channel (Ca^{2+}-sensitive K^+ cationic channel) transient activation induced by the membrane deformation upon seal formation, resulting from a sudden increase in calcium permeability. Moreover, it appears that the combined effects of the Gardos and anionic channels make possible rapid and reversible changes of ionic composition and cell volume, which

consequently may effectively influence the respiratory function of RBCs (Thomas et al., 2011). It also appears that changes in the membrane properties play a determining role in the activation of channels.

In this context, the present PhD work focused on channels in the RBCs membrane, concentrating on: 1/ description of the electrophysiological activity of anionic membrane conductance in human erythrocyte, 2/ identification of the molecular nature of the anionic channels, 3/ determination of the mechanisms of their activation with the use of the pathophysiological conditions of malaria infection, 4/ determination of the participation of channel activity in: i) the process of senescence of red blood cells, ii) the pathophysiological situation induced by malaria infection. Therefore, the four objectives of this thesis, developed in four different chapters, were:

First objective:

Further clues on electrophysiological characterization of anionic channels in human red cell membrane.

Because the previous electrophysiological studies have shown the presence of anionic channels in the red cell membrane it was necessary to go further with their characterization. Moreover, the status of anionic channels proper has never been clarified, and the informations obtained by different groups of electrophysiologists were confusing. This part of the thesis was aimed at rationalizing and explaining earlier confusing data.

Second objective:

The molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells.

Because the results obtained in the first objective showed the existence of maxi-anion channels in the human red cell membrane the question was now of the molecular identity of such maxi-anion channels? With regards to our observations

(selectivity, complex gating, pharmacological properties) we made the hypothesis that anionic conductance of the human RBC is a voltage dependent anion channel (VDAC). Originally such a channel is characterized as mitochondrial porin, but it can also be expressed in plasma membranes alone or as a component of a complex like the peripheral-type benzodiazepine receptor (PBR) consisting three components: a 18-kDa translocator protein with the acronym TSPO, a 32-kDa VDAC and a 30-kDa adenine nucleotide transporter (ANT).

Third objective:

The activation of anionic channels by *P. falciparum* and possible involvement of RESA1 protein in this process.

Because the previous part of PhD thesis demonstrates that the three components of the peripheral-type benzodiazepine receptor (PBR) including the voltage dependent anion channel (VDAC) are present in the human RBC membrane and have an important implication in the permeability of *Plasmodium*-infected human RBCs, the question was then by which gating mechanism and triggering factors the parasite is able to activate these endogenous anionic channels. One possibility was the involvement of some parasite proteins exported to the RBC membrane. It has been shown that one such protein, that plays crucial role in red cell membrane modification already at early parasite-stage Pf155/RESA1, interacts with spectrin and stabilises the infected red blood cell cytoskeleton, thereby allowing it to overcome membrane fragilisation upon exposure at febrile temperatures (Silva et al., 2005; Pei et al., 2007; Mills et al., 2007). The Pasteur Institute team (collaborator for this part of work) also has observed that RESA1 confers resistance of the infected erythrocytes against osmotic lysis under febrile mimicking conditions. The question addressed in this part of this PhD thesis was if RESA1 participates or not in the activation of NPPs? And if so, by what mechanism?

Forth objective:

Physiological role of human erythrocyte channels: A unifying hypothesis for senescence, sickle cells and malaria.

As said above, anionic channel activity is secondarily generated by Gardos channel transient activation induced by the membrane deformation. Because it was shown that the Gardos channel is inactive in the 'resting cell', its physiological role *in vivo*, if any, is still unknown. However, its obvious contribution to the process of RBC senescence and in the process of sickling in sickle cell anemia, together with the results obtained in the three first parts of this work prompted us to suggest a unifying hypothesis for a physiological and pathophysiological role of channels in healthy cells in a correlation with cell aging and in disease (sickle cell anemia and malaria). A major task for this part of the thesis was to address this issue. Some experiments were carried out in order to strengthen the validity of this hypothesis.

I. 3. Summary of results.

First objective

Further clues on electrophysiological characterization of anionic channels in human red cell membrane *(maxi-anion channels).*

This study, using the *cell-attached* configuration of the patch-clamp electrophysiological technique, demonstrates that the global conductance of a red cell in suspension, 'dormant' under normal physiological conditions, could be potentially activated and give a very high membrane conductance under various experimental conditions, far higher than the ground leak mediated by band 3. This conductance as well as the diversity of activities recorded in early studies corresponds to the activation of *maxi-anion channels*, 600 pS maximum conductance, with multiple conductance levels, gating properties and pharmacology, depending on conditions. We demonstrate the role of activator played by human serum in the recruitment of multiple new conductance levels. The modes at which electrical activity was activated have been classified in three patterns: A, between 0 and 50 pS; B, between 0

and 100 pS; C, between 0 and 600 pS (at positive potentials only); all correspond to different conductance levels of a single channel present in the patch of membrane.

According to these observations, the effect of partial replacement of Cl^- ions by SCN^- ions was studied for checking the possible preferences for anions. An obvious preference for SCN^- ions at least at the low conductance levels and at low concentrations of this chaotropic anion (10 mM) was observed. Data obtained at high SCN^- (69 mM) fit closely with the channel activity described in *cell-attached* configuration in *P. falciparum*-infected RBCs with Cl^- ions (Egee et al., 2002; Bouyer et al., 2006).

Second objective

The molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells (*VDAC*).

With regards to our observations (selectivity, complex gating, pharmacological properties) we made the hypothesis that anionic conductance of the human RBC is a voltage dependent anion channel (VDAC). We used western blotting and immunofluorescence to demonstrate that the three components of the peripheral-type benzodiazepine receptor (PBR), including the voltage dependent anion channel (VDAC), are present in the human RBC membrane. Quantitative RT-PCR made in Department of Cellular and Molecular Physiology (Yale Medical School, USA) confirmed that all three VDAC genes are expressed in erythroid cells, predominantly VDAC2 and 3; both TSPO genes are highly expressed; we observed also low levels of ANT1 expression and higher levels of ANT2 and ANT3.

Because the NPPs displays many common characteristic features with the PBR/VDAC and because the ligands of PBR are known as potent inhibitors of *P. falciparum* growth in infected RBCs *in vitro* (Dzierszinski, 2002; Bah, 2007), we next considered if the antiplasmodial effects of PBR ligands could correspond to an interaction of the parasite with this native PBR complex through up-regulation of PBR/VDAC activity. We used growth assays, sorbitol haemolysis and electrophysiology to demonstrate that the PBR ligands PK11195, Ro5-4864 and diazepam

block *in vitro* intraerythrocytic parasite growth and reduce membrane transports and conductance.

These data support the hypothesis that peripheral-type benzodiazepine receptors are dormant in RBC and after *P. falciparum* infection they are up-regulated and become the so-called "new permeability pathways". It should be underlined that these channels are obvious targets for selective inhibition in anti-malarial therapies, as well as potential routes for drug delivery in pharmacologic applications, but their activation process still remain unknown.

Third objective

The activation of anionic channels by *P. falciparum* and possible involvement of RESA1 protein in this process.

As mentioned above, channel activation might result from a direct interaction between parasite proteins and the PBR/VDAC complex. We tested the hypothesis that Ring infected Erythrocyte Surface Antigen (RESA), more precisely RESA1 also called Pf155, could be involved in the activation process of anionic channels. Experiments were performed on a large number of erythrocytes infected with strains RESA1-wild type, RESA1-KO mutant, RESA1-KI revertant.

Using western blotting and immunofluorescence we have confirmed that RESA1 is exported to the host erythrocyte during first 12 hpi and can be visualized in the red cell membrane of WT and KI, but not KO at early ring-stage. In the late schizont-stage the protein is no more present in erythrocyte membrane but in the parasites (again WT and KI, but not KO) which are preparing to be released and invade new cells.

Whole-cell configuration of patch-clamp technique was used to test membrane global conductance of different RESA1 strains, since this configuration allows an access to all ionic channels present in the erythrocyte membrane. The first series of electrophysiological experiments indicate that cultivation conditions play very important role for RESA1 protein effect on channel activity. The data reported here indicate that a component of normal human serum needs to be present in the me-

dium when RESA1 is being presented at the host cell membrane. This suggests also the possibility that serum has direct effect on the RESA1 delivery, the pathway for its trafficking and presentation in the erythrocyte membrane. The second series of electrophysiological experiments tested the modulating effect of serum on basic membrane current of RESA1 WT, KI and KO. Differences between WT, KI and KO appeared in control recordings as well as in serum effect but the effect of serum was larger for RESA1-WT than for RESA1-KO, providing the first demonstration that RESA1 protein participates in the modulation process of NPPs.

If the hypothesis that parasite exported RESA1 protein could be involved in the modulation of anionic channels is true, we should expect a differences in haemolysis when cells containing new permeability pathways are exposed to isotonic sorbitol solution. Our results showed that there is difference in haemolysis between RESA1-WT and RESA1-KO but only for strains cultivated in the presence of serum, which confirmed that RESA1 protein exported by the parasite to the host membrane participates in the modulation process of anionic channels, dormant in physiological erythrocyte membrane and up-regulated by *P. falciparum* after infection.

Forth objective

Physiological role of human erythrocyte channels: A unifying hypothesis for senescence, sickle cells and malaria.

As previously reported, ionic channels are apparently dormant in steady-state conditions, but their activity has been shown in some experimental or pathological situations such as senescence (Lew et al., 2007), sickle cell anemia (Lew and Bookchin, 2005) and malaria (Ginsburg, 1983; Ginsburg and Kirk, 1998).

That most or all processes involved in these situation may be mediated by a single cascade (or chain) of transporters is a tempting unifying hypothesis. The permeability pathways involved in this process are not yet well defined but the results obtained in the framework of this thesis make it reasonable to consider that calcium entry through a calcium permeability pathway, followed by Gardos channel activation, inducing PBR/VDAC activation in either of its two modes (cationic or

anionic) provide a satisfactory explanation for all changes in ionic compositions and gradients observed. The experiments performed in this part of the thesis were designed to reinforce the theoretical model on two points: 1/ Is the activity of the Gardos channel declining with age? 2/ Is the Gardos channel still activatable in *P. falciparum*-infected cells? For this purpose, erythrocytes were separated into five fractions, 1 contains young cells; 2, 3 and 4, correspond to the mature erythrocytes; 5, contains the oldest cells, using gradient centrifugation in Percoll buffer solution. Using *cell-attached* configuration of the patch-clamp the activity of the Gardos channel was monitored during 3 days following separation to check a possible change in activity with time. The results obtained for these series of experiment indicate no differences in channels activity, neither between fractions nor with time of *in vitro* storage. Secondly, we monitored the changes in channel activity in malaria infected erythrocytes. For that purpose we used the common *P. falciparum* strain 3D7, as well as RESA1-WT and RESA1-KO. The results demonstrate that Gardos channel is still active in infected cells.

I. 4. Scientific communication.

I. 4. 1. Publications.

1. Ion channels in human red blood cell membrane: Actors or relics? (Blood Cells, Molecules and Diseases, 2011).

Serge L.Y. Thomas, Guillaume Bouyer, Anne Cueff, Stéphane Egée, Edyta Glogowska, Céline Ollivaux.

Contribution: SLYT wrote the manuscript; J.F. Hoffman, P. Bennekou and P. David read and revised the manuscript.

2. Erythrocyte Peripheral Type Benzodiazepine Receptor /Voltage-Dependent Anion Channels are up-regulated by *Plasmodium falciparum* (Blood, 2011).

Guillaume Bouyer, Anne Cueff, Stéphane Egée, Justyna Kmiecik, Yelena Maksimova, Edyta Glogowska, Patrick G. Gallagher, Serge L.Y. Thomas.

Contribution: AC, GB contributed equally in experimental design, execution, and in-

terpretation and must be considered as co-first authors. JK, EG, SE, YM, and PGG contributed to experimental design, execution and interpretation. SLYT conceived the project and analyzed data. SLYT and PGG wrote the manuscript.

3. Anion conductance of the human red cell is carried by maxi-anion channel (Blood Cell, Molecules and Diseases, 2010).

Edyta Glogowska, Agnieszka Dyrda, Anne Cueff, Guillaume Bouyer, Stéphane Egée, Poul Bennekou, Serge L.Y. Thomas.

Contribution: SLYT conceived and designed project and experiments, contributed to their performance, analyzed data and wrote the manuscript; PB analyzed data and revised the manuscript; EG and AD contributed equally to the majority of experiments and analysed data; AC, GB and SE contributed to experimental design and to experiments.

4. Parasite membrane proteins modulate NPPs activity by changes of membrane rigidity and deformability.

Glogowska et al., *in preparation*

5. Gardos channel is still active in *Plasmodium falciparum* infected human red blood cells.

Glogowska et al., *in preparation*

I. 4. 2. Presentations on international conferences.

As a speaker:

∗ 18th meeting of European Association for Red Cell Research in Wroclaw - Piechowice, Poland (12 - 15 May 2011). 'New insights into red blood cell ionic channels in health and disease'.

As a contributor:

∗ Third Young Scientist Day, Station Biologique, Roscoff, France (24 November 2011). 'New insights into red blood cell ionic channels in health and disease'. (poster: E. Glogowska)

∗ Red Cell Club Conference, Philadelphia, USA (14 - 15 October 2011). 'Role of ionic channels in health and disease: a unifying hypothesis'. (oral: SLY. Thomas)

∗ Ephitelia & Membrane Transport Themed Meeting (1 - 3 September 2011). 'Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are up-regulated by *Plasmodium falciparum*. (oral+poster: G. Bouyer)

∗ Red Cell Club Conference, Cincinnati, USA (8 - 9 October 2010). 'Anionic conductance of human erythrocyte is carried by a peripheral-type benzodiazepine receptor/voltage-dependent anion channel susceptible of up-regulation by *Plasmodium falciparum*'. (oral: SLY. Thomas)

∗ BioMaIPar annual meeting, EMBL Heidelberg, Germany (6 - 7 May 2010). 'Further clues on the identity of the permeation pathways activated in *Plasmodium falciparum*-infected erythrocytes'. (oral: SLY. Thomas)

∗ Red Cell Club Meeting, American Society for Hematology, New-Haven Yale, USA (16 - 17 October 2009). 'Further clues on the identity and dynamical behaviour of ionic channels in human red blood cell'. (oral: SLY. Thomas)

∗ BioMaIPar annual meeting, EMBL Heidelberg, Germany (18 - 20 May 2009). 'Further clues on the identity of the permeation pathways activated in *Plasmodium falciparum*-infected erythrocytes'. (poster: SLY. Thomas)

∗ 17th meeting of European Association for Red Cell Research in Truggio, Italy (23 - 27 April 2009). 'Gardos and anionic channels activation induced by local membrane deformation in intact human red blood cells'. (oral: A. Dyrda)

I. EN BREF.

I. 1. Thème de recherche.

L'érythrocyte humain (hématie) est, d'un point de vue structurel, la plus simple des cellules d'eucaryotes. Dépourvu d'organelles intracellulaires, il constitue un modèle biologique classique pour l'étude des mouvements d'ions, de nutriments et d'autres solutés à travers la membrane cellulaire. Bien qu'abondamment étudié depuis de nombreuses années, ce modèle pose encore de multiples questions qui restent sans réponse (pour revue voir: Bernhardt and Ellory, 2003). Les érythrocytes assurent leur fonction respiratoire (transport de l'oxygène des poumons ou branchies vers les tissus et transport inverse pour le dioxyde de carbone) grâce à deux structures moléculaires spécialisées: l'hémoglobine et un échangeur d'anions (Cl^-/HCO_3^-). Tous les autres transporteurs de la membrane (pompes, échangeurs, cotransporteurs) sont là pour assurer le maintien de l'homéostasie cellulaire. Ils maintiennent la constance du volume et les propriétés d'élasticité qui permettent les déformations nécessaires au franchissement des capillaires les plus fins. Pendant longtemps, la plupart des informations disponibles sur les transports membranaires provenaient de mesures de flux isotopiques et il a été montré au cours des trois dernières décades que la membrane érythrocytaire est pourvue d'une grande variété de canaux ioniques (anioniques et cationiques); cependant les protocoles de protéomiques standards n'ont pas permis la détection de ces canaux du fait de la très faible quantité de protéines que représentent 100 à 200 canaux par cellule. La technique électrophysiologique du 'patch-clamp', qui permet l'enregistrement unitaire des courants résultant de l'activité des canaux était donc la meilleur méthode pour mettre en évidence ces voies de conduction. Cependant, du fait des difficultés techniques liées à la petite taille des cellules et à leur remarquable déformabilité, la méthode ne fut que très peu utilisée et les informations disponibles sur les canaux

sont longtemps restées fragmentaires. En particulier, leur identité, leur rôle physiologique éventuel et leur régulation commence seulement à apparaître. Il semblait peu probable qu'ils participent directement au maintien de l'homéostasie plutôt assuré par les cotransporteurs et par la quasi- absence de perméabilité cationique (pump-leak concept, Tosteson and Hoffman, 1960). Lorsque ce travail de thèse a été entrepris, il était par contre acquis que les canaux participent au processus de sénescence et sont actifs dans certaines situations pathologiques comme la drépanocytose et le paludisme. Il n'était donc pas sûr que les canaux jouent un rôle physiologique mais il était certain qu'ils constituent une menace pour l'homéostasie cellulaire.

Depuis 1999, le groupe de recherche “Physiologie comparée des érythrocytes” de la Station Biologique de Roscoff (France) travaille à la description au niveau moléculaire de l’activité des canaux de la membrane des érythrocytes, érythrocytes nucléés (de poissons et d’oiseaux) ou non nucléés (humains) pour déterminer leur régulation et leur rôle physiologique ou pathophysiologique (malaria). Leurs premières observations ont indiqué que les canaux de l’érythrocyte humain sont inactifs dans la cellule non stimulée mais qu’ils peuvent, dans certaines situations expérimentales, générer des courants puissants capables d’induire des désordres électrolytiques tells que la déshydratation cellulaire et la dissipation des gradients. Comment donc expliquer la présence de ces canaux qui menacent l’homéostasie cellulaire?

Du fait que ces premiers travaux avaient déjà fourni des indices quant à caractérisation électrophysiologique des canaux en conditions ‘normales’ ou après infection par le parasite du palud (Egee et al., 2002; Decherf et al., 2004; Bouyer et al., 2006; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009; Dyrda et al., 2010), l’objectif de cette thèse a été de poursuivre dans cette voie afin de préciser la nature moléculaire, la régulation et le rôle physiologique des canaux de la membrane de l’érythrocyte humain. Ce travail a été réalisé à la Station Biologique de Roscoff sous la direction du Dr Serge Thomas.

I. 2. Situation du sujet et objectifs.

Globalement, compte tenu de l'état d'avancement des recherches sur les érythrocytes humains, il apparaît clairement que les hypothèses sur le rôle physiologique des canaux dépendront directement de la détermination précise de la nature moléculaire des canaux. Les canaux cationiques sont déjà assez bien décrits, contrairement aux canaux anioniques qui n'ont suscité l'intérêt que très récemment. En effet, il était admis depuis bien longtemps que la conductance anionique résultait d'une particularité fonctionnelle de l'échangeur électroneutre Cl^-/HCO_3^- (band 3, AE1) qui dans une fraction infime de son fonctionnement pouvait comporter une activité électrogénique. Plusieurs groupes d'électrophysiologistes dont le notre ont remis ce dogme en question et ont pu montrer la présence de véritables canaux ioniques dans la membrane. Utilisant les configurations '*cell-attached*' et '*excised inside-out*' de la technique du patch-clamp, le groupe de Roscoff a identifié électrophysiologiquement trois types de canaux ioniques: un canal de faible conductance linéaire (SCC, ~5 pS), un canal de conductance moyenne présentant une rectification entrante (IRC, ~15 pS) et un canal de grande conductance présentant une rectification sortante (ORC, 75 - 85 pS). Ces canaux n'ont pas été caractérisés an plan moléculaire mais il a par contre été clairement établi que ces canaux sont susceptibles d'activation par le parasite *Plasmodium falciparum* (Desai et al., 2000; Egee et al., 2002; Huber et al., 2002 ; Thomas and Lew, 2004; Bouyer et al., 2006; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009). On savait depuis longtemps qu'à la suite de l'invasion de l'érythrocyte par le parasite une forte activité électrique était induite dans la membrane par l'intervention de nouvelles voies de perméabilité (New Permeability Pathways, NPPs), montrant des caractéristiques de canaux anioniques (Ginsburg et al., 1983; Ginsburg, 1994; Kirk, 2001) sans que l'on sache si cette activité résultait de canaux endogènes constitutifs de la membrane érythrocytaire ou de canaux d'origine parasitaire et adressés à la membrane érythrocytaire. Par ailleurs, un travail récent du groupe de Roscoff (Dyrda et al., 2010) a montré que la déformation de la membrane de l'érythrocyte humain provoque une entrée de calcium qui induit l'activité du canal K^+ activable par les ions Ca^{2+} (dit canal Gardos) et que des canaux anioniques peuvent être activés

secondairement à l'activation du canal Gardos. Il est apparu alors que cette activation combinée de canaux cationiques et anioniques rendait possibles des variations larges mais rapidement réversibles de la composition ionique et du volume cellulaire capables d'influer sur la fonction respiratoire des érythrocytes (Thomas et al., 2011).

Dans ce contexte, le présent travail de thèse a porté sur: 1/ la description de l'activité électrophysiologique anionique de la membrane érythrocytaire, 2/ l'identification de la nature moléculaire des canaux correspondants, 3/ la détermination des mécanismes de leur activation lors de l'infection palustre, 4/ la détermination de leur rôle dans: i) le processus de sénescence des érythrocytes, ii) l'infection palustre.
Les quatre objectifs de cette thèse, développés dans quatre chapitres distincts sont donc:

Premier objectif:

Poursuite de la caractérisation électrophysiologique des canaux anioniques de la membrane de l'érythrocyte humain.

Cette première partie prolonge les travaux déjà engagés dans l'équipe qui s'ajoutent aux travaux d'autres équipes dans le monde et qui jusqu'alors ne permettaient pas une vision très claire de la nature des canaux. Ce travail avait donc pour premier but de mettre fin à une certaine confusion dans le domaine.

Second objectif:

Détermination de l'identité moléculaire et de la régulation des canaux anioniques en situation normale ou en situation pathologique.

Comme les résultats du premier objectif ont démontré que la grande diversité des canaux décrits dans la littérature correspondait en fait à des sous-états d'un maxi-canal anionique, et compte tenu de nos observations sur la sélectivité et sur les propriétés du canal, nous avons fait l'hypothèse, puis démontré l'existence d'un 'voltage dependent anion channel' (VDAC). Caractérisé à l'origine comme une

porine mitochondriale, cette protéine peut également exister dans toute membrane cellulaire soit seule soit en formant un complexe de type 'peripheral-type benzodiazepine receptor' (PBR) constitué de trois composantes: une protéine de 18-kDa 'tranlocator protein' portant l'acronyme TSPO, la protéine VDAC de 32-kDa et une protéine de 33-kDa 'adenine nucleotide transporter' (ANT).

Troisième objectif:

Détermination du possible rôle de la protéine parasitaire RESA1 dans l'activation des canaux anionique par *P. falciparum.*

La présence d'un complexe (PBR) incluant un VDAC ayant été démontrée, la question se posait de savoir comment le parasite peut activer le canal VDAC après l'invasion. Une possibilité était l'exportation à la membrane érythrocytaire de protéines parasitaires. De telles protéines (Pf155/RESA1) jouent très tôt après l'invasion, un rôle crucial dans les modifications de la membrane en interagissant avec la spectrine et en stabilisant le cytosquelette pour permettre la résistance aux températures fébriles (Silva et al., 2005; Pei et al., 2007; Mills et al., 2007). Une équipe de l'Institut Pasteur avec qui nous collaborons a observé que RESA1 confère une résistance érythrocytes infectés contre la lyse osmotique en conditions fébriles. La protéine RESA1 participe-t-elle également à l'activation des canaux?

Quatrième objectif:

Rôle physiologique des canaux ioniques de l'érythrocyte humain: une hypothèse unificatrice concernant la sénescence, la drépanocytose et le paludisme.

Comme la déformation de la membrane de l'érythrocyte humain provoque une entrée de calcium qui induit l'activité du canal K^+ activable par les ions Ca^{2+} (dit canal Gardos) et que des canaux anioniques peuvent être activés secondairement à l'activation du canal Gardos. Comme également le canal Gardos est inactif en condition 'normale' mais participe au processus de sénescence et de déshydratation cellulaire lors de drépanocytose, les résultats des trois volets précédents incitaient à proposer une hypothèse unificatrice basée sur la cascade

'déformation/entrée de calcium/activation du canal Gardos/activation du VDAC' Quelques enregistrements supplémentaires étaient nécessaires pour renforcer cette hypothèse.

I. 3. Résumé des résultats.

Premier objectif

Poursuite de la caractérisation électrophysiologique des canaux anioniques de la membrane de l'érythrocyte humain.

Cette étude fait appel à la configuration *cell-attached* de la technique du patch-clamp pour démontrer que la conductance globale de la membrane d'un érythrocyte 'dormant', en suspension en conditions physiologiques, peut être activée considérablement dans certaines conditions expérimentales. Cette conductance, aussi bien que celles, très diverses, décrites par d'autres auteurs correspond à l'activation de maxi-canaux anioniques, de 600 pS de conductance maximale, montrant de très nombreux niveaux de conductance et des propriétés cinétiques et pharmacologiques variables selon les conditions expérimentales. Nous démontrons le rôle d'activateur joué par le sérum humain dans le recrutement de multiples niveaux de conductance et avons classé les types d'activité en trois catégories : A, entre 0 and 50 pS; B, entre 0 and 100 pS; C, entre 0 and 600 pS; Tous correspondent aux différents niveaux de conductance d'un seul et unique type de canal présent dans la membrane à environ 100 à 150 copies. Ce canal montre une préférence nette pour les ions SCN^- aux faibles conductances et aux faibles concentrations (10 mM) et les enregistrements effectués aux fortes concentrations (69 mM) correspondent à ces effectués sur des cellules infectées *P. falciparum* (Egee et al., 2002; Bouyer et al., 2006).

Second objectif

Détermination de l'identité moléculaire et de la régulation des canaux anioniques en situation normale ou en situation pathologique.

Pour tester l'hypothèse que ce maxi-canal anionique pouvait être, compte tenu de ses caractéristiques, un canal VDAC nous avons mis en œuvre des techniques de 'western blotting' et d'immunofluorescence qui nous ont permis de démontrer la présence des trois protéines composant le complexe 'peripheral-type benzodiazepine receptor (PBR)'. L'utilisation de la RT-PCR quantitative a permis à nos collègues du 'Department of Cellular and Molecular Physiology' (Yale Medical School, USA) de confirmer que les gènes codant pour les trois types de VDAC sont bien exprimés dans les cellules érythroïdes, surtout VDAC2 and 3, ainsi que les gènes codant pour les deux TSPO et ceux codant pour les trois ANT.
Comme les nouvelles voies de perméabilité (NPPs) induites par le parasite *P. falciparum* présentent de nombreux points communs avec l'activité électrique du complexe PBR/VDAC et comme les ligands du PBR sont connus pour être de puissants inhibiteurs de la croissance des parasites *in vitro* (Dzierszinski, 2002; Bah, 2007), nous avons fait l'hypothèse que cette activité NPP pouvait résulter d'une interaction entre le parasite et ce complexe PBR par le biais d'une suractivation de l'activité VDAC. L'utilisation de tests de croissance, d'hémolyse au sorbitol et d'électrophysiologie a permis de valider l'hypothèse que le complexe PBR/VDAC est dormant en situation normale mais qu'après l'invasion il s'active en NPP. Ces canaux deviennent dès lors des cibles pour une inhibition sélective ou comme route pour l'acheminement de drogues dans une thérapie antipaludique.

Troisième objectif

Détermination du possible rôle de la protéine parasitaire RESA1 dans l'activation des canaux anionique par *P. falciparum.*

Comme mentionné plus haut, la protéine RESA1 (Ring infected Erythrocyte Surface Antigen) également appelée Pf155 pourrait être impliquée dans le processus d'activation des canaux (VDAC) constituant les NPPs. Des approches biochimiques et électrophysiologiques ont été effectuées sur un grand nombre d'érythrocytes

infectés par les souches 'RESA1-wild type', 'RESA1-KO mutant', 'RESA1-KI revertant'. Des technique de 'western blotting' et d'immunofluorescence ont confirmé que RESA1 est bien exportée à la membrane érythrocytaire pendant les 12 premières heures suivant l'invasion par les souches 'RESA1-wild type' et 'RESA1-KI revertant' , ce qui n'est pas le cas pour la souche 'RESA1-KO mutant'. La configuration *whole-cell* du patch-clamp qui permis une mesure des courants globaux a montré tout d'abord que les conditions de culture influence considérablement l'effet de RESA1 sur l'activation des canaux. Un composé du sérum humain est nécessaire à cette activation et pourrait donc intervenir au niveau de l'adressage de la protéine RESA1 à la membrane. Ensuite et surtout des différences sont apparues entre les enregistrements correspondant aux trois souches lors de l'activation des canaux par le sérum (selon un protocole comparable à celui de l'objectif 1). L'activation des canaux était nettement supérieure pour RESA1-WT que pour RESA1-KO, ce qui fournit la démonstration que RESA1 participe au processus modulation des NPPs. Si tel est le cas, nous pouvions attendre une différence comparable dans les tests d'hémolyse au sorbitol puisque des cellules comportant des NPPs actifs dans leur membrane subissent l'hémolyse contrairement aux cellules non-infectées. Nos résultats démontrent en effet que les cellules infectées par RESA1-WT présentent plus d'hémolyse que celles infectées par RESA1-KO.

Quatrième objectif

Rôle physiologique des canaux ioniques de l'érythrocyte humain: une hypothèse unificatrice concernant la sénescence, la drépanocytose et le paludisme.

Il était connu que des canaux ioniques érythrocytaires sont impliqués dans des processus comme la sénescence (Lew et al., 2007), l'anémie falciforme de la drépanocytose (Lew and Bookchin, 2005) et le paludisme (Ginsburg, 1983; Ginsburg and Kirk, 1998). Qu'une seule chaine de transporteurs soit impliquée selon une séquence (1: déformation, 2: entrée de calcium, 3: activation du canal Gardos, 4: activation du VDAC en mode cationique puis en mode anionique) identique dans ces différents cas est une hypothèse séduisante. Les expériences

entreprises ici avaient pour objectif de renforcer le modèle sur deux points: 1/ La sensibilité du Gardos à la déformation membranaire décline-t-elle avec l'âge? 2/ Ce canal Gardos est-il activable après l'infection par *P. falciparum*?
Des érythrocytes ont été séparés en cinq fractions; la fraction 1 contenant les plus jeunes, la fraction 5 contenant les plus âgées. La configuration *cell-attached* qui permet l'enregistrement unitaire de l'activité des canaux n'a pas révélé de différence significative dans la capacité du canal à s'activer lors d'une déformation de la membrane. Concernant l'activité après l'infection palustre nos enregistrements montrent que le canal reste potentiellement activable après l'infection.

Conclusion

Les érythrocytes sont pourvus de transporteurs membranaires puissants capables de provoquer des variations très rapides du volume cellulaire et des équilibres acido-basiques et électrolytiques susceptibles d'influencer efficacement leur fonction respiratoire. Nos travaux ont contribué à faire avancer la connaissance du rôle spécifique joué par les canaux ioniques dans la membrane de l'hématie humaine. Ils démontrent qu'il serait simpliste de considérer, comme ce fut longtemps le cas, que les canaux ioniques ne sont que des reliques ou des résidus. Même s'ils paraissent, dans l'état actuel des connaissances, très peu spontanément actifs, cette apparente inactivité n'est probablement que le résultat de conditions expérimentales *in vivo* qui ne reproduisent que très peu les sollicitations mécaniques et physico-chimiques auxquelles un érythrocyte doit constamment faire face. L'activation du canal Gardos seul constituerait donc une menace pour l'homéostasie cellulaire; les effets conjugués du canal Gardos et du VDAC rendent, au contraire, possibles des variations rapides et réversibles qui, combinées à la puissance des pompes Ca^{2+}-ATPase et Na^{+}/K^{+}-ATPase constituent bien plus un avantage qu'une menace pour l'homéostasie cellulaire.

I. 4. Communications scientifiques.

I. 4. 1. Publications.

1. Ion channels in human red blood cell membrane: Actors or relics? (Blood Cells, Molecules and Diseases, 2011).

Serge L.Y. Thomas, Guillaume Bouyer, Anne Cueff, Stéphane Egée, Edyta Glogowska, Céline Ollivaux.

Contribution: SLYT wrote the manuscript; J.F. Hoffman, P. Bennekou and P. David read and revised the manuscript.

2. Erythrocyte Peripheral Type Benzodiazepine Receptor /Voltage-Dependent Anion Channels are up-regulated by *Plasmodium falciparum* (Blood, 2011).

Guillaume Bouyer, Anne Cueff, Stéphane Egée, Justyna Kmiecik, Yelena Maksimova, Edyta Glogowska, Patrick G. Gallagher, Serge L.Y. Thomas.

Contribution: AC, GB contributed equally in experimental design, execution, and interpretation and must be considered as co-first authors. JK, EG, SE, YM, and PGG contributed to experimental design, execution and interpretation. SLYT conceived the project and analyzed data. SLYT and PGG wrote the manuscript.

3. Anion conductance of the human red cell is carried by maxi-anion channel (Blood Cell, Molecules and Diseases, 2010).

Edyta Glogowska, Agnieszka Dyrda, Anne Cueff, Guillaume Bouyer, Stéphane Egée, Poul Bennekou, Serge L.Y. Thomas.

Contribution: SLYT conceived and designed project and experiments, contributed to their performance, analyzed data and wrote the manuscript; PB analyzed data and revised the manuscript; EG and AD contributed equally to the majority of experiments and analysed data; AC, GB and SE contributed to experimental design and to experiments.

4. Parasite membrane proteins modulate NPPs activity by changes of membrane rigidity and deformability.

Glogowska et al., *en préparation*

5. Gardos channel is still active in *Plasmodium falciparum* infected human red blood cells.

Glogowska et al., *en préparation*

I. 4. 2. Présentations lors de conférences internationales.

Comme présentateur:

∗ 18th meeting of European Association for Red Cell Research in Wroclaw - Piechowice, Poland (12 - 15 May 2011). 'New insights into red blood cell ionic channels in health and disease'.

Comme contributeur:

∗ Third Young Scientist Day, Station Biologique, Roscoff, France (24 November 2011). 'New insights into red blood cell ionic channels in health and disease'. (poster: E. Glogowska).

∗ Red Cell Club Conference, Philadelphia, USA (14 - 15 October 2011). 'Role of ionic channels in health and disease: a unifying hypothesis'. (oral: SLY. Thomas)

∗ Ephitelia & Membrane Transport Themed Meeting (1 - 3 September 2011). 'Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are up-regulated by *Plasmodium falciparum*. (oral+poster: G. Bouyer)

∗ Red Cell Club Conference, Cincinnati, USA (8 - 9 October 2010). 'Anionic conductance of human erythrocyte is carried by a peripherial-type benzodiazepine receptor/voltage-dependent anion channel susceptible of up-regulation by *Plasmodium falciparum*'. (oral: SLY. Thomas)

∗ BioMaIPar annual meeting, EMBL Heidelberg, Germany (6 - 7 May 2010). 'Further clues on the identity of the permeation pathways activated in *Plasmodium falciparum*-infected erythrocytes'. (oral: SLY. Thomas)

∗ Red Cell Club Meeting, American Society for Hematology, New-Haven Yale, USA (16 - 17 October 2009). 'Further clues on the identity and dynamical behaviour of ionic channels in human red blood cell'. (oral: SLY. Thomas)

∗ BioMaIPar annual meeting, EMBL Heidelberg, Germany (18 - 20 May 2009). 'Further clues on the identity of the permeation pathways activated in *Plasmodium falciparum*-infected erythrocytes'. (poster: SLY. Thomas)

∗ 17th meeting of European Association for Red Cell Research in Truggio, Italy (23 - 27 April 2009). 'Gardos and anionic channels activation induced by local membrane deformation in intact human red blood cells'. (oral: A. Dyrda)

CHAPTER II

THEORETICAL BACKGROUND

II. THEORETICAL BACKGROUND.

II. 1. Human red blood cells.

Red blood cells (RBCs), also called erythrocytes make up the most plentiful morphotic elements of the blood population, numbering of (4 - 6) x 10^6 per one microliter of this tissue. From stem cells in bone marrow, human erythroid cells are differentiated through a process named erythropoiesis to become mature erythrocytes. A typical human erythrocyte measures about 6 - 8 μm in diameter and 2 μm in thickness, and has flattened biconcave shape. Circulating human RBCs are relative simple cells due to the lack of intracellular organelles (mitochondria, ribosomes, Golgi apparatus, endoplasmic reticulum, lysosomes) and nucleus. These characteristic properties allow to optimize their two main duties: i) the delivery of oxygen from lungs to tissues, and ii) removal of waste such as carbon dioxide, both caused by increasing in capacity of transported O_2 (lack of organelles let more space for haemoglobin, Hb, universal respiratory oxygen-fixing pigment) and area-to-volume ratio (greater for biconcave shape than a sphere of the same diameter). These functions of RBCs are realized by two specialized molecular machines: Hb (normal erythrocytes contain 5 mM of this metalloprotein, constituting 97.5% of the total cell protein by weight) and membrane anion exchange carrier (AE1).

From the structural point of view human red blood cells are the simplest of all eukaryotic cells and this makes them very useful tool for studies on plasma membrane transport systems.

Figure II. 1

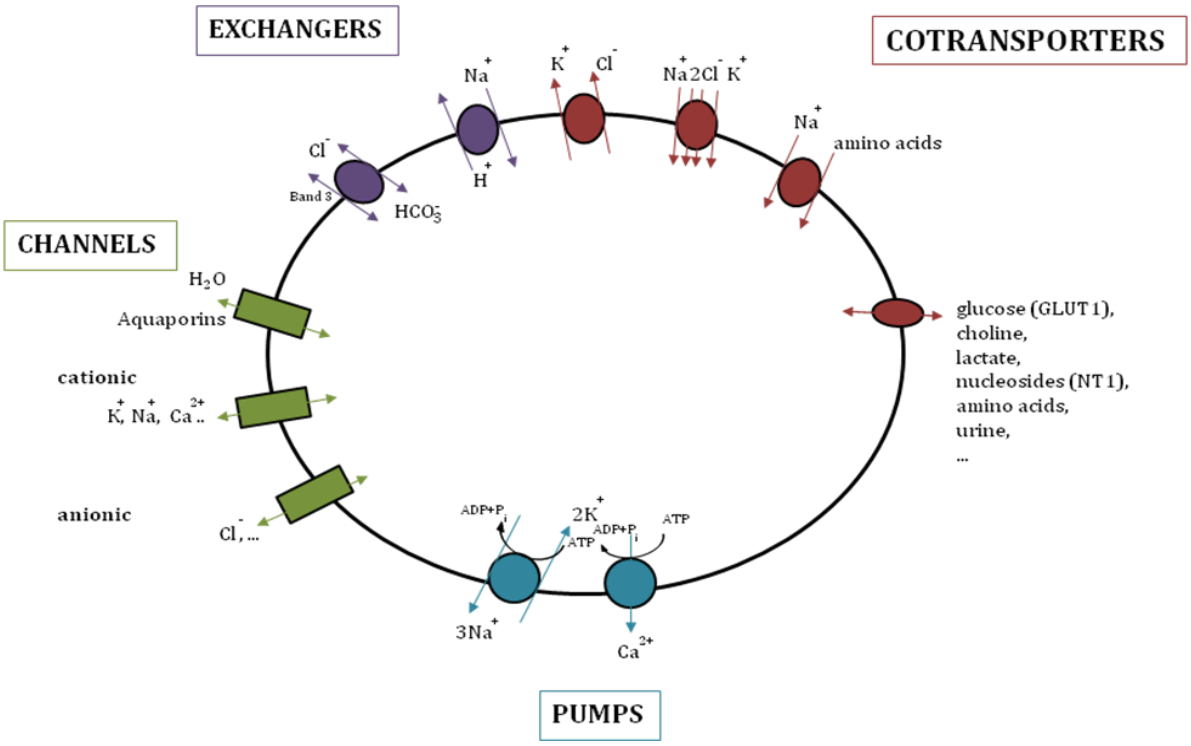


Figure II. 1: **Overview of the principal transport pathways of the human red blood cell membrane.** Ions transport across the red cell membrane is realized by specific mechanisms: pumps (using the energy of ATP hydrolysis to transport ions against their electrochemical gradient), channels (specific proteins allowing ions to cross the membrane by the use of passive flow down their electrochemical gradients) and cotransporters: antiporters, symporters and uniporters (movement of one ion species against its electrochemical gradient is powered by the downhill movement of another). The principal transporters in the human RBCs are shown. Cationic (Ca^{2+}-activated K^+ channel known as a Gardos channel, and non-selective cationic channel NSC) and anionic channels are pointed out. Up to-date only cationic channels have been well characterized.

II. 2. Membrane transporters of the human RBCs in health.

Whereas the molecules such as O_2 and CO_2 pass through the red cell membrane by diffusion according to their partial pressure gradients, organic and inorganic compounds (among them ions as the most interesting from the point of this thesis), influencing the electrolyte and acid-base intracellular equilibrium, need other specific pathways. Different transport systems have been characterized in the membrane of normal (non-infected) human red blood cells:

- *pumps*, using the energy of ATP hydrolysis to transport ions against their electrochemical gradient;
- *channels*, specific proteins allowing ions to cross the membrane by the use of passive flow down their electrochemical gradients;
- *cotransporters*: antiporters, symporters and uniporters, in which movement of one ion species against its electrochemical gradient is powered by the downhill movement of another (summary in Fig. II. 1).

Interaction of membrane transporters, cytoplasmic buffer (Table II. 1 provides the electrolyte composition of plasma), charge and osmotic properties of haemoglobin and other impermeable solutes assure the control of RBC volume, pH, membrane potential and ion content. Therefore, transporters (together with membrane cytoskeleton) contribute to maintenance of the cell integrity, its stability and deformability in response to shear forces of blood circulation.

This work focuses on ionic channels in the human red cell membrane and, because up to-date only cationic channels (Ca^{2+}-activated K^+ channel known as a Gardos channel, and non-selective voltage-dependent cationic channel NSVDC, called further NSC) have been well characterized, it is aimed at describing anionic conductive pathways, clarifying some controversial and unanswered aspects and verifying their physiological role.

Table II. 1

		PLASMA	ERYTHROCYTE
Cations:		[mM]	[mM]
Sodium	Na^+	142	11
Potassium	K^+	4.5	140
Calcium	Ca^{2+}	2.5	0.0001
Magnesium	Mg^{2+}	1	1.5 - 2.4
		total: 150 mM/L	
Anions:			
Chloride	Cl^-	104	80
Bicarbonate	HCO_3^{2-}	24	variable
Phosphate	HPO_4^{2-}	2	
Proteins		14 (60 - 80 g/L)	5 (340 g/L)
Others		6	
		total: 150 mM/L	

Table II. 1: **Values of the major electrolytes of the human plasma and erythrocyte** (according to Blacque-Belair, 1991).

II. 2. 1. Exchangers, pumps, cotransporters.

Among the different types of RBC membrane transporters, the major protein Cl^-/HCO_3^- anion exchange carrier AE1 (also called band 3, SLC4A1) plays an essential role in the increasing of blood CO_2-carrying capacity and supports acid-base homeostasis (LaCelle and Rothsteto, 1966; Gunn et al., 1973; Cabantchik, 1999). Due to the strong interaction with lipids and proteins of membrane cytoskeleton it assures mechanical integrity and viscoelasticity of RBCs, allowing them resistance to the shear forces of circulation and squeeze through the narrow capillaries (Jay, 1996).

Maintenance of the cell volume is mainly realized by well characterized primary active and energy consuming pumps: Na^+/K^+ ATPase and Ca^{2+} ATPase. Indeed, pump-leak mechanism (Tosteson and Hoffman, 1960) sustains a high intracellular K^+ concentration (around 140 mM) and low intracellular Na^+ concentration (around 5 mM), by pumping sodium out of the cell and potassium into the cell and thus generating electrochemical gradients for both ions (see Table II. 1 for electrolytes value). In steady-state cytoplasmic Na^+/K^+ ratio is 0.12 - 0.16 (Bernstein, 1954). The 'leak' of sodium and potassium results from other exchangers, transporters and channels present in the red cell membrane.

The electrolytes gradient can be used by erythrocytes to facilitate the movement of different solutes through the membrane via secondary active transporters, labelled as cotransporters (symporters or antiporters according to the relative direction of solutes). The Na^+/H^+ exchanger is an example of such transporter, which plays a key role in the regulation of intracellular pH, using the energy of the Na^+ gradient to extrude H^+ (Kaloyianni et al., 2001).

Furthermore, other shown principal membrane transporters are: aquaporin 1 (a water channel), K^+-Cl^- cotransporter, a Na^+-$2Cl^-$-K^+ cotransporter, some amino acids transporters, a glucose transporter (Glut1), an oxidized gluthatione (GSSG) transporter (GSTP), a nucleoside transporter (NT1), choline, lactate, urea … transporters.

The human erythrocyte possesses also conductive pathways for ions which are cationic and anionic channels.

II. 2. 2. Ionic channels.

For a long time the studies on ions passing the red cell membrane through the channels were very poor, due to the limitations of available techniques. Most of the early information about these conductive pathways came from flux experiments, unfortunately restricted in molecular details. The knowledge of RBC membrane permeabilities at the molecular level has evolved due to the successful application of the patch-clamp electrophysiological technique, allowing research on channel-mediated transport of charged solutes across erythrocyte membrane. This led to characterization of ionic channels in nucleated erythrocytes e.g. fish (Egee et al., 1998; Lapaix et al., 2002), and avian (Lapaix et al., 2008). In contrast, human red blood cells brought more difficulties caused by the fragility and a small size of these cells limiting the use of *whole-cell* recordings.

II. 2. 2. 1. Cationic channels.

However, in the human red cell membrane two different cationic channels have been characterized:

- Ca^{2+}-sensitive K^+ channel (also called Gardos channel, Hamil, 1981; Grygorczyk et al., 1984; Grygorczyk and Schwarz, 1985; Shields et al., 1985; Stampe and Vestergaard-Bogind, 1985; Alvarez and Garcia-Sancho, 1987; Grygorczyk, 1987; Fehlau et al., 1989; Bennekou and Christophersen, 1990; Christophersen, 1991; Leinders et al., 1992; Romero and Rojas, 1992; Pellegrino and Pellegrini, 1998; Pellegrino et al., 1998; Fanger et al., 1999; Del Carlo et al., 2003; Low et al., 2008; Tharp and Bowles, 2009);
- non-selective cationic channel (NSC) (Christophersen and Bennekou, 1991; Bennekou, 1993; Kaestner et al., 1999; Kaestner et al., 2000; Huber et al., 2001; Kaestner and Bernhardt, 2002; Duranton et al., 2002; Lang et al., 2003; Rodighiero et al., 2004).

Patch-clamp electrophysiological experiments allowed describing Gardos channel as: highly-K^+ selective, voltage-independent (Tharp and Bowles, 2009), inwardly rectified with a conductance of about 20 pS. Four Ca^{2+} cations have been shown to activate this channel via interaction with calmodulin (Fanger et al., 1999). Sensitiv-

ity for Ca^{2+} has been reported as pH dependent (Stampe and Vestergaard-Bogind, 1985). Furthermore, open probability of Gardos channel increases with the reduction of temperature (Grygorczyk, 1987) and with an increase of intracellular calcium (Leinders et al., 1992b). When fully activated, Gardos cause membrane hyperpolarization near the K^+ equilibrium potential, providing a driving force for rapid KCl and water loss, rate-limited by Cl^- permeability. In addition, intracellular antioxidant system of erythrocytes has been suggested as a modulator of Gardos channel behaviour (Low et al., 2008).

By using the patch-clamp technique the activity of another cationic conductance has been also depicted, corresponding to a non-selective voltage-dependent cationic channel, proposed as a nicotinic type of acetylcholine receptor (Bennekou, 1993) and activated at depolarized membrane potential. On the other hand, it has been shown that oxidation of the RBC membrane or energy depletion (Duranton et al., 2002) stimulates this channel. Further, NSC can be activated by removal of intracellular and extracellular Cl^- (Huber et al., 2001; Duranton et al., 2002; Rodighiero et al., 2004), by incubation in low ionic strength (LIS) medium (LaCelle and Rothsteto, 1966; Jones and Knauf, 1985; Bernhardt et al., 1991) or by prostaglandine PGE_2 (Kaestner et al., 1999; Kaestner and Bernhardt, 2002; Kaestner et al., 2004). The non-selective cationic channel has been reported to be permeable to mono- and divalent cations, especially Ca^{2+} (Kaestner et al., 2000; Huber et al., 2001; Duranton et al., 2002) and inhibited by amiloride, EIPA and gadolinium (Huber et al., 2001; Duranton et al., 2002; Lang et al., 2003). However, the exact molecular nature of such cationic channel is not completely solved, especially NSVDC and NSC moiety.

II. 2. 2. 2. Anionic channels.

Already 50 years ago, Dan Tosteson calculated the anion self-exchange rate and pointed alternative pathways for Cl^- passing the red cell membrane different from its free electrodiffusion (Tosteson and Hoffman, 1960). However, as a result of technical difficulties related to the small size of erythrocytes and their remarkable deformability these conductive pathways in the human red cell membrane were

almost out of reach. Whereas tracer flux experiments showed membrane permeability of ~ 10^{-7} cm/s corresponding to anionic channels numbering in 10^{-4} – 10^{-6} of the total chloride exchange, just a few electrophysiological reports suggested their presence in the human red cell membrane.

These experiments brought however important informations to this subject. For instance, the membrane potential has been defined for about -10 to -12 mV, close to the Nernst potential for chloride (Hoffman and Laris, 1974). This was in agreement with a theory that diffusible anions are distributed in accordance with a Donnan equilibrium and that the RBC membrane was relatively impermeable to cations compared to anions (Warburg et al., 1922; Van Slyke et al., 1923; Funder et al., 1966). Limitation by anions of the salt efflux from the RBC has been shown by experiments using components (gramicidin, valinomycin) increasing the membrane permeability for cations (Harris and Pressman, 1967; Scarpa et al., 1970). They provided a model of the two-parts anion transporter: i) a large electroneutral exchanger fundamental to the CO_2-carrying capacity of the blood (Dalmark and Wieth, 1972; Gunn et al., 1973), and ii) a smaller electrogenic component that determines the RBC resting potential (Hunter 1971; Lassen et al., 1978) and could be important as the rate-limiting step for electrolyte and water movements through the RBC membrane.

The exchange concept has been strongly confirmed by Hunter, who showed the net permeability (conductance) 4 orders of magnitude smaller than the tracer permeability (Hunter, 1971; Hunter, 1977). The same author estimated the human RBC anionic permeability proper (conductance) of about 10 μS/cm^2. A similar value was obtained by Hoffman and co-workers (Hoffman et al., 1980) measuring membrane potential- dependent fluorescence, and flux from the electrogenic fraction of the Na^+/K^+-pump.

Furthermore, in 1972 Cabantchik and Rothstein identified a 100 kDa red cell membrane protein, called band 3 (AE1, SLC4A1) as the pathway for anion transport (Cabantchik and Rothstein, 1972). In spite of differences in pH dependence, energy of activation, selectivity, etc… both components, *i.e.* the electroneutral anion exchange and the conductance proper have been attributed to be mediated by band 3. Concerning the mechanism of the assumed conductive pathway it has not

been identified yet. Although band 3 has been cloned and successfully expressed in oocytes with regard to exchange, the concomitant conductance has not been observed in human band 3 probably due to a low level of expression (Alper et al., 2002). Moreover, band 3-mediated conductance has been shown to be not very important (Bennekou et al., 2001) and if assuming that the ground anion leak in human RBCs is mediated by 1 million copies of band 3, the unit conductance should be very small, and if gated, the mean open time very short.
This raises the question of whether anionic channels proper are present in the red cell membrane.

Although patch-clamp have been applied to human RBCs for a long time, until year 2000 the only report of red cell anionic conductance came from Schwarz and coworkers reporting a 10 pS anionic channel in normal cells (Schwarz and Passow, 1983) and from another study, where electrophysiological events could almost certainly be attributed to anion transport (Schwarz et al., 1989). After year 2000, successful application of this electrophysiological technique brought new light in this field. The group 'Comparative Physiology of Erythrocytes' from Station Biologique in Roscoff (France), which during the last ten years has focused at studying ionic channels in red cell membrane, and is now one of the leaders in this area, identified three types of anionic channels in human RBCs:

- a small conductance channel (~ 5 pS, SCC);
- a medium inwardly rectifying (~ 15 pS, IRC) linear conductance channel, activated either by adenylate cyclase using forskolin in the *cell-attached* configuration or by exposure to the catalytic subunit of cAMP-dependent protein-kinase in the excised *inside-out* patches;
- and an outwardly rectifying channel (75 - 85 pS at positive membrane potentials, ORC)(Egee et al., 2002; Decherf et al., 2004; Bouyer et al., 2006; Decherf et al., 2007; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009).

Interestingly, that channel activity was often observed as simultaneous gating of two channels (Decherf et al., 2007) and appeared at high membrane potentials (less than -80 mV, > 80 mV). Moreover, IRC channel was visible in more than 80%

of membrane patches, whereas ORC was only present in less than 5% of cell patches. In excised *inside-out* configuration, the activity of this large conductance channel was not stable with time and run-down was always rapidly observed leading to disappearance of channel openings within few minutes. Huge studies made by the group from Roscoff have also shown that total inhibition of channel activity could be obtained by treatment with 100 μM NPPB, 100 μM niflumic acid, 100 μM 9-AC, 1 mM DPC, and 10 μM tamoxifen (typicall inhibitors of band 3, chloride channels and cotransporter K^+-Cl^-). DIDS, at a concentration of 100 μM induced a variable partial block in all cases.

Additionally, their recent report (Dyrda et al., 2010) gave evidence for anionic channel activity subsequently generated by transient Ca^{2+}-activated K^+ channels (Gardos) upon membrane deformation. Touching with the pipette modify red cell membrane which in experimental conditions allows Ca^{2+} entry to the cell, Gardos channel activation and anionic as well, a phenomenon observed 15 min following seal formation.

Interestingly, anionic channels in the human red blood cell membrane in normal physiological experimental conditions did not exhibit activity, but it has been noted that under stimulation during experiments e.g. mechanical stress, phosphorylation, oxidation etc., they could be potentially activated. On the other hand, they have been well described in pathological conditions, since their manifestation was observed in *Plasmodium falciparum*-infected human red blood cells.

What does this dormancy of anionic channels and they stimulated activation mean therefore? What are the molecular identity and regulators of such a conductance? Finally, what is the physiological role of anionic channels of the human red blood cell membrane in health and disease? The present PhD thesis is aimed at answering, at least partly, these questions.

Figure II. 2

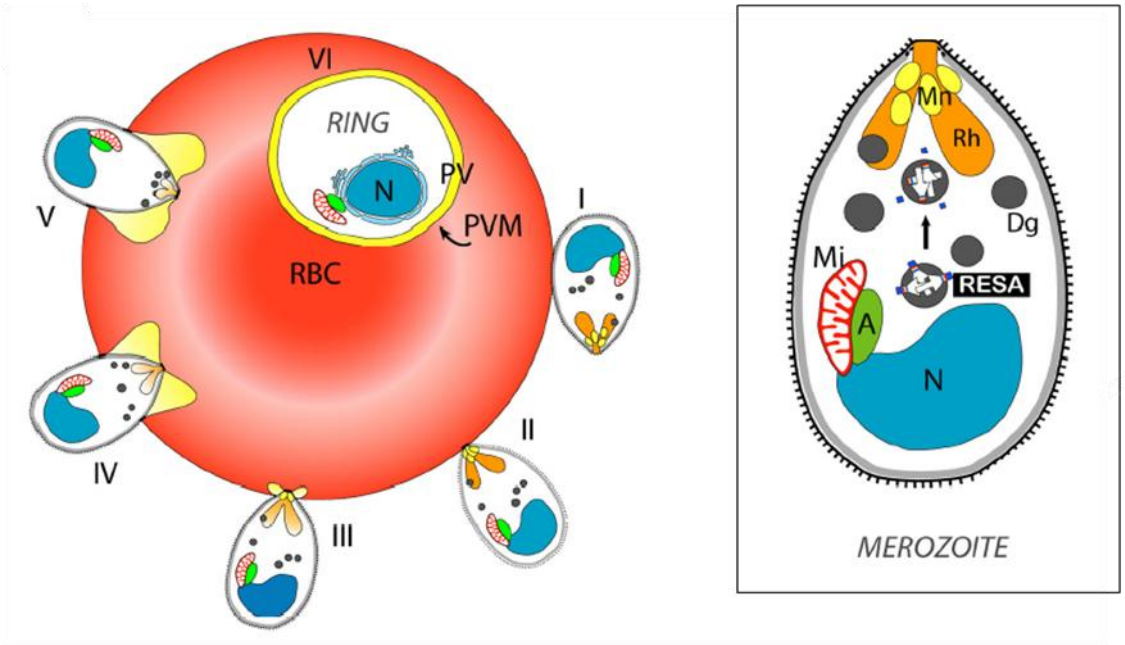


Figure II. 2: **Infection of the human red blood cells by malaria parasite.** *Plasmodium* prepares for the invasion of a new host red blood by transporting crucial molecules to the three prototypic apicomplexan organelles (the microneme [Mn], the rhoptry [Rh], and the dense granule [Dg]) located close to the apical end of the merozoite. Microneme proteins are mainly involved in initiating invasion (stages I–III), rhoptry and dense granule proteins are implicated in establishment of the parasite in the newly invaded host cell (stages III–VI e.g. RESA, Ring Infected Erythrocyte Surface Antigen). Mi, mitochondrion; A, apicoplast; N, nucleus; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane (from Marti et al., 2005).

II. 3. Human RBC membrane in disease.

As mentioned, anionic channels in the human red blood cell membrane have been firstly recognized and described, at least partly, in pathological conditions, such as in malaria after *Plasmodium falciparum* invasion. In contrast to healthy human RBCs, in this situation changes in erythrocyte membrane have been indicated.

II. 3. 1. Infection of the human RBCs by malaria parasite.

From the four known intracellular protozoan parasites of the genus *Plasmodium: P. falciparum*, *P. vivax*, *P. ovale* and P. *malariae* which caused an endemic disease called malaria transmitted by the female *Anopheles* mosquito, the first one, invading the host human RBCs during its asexual life cycle, is responsible for enormous morbidity and mortality. Infection of the human erythrocytes by malaria parasite is a complex and dynamic process. The invading forms of *P. falciparum* called merozoites interact with the red cell membrane by their 'apical' end consisting in specialized secretory organelles, such as the micronemes, rhoptries and dense granules. This leads to erythrocyte membrane deformation and formation of a stable parasite-host cell junction (Dvorak et al., 1975; Marti et al., 2005; see Fig. II. 2). Microneme proteins are mainly involved in initiating invasion, rhoptry and dense granule proteins drive vacuole formation and are implicated in establishment of the parasite in the newly invaded host cell (Cowman and Crabb, 2006). Invagination of the erythrocyte bilayer results after in establishment of the intracellular parasite surrounded by a vacuolar membrane (PVM, parasitophorous vacuole membrane), in its 'ring' stage in the host (Bannister et al., 2000). Once the merozoite has entered the erythrocyte, it differentiates from ring form to trophozoite (~ 15 h after invasion). At this stage, the erythrocyte is loosing its smooth biconcave discoid shape to become more spherical and small electro-dense protrusions on its surface called 'knobs' are formed (Sherman et al., 2004). The parasite then enters the schizont stage corresponding to a rapid DNA/RNA amplification phase leading to the formation of 8 to 32 daughter merozoites. The infected erythrocytes finally

Figure II. 3

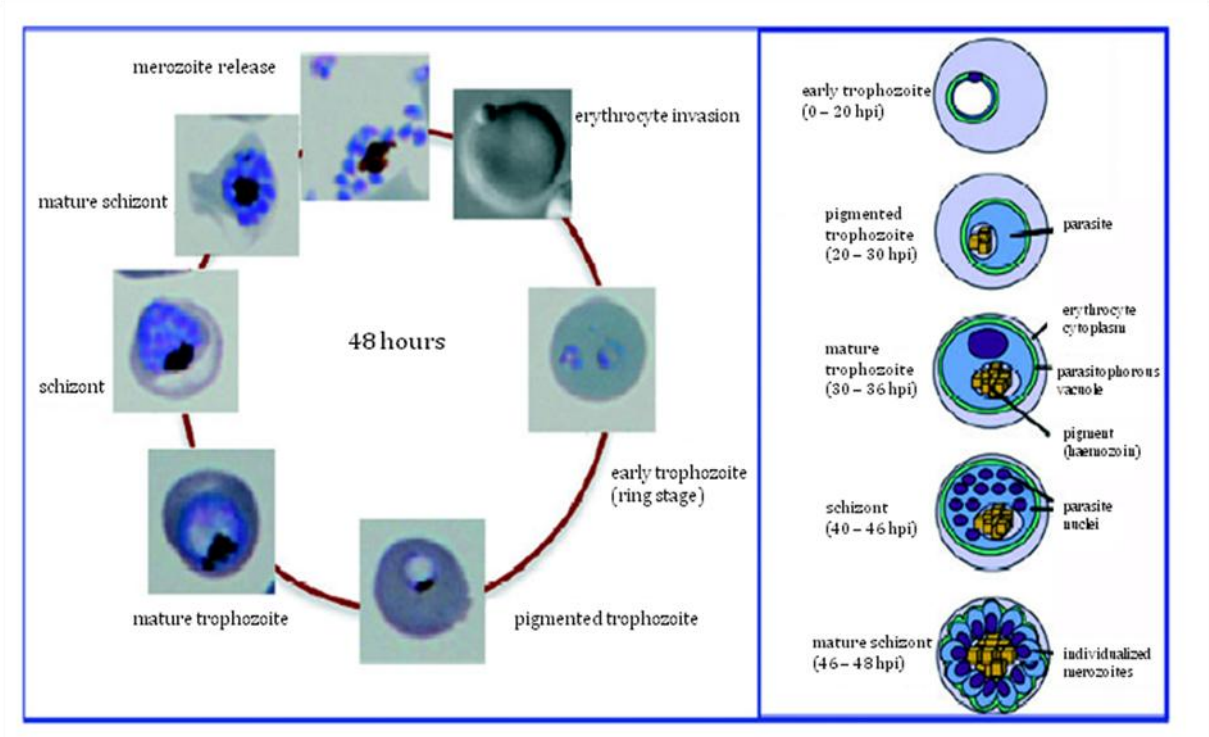


Figure II. 3: **The 48 h intraerythrocytic development of *Plasmodium falciparum*.** The different stages of the parasite intraerythrocytic development are presented as Giemsa-stained infected RBCs (from early trophozoite ring stage to merozoite release). 48 hours of malaria-parasite asexual life cycle is showing in left panel; right panel: scheme of different parasite stages. Following entry into the RBC, the merozoite differentiates into a trophozoite, which grows during the first 30–35 h of development. Haemoglobin digestion by the parasite results in the accumulation of haemozoin, also known as malaria pigment. Mature trophozoites differentiate into multinucleate schizonts. Following nuclei divisions, merozoites are individualized and further released in the external milieu. hpi, hours post-invasion (from Mbengue et al., 2012).

rupture and release merozoites ready to invade new red blood cells. Asexual life cycle of *P. falciparum* (Fig. II. 3) takes approximately 48 h.
It should be also noticed, that inside the red blood cells, the malaria parasite develop either into an asexual forms (ring, trophozoite, schizont) or a sexual (micro- and macrogametocytes, males and females, respectively). This strategy helps the parasite to protect from the harmful or lethal effects of antibodies or immune defence mechanisms of host.

During invasion and intracellular development of malaria parasite different morphological changes accompanied by metabolic and biosynthetic activity alterations occur in the host erythrocyte.

II. 3. 2. Remodelling of the host erythrocyte membrane by *Plasmodium falciparum*.

Plasmodium falciparum invades mature RBCs nearly metabolically inert and devoid of functional trafficking machinery. From the pathogenetical point of view this may seem like a perfect hideaway from the host's immune system. However, firstly the erythrocyte's environment appears for the parasite insufficient. Although the intracellular parasite is using most of the nutrients mainly from the digestion of the host haemoglobin (Rosenthal and Meshick, 1998; Krugliak et al., 2002; Lew et al., 2003), several essential components necessary for its growth have to be supplied from the outside of the infected cell. For instance, parasite survival is totally dependent on isoleucine (Sherman, 1997) and human haemoglobin does not contain this amino acid. To reach the parasite, this substrate must first cross the host red blood cell plasma membrane (Martin and Kirk, 2007). In some cases, the endogenous specific transport systems of erythrocyte membrane (described above) are able to maintain an adequate supply. This takes place, for example, for glucose, the primary energy source for the parasite, transported through endogenous receptor GLUT1. Nevertheless, for some other essential nutrients including, e.g. the vitamin B5 (panthothenic acid) (Saliba et al., 1998) or the amino acid glutamate (Divo et al., 1985) the transport pathways do not exist in the human red cell membrane.

Figure II. 4

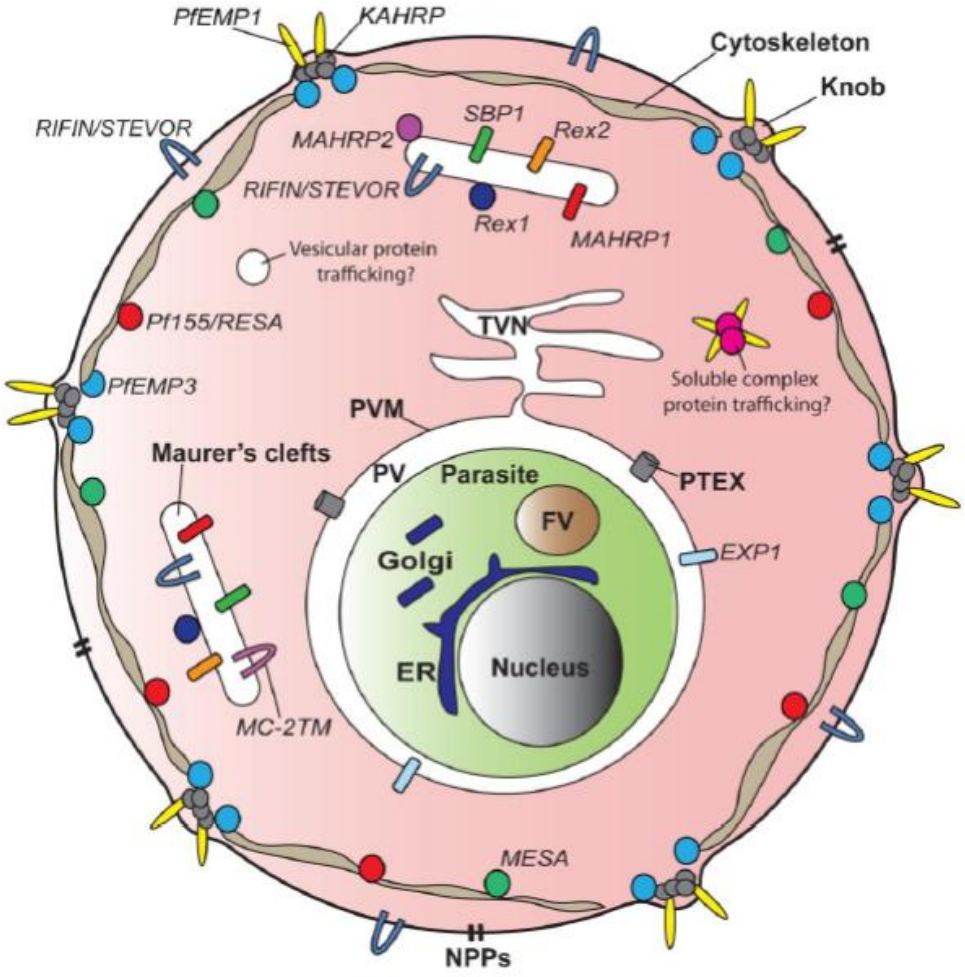


Figure II. 4: **Host cell modifications induced by *P. falciparum* parasites.** (TVN, tubulovesicular network; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; PTEX, *Plasmodium* translocon of exported proteins; NPPs, new permeability pathways; FV, food vacuole; ER, endoplasmatic reticulum; PfEMP1(3), *P. falciparum* erythrocyte membrane protein 1(3); KAHRP, knob-associated histidine-rich protein; MAHRP1(2), membrane-associated histidine-rich protein 1(2); SBP1, skeleton binding protein 1; REX1(2), ring exported protein 1(2); Rifin, repetitive interspersed protein; STEVOR, subtelomeric variable open reading frame protein; Pf155/RESA, ring-infected erythrocyte surface antigen; MESA, mature-infected erythrocyte surface antigen; MC-2TM, Maurer's cleft two transmembrane domain; EXP1, *P. falciparum* blood-stage antigen) (from ki.se/content/1/c6/08/93/.../Nilsson%202011.pdf).

Secondly, as the parasite matures inside the erythrocyte, the infected cells become vulnerable to splenic clearance mechanisms.

To overcome all these problems, the parasite dramatically remodels its host cell properties by: i) induction of new permeability pathways, ii) establishing a parasite derived trafficking machinery in the host cell cytosol, iii) interacting with the host cell cytoskeleton, and iv) expressing parasite-derived adhesins on the RBC surface. Most of these host modifications are mediated by a subset of parasite- derived proteins. General summarizing view of the host cell remodelling is shown in Fig. II. 4 (description below).

Before the description of the strategies used by malaria-parasite in the red cell membrane remodelling, it should be noticed that during intracellular development of *P. falciparum* changes occur also in erythrocyte volume, pH and ions concentration. Parasite enters red cell cytosol defined by high K^+ (140 mM), low Na^+ (10 mM) and Ca^{2+} (0.1 μM). Following the model proposed by Lew and Bookchin (Lew and Bookchin, 1986), during the first 24 hpi inversion of Na^+ and K^+ gradient arises, leading to change of the host cell cytosol composition to that remains external fluids (Staines et al., 2001; Lew et al., 2003; see Fig. II. 5).

On the other hand, the parasite's internal milieu is maintained by the combined performance of active and passive membrane transporters in the parasitophorous vacuole membrane (Allen and Kirk, 2004).

Malaria-parasite occupies a significant fraction of the intracellular compartment. Haemoglobin degradation produces large amounts of amino acids which associated to other waste products exert significant osmotic pressure in infected red blood cells. Therefore, it seems that induced changes in the host erythrocyte play also very important role in the cell volume regulation to prevent cell rupture before finishing of parasite multiplication (Krugliak et al., 2002; Lew et al., 2003).

Figure II. 5

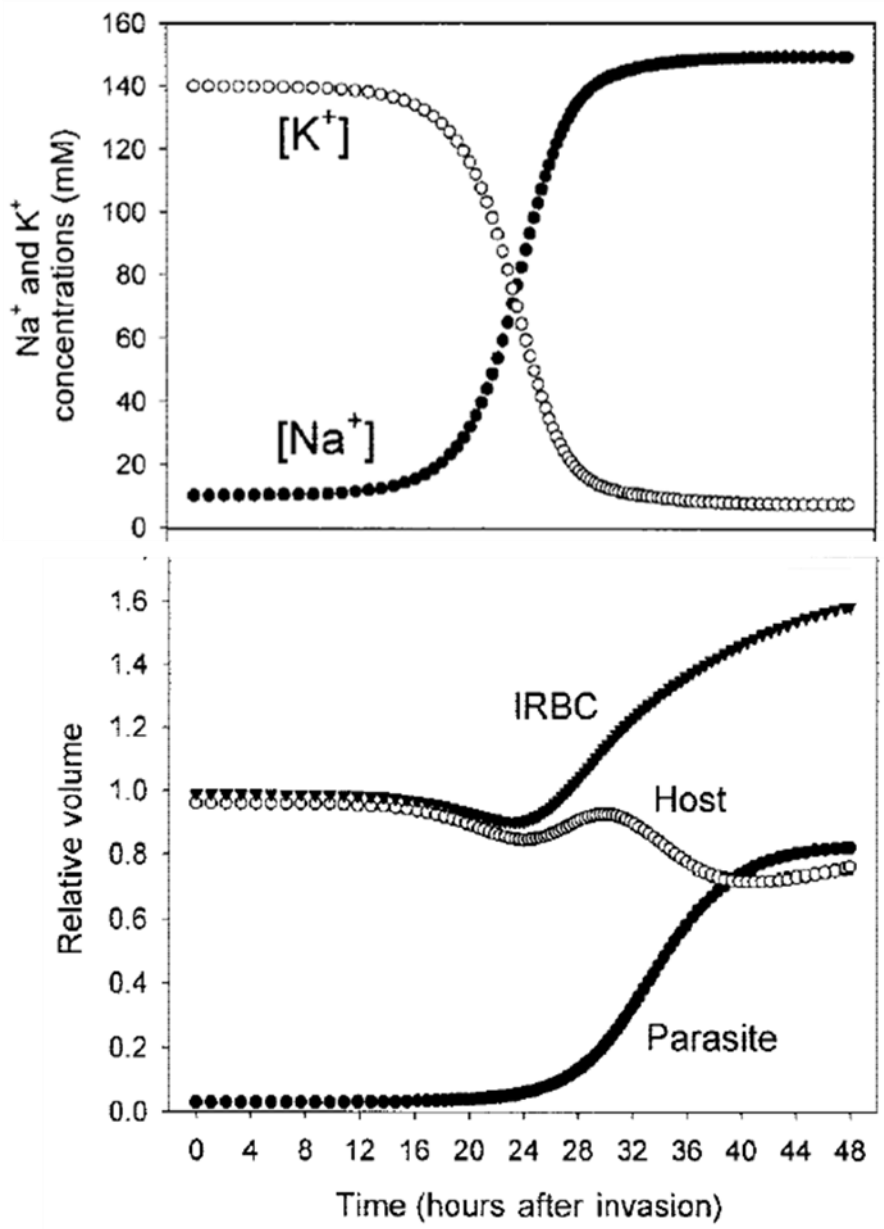


Figure II. 5: **Predicted changes in ionic concentration (Na^+, K^+) and volume occur after *P. falciparum* infection of human red blood cells** (Lew et al., 2003). Top panel: evolution of cytoplasmic concentration of Na^+ and K^+ in the host erythrocyte infected by malaria-parasite. Bottom panel: evolution of relative volume of: infected RBCs (IRBC), host erythrocyte and parasite. All volumes were expressed relative to RBC volume at the time of invasion, defined as 1. The changes have been monitored during 48-hours of asexual life cycle of *P. falciparum*.

II. 3. 2. 1. New permeability pathways (NPPs).

Since the 80s, large number of studies tried to describe modifications in membrane permeability after malaria infection. Despite the above-mentioned intracellular conditions, unfavourable for the parasite, it has been demonstrated that the transport properties of the *P. falciparum* infected red cell membrane become obviously different from the non-infected cells (Staines and Kirk, 1998; Staines et al., 2002): i) 100-fold increase of the glycolitic rate of the infected RBCs, ii) generation of new metabolic processes, iii) increase in the traffic of nutrients, waste products and cations, iv) dissipation of the normal Na^+ and K^+ gradients across the host cell membrane. The question was which strategies is malaria parasite using to be able to grow and replicate without the red blood cell swelling and bursting prematurely? It has been shown, that when red cell endogenous system do not exist or cannot maintain proper supply, the parasite activates other transporters, referred as 'New Permeability Pathways' (NPPs), approximately 10 - 20 h post-invasion (hpi), thus allowing permeation of low-molecular-weight solutes. Radiotracer flux and haemolysis experiments performed by Hagai Ginsburg and Kiaran Kirk indicate functional and pharmacological properties of NPPs (Ginsburg et al., 1983; Ginsburg et al., 1985; Ginsburg, 1994; Ginsburg and Kirk, 1998; Kirk et al., 1994; Kirk et al., 1999; Kirk, 2001). They characterized the nature of the transported substrates, their rates of transport, selectivity properties and inhibitors of these new pathways, as follow:

- NPPs allow both organic and inorganic anions (i.e., negatively charged lactate or Cl^- ions), electroneutral molecules (i.e., polyols, amino acids, nucleosides), and organic and inorganic cations (i.e., positively charged Na^+ and K^+) to pass;
- they display preferentially anionic selectivity; indicated permeability for solutes is presented in Fig. II. 6;
- NPPs are sensitive for the known anion-selective transport pathways blockers such a: 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), furosemide, niflumic acid and glybenclamide.

Furthermore, their energy of activation has been calculated of 10 kcal/mol. NPPs have been shown to be non-stereospecifique and non-saturable for physiologic

Figure II. 6

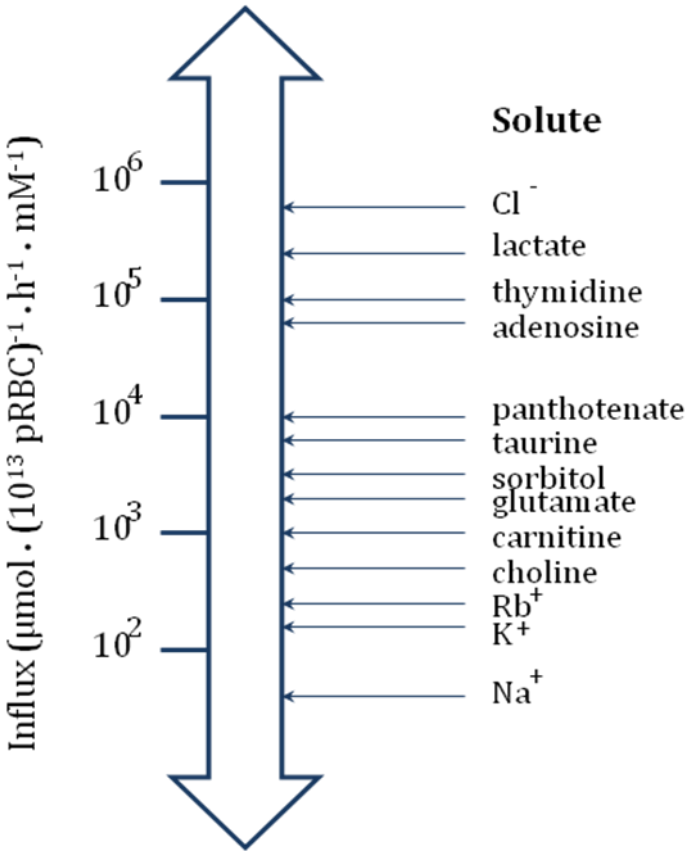


Figure II. 6: **Relative rates of transport of different solutes *via* the new permeability pathways (NPPs) in the red blood cell membrane infected by *Plasmodium falciparum* malaria parasite** (from Kirk, 2001).

concentrations of choline (Kirk et al., 1991) or panthothenic acid (Saliba et al., 1998).

Taken together, NPPs have been characterized as a single type of pathway with the general properties of a channel or a pore, i.e. linear concentration dependence, low energy of activation, the inability to distinguish between stereoisomers of permeant solutes, supported by pharmacological data. Because of a dramatic gap in original knowledge of endogenous red blood cell anionic channels this transport has been attributed to a large, poorly selective anionic channel.

II. 3. 2. 2. Morphological changes.

Malaria parasites grow and replicate inside a parasitophorous vacuole (PV) generated upon RBC invasion (for review Zuccala and Baum, 2011) surrounded by parasitophorous vacuole membrane (PVM), which initially contains host cell plasma membrane phospholipids (Ward et al., 1993). As mentioned, between 12 and 16 hpi an increase in the host erythrocyte membrane permeability and the intraerythrocytic parasite metabolic and biosynthetic activity have been observed. Various RBC changes appear to ensure the supply of nutrients to the parasite: i) alteration of RBC membrane induced by the parasite by creating new permeability pathways (Kirk and Saliba, 2007), and ii) exporting by the parasite a network of tubulovesicular membranes (TVN) that extends from the parasitophorous vacuole to the RBC periphery and might provide the parasite with efficient access to nutrients complementary to NPPs (Lauer et al., 1997). Moreover, proteins from the parasite's rhoptries and dense granules are secreted into the PV and trafficked, along with early ring-stage proteins, to the RBC cytoplasm to initiate the cascade of events that are required for remodelling of the host cell (Culvenor et al., 1991; Vincensini et al., 2008).

Whereas ring-stage infected erythrocytes circulate in the blood, mature stages are sequestered in different organs and do not circulate. The adhesive properties of infected cells are associated with changes in the host membrane, caused in the presence of knobby protrusions (Kilejian, 1979; Crabb et al., 1997), at the RBC surface.

Knobs, composed mainly of the knob-associated histidine-rich protein (KAHRP) are the platforms for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), cytoadherence components responsible for adhesion to endothelial and other cells in the host vasculature (Fig. II. 4). The adhesion prevents phagocytic clearance in the spleen and can be associated with lethal complications, such as cerebral and placental malaria (Duffy and Fried, 2003; Haldar and Mohandas, 2007).

Beyond parasitophorous vacuole, parasitophorous vacuole membrane and tubulovesicular membrane another organelles appear in the host cell cytoplasm as the parasite develops in its RBC. These flat, disc-shaped with irregular edges, ~ 30 nm wide structures, known as the Maurer's clefts, appear to act as secretory organelles. They concentrate virulence proteins (e.g. MAHRP1(2), membrane-associated histidine-rich protein 1(2); SBP1, skeleton binding protein 1; REX1(2), ring exported protein 1(2); Rifin, repetitive interspersed protein; STEVOR, subtelomeric variable open reading frame protein; see Fig. II. 4; *note*: Rifin and STEVOR are also exported to the RBC membrane) for delivery to the host RBC membrane (Wickham et al., 2001; Kriek et al., 2003; Bhattacharjee, et al., 2008). However, the trafficking machinery remains not fully understood as yet and it is suggested that the parasite develops novel system for trafficking proteins through the RBC cytoplasm. Moreover vesicles observed in the infected RBC cytoplasm (Fig. II. 4), proposed as being involved in the transport between membrane-bound compartments, seem not to play this role since they do not possess the characteristic morphology of the transport vesicles of other eukaryotes.
This novel secretory system for the export of parasite's proteins to the RBC cytoplasm and membrane (Charpian and Przyborski, 2008) has been indicated as pentameric amino acid sequence motif called '*Plasmodium* export element' (PEXEL) or vacuole transport signal (VTS) (Marti et al., 2004; Hiller et al., 2004). Up to 8% of the parasite gene products contain this predicted motif (Sargeant et al., 2006).

As mentioned, maturation of the intraerythrocytic parasite is accompanied by striking changes in the surface topology of the infected RBC (Atkinson and Aikawa, 1990; Aikawa, 1997) and by a marked loss of cellular deformability (Nash et al., 1989; Glenister et al., 2002). Parasite establishes an elaborate membrane system in

Table II. 2

Protein	Location	Putative function	References
GBP130	RBC cytoplasm, PVM	unknown; deletion increases RBC rigidity	Nolte et al., 1991; Maier et al., 2008;
KAHRP	RBC skeleton	binds to RBC spectrin-actin; essential for knob formation and attachment of PfEMP1; deletion decreases RBC rigidity and adhesion under flow conditions	Crabb et al., 1997; Waller et al., 1999; Oh et al., 2000; Wickham et al., 2001.
MESA (PfEMP2)	RBC skeleton	binds to protein 4.1	Petersen et al., 1989; Waller et al., 2003.
RESA/Pf155	RBC skeleton	binds to spectrin; deletion increases heat-induced membrane vesiculation; may stabilize RBC membrane; may prevent invasion of already parasitized RBCs	Da Silva et al., 1994; Diez Silva et al., 2005; Pei et al., 2007; Mills et al., 2007.
PfEMP3	MC, RBC skeleton	binds to spectrin; disrupts spectrin-actin-4.1 interaction; appears to be involved in PfEMP1 trafficking	Waterkeyn et al., 2000; Pei et al., 2007.
HRP2	RBC cytoplasm	binds haem; may promote haem detoxification	Sullivan et al., 1996; Papalexis et al., 2001.
REX1	MC	associates with the edges of Maurer's cleft bodies; affects MC morphology	Hawthorne et al., 2004; Spielmann et al., 2006.
REX3	RBC cytoplasm	unknown; highly expressed PEXEL-containing protein	Day et al., 1993; Bozdech et al., 2003; Le Roch et al., 2003; Spielmann et al., 2006.
MAL7P1.172	MC	involved in PfEMP1 trafficking	Maier et al., 2008.
MAL7P1.171	unknown	involved in PfEMP1 trafficking; deletion decreses RBC rigidity	Maier et al., 2008.
Exoantigen PF70	unknown	involved in PfEMP1 trafficking; deletion decreses RBC rigidity; antibodies to Pf70 may have a protective role	Tshefu and James, 1993; Maier et al., 2008.
PFD1170c	unknown	deletion affects knobs, may promote KAHRP self-assembly	Wu and Craig, 2006; Maier et al., 2008.
PF10_0381	unknown	deletion affects knobs	Maier et al., 2008.
PFB0920w	unknown	deletion increases RBC rigidity; may promote host RBC survival	Maier et al., 2008.
PF13_0073	unknown	deletion increases RBC rigidity	Maier et al., 2008.
PFD1160w	unknown	deletion decreases RBC rigidity	Maier et al., 2008.
MAL8P1.154	unknown	deletion decreases RBC rigidity	Maier et al., 2008.
PF14_0018	unknown	deletion decreases RBC rigidity	Maier et al., 2008.
PFC0435w	TVN	junctional TVN protein	van Ooij et al., 2008

Table II. 2: **Characteristics of some soluble exported *Plasmodium falciparum* proteins** (from Maier et al., 2009).

the RBC cytosol (Hanssen et al., 2008), alters the host erythrocyte's fluidity, permeability and adhesiveness (reviewed in Foley and Tilley, 1995; Cooke et al., 2001) and increased ability to vary the antigenic coat of the infected RBC to avoid protective antibodies (Cooke et al., 2004; Rowe et al., 2009). These changes are initiated in the ring stage of infection and are increased during the trophozoite stage of growth (Glenister et al., 2002; Parker et al., 2004). Furthermore, it has been reported that proteins exported by the parasite, located in the cytoplasm, at the Maurer's cleft or in the RBC plasma membrane, play a crucial role in these changes. The contributions of parasite proteins to alterations in RBC membrane properties are indicated in Table II. 2 and Table II. 3. Among them, there are proteins that modify host cell architecture and RBC deformability. For instance, Ring Infected Erythrocyte Surface Antigen (RESA), produced in the final stages of schizont development and stored in individual merozoites, is probably one of the earliest exported protein. Detailed characterization of RESA and its crucial role played in erythrocyte modifications during the first 12 hours after invasion will be presented in chapter IV. 3.

It should be noticed, that from approximately 400 parasite's proteins, 160 may be involved in erythrocyte remodelling (Hiller et al., 2004; Marti et al., 2005; Sargeant et al., 2006), and only few are well studied up to-date (Pei et al., 2007) like e.g.: PfEMP1 (*P.falciparum* Erythrocyte Membrane Protein 1), KAHRP (Knob-Associated Histidine-Rich Protein), MESA (Mature-infected Erythrocyte Surface Antigen), RESA ... Some of these proteins have been proposed as suitable drug targets in their own right (Patel et al., 2008), others play a key role in antimalarial drug resistance, but the role of some of them is still poorly understood.

PfEMP1, a transmembrane protein encoded by members of a multigene family is exposed at the red cell surface and attached to the membrane skeleton through interactions with spectrin, actin and KAHRP (Baruch et al., 1996; Oh et al., 2000; Waller et al., 2002). This protein mediates adhesion to the host molecule appears to be a target of protective immunity in humans (Bull et al., 1998). Like KAHRP, other parasite and RBC proteins affect the amount and distribution of PfEMP1 at the RBC surface (Fairhurst and Wellems, 2006).

Table II. 3

Protein	Location	Putative function	References
PfEMP1	MC, RBC membrane, RBC surface	cytoadherence ligand; involved in antigenic variation; interacts with KAHRP	Smith et al., 1995; Su et al., 1995; Oh et al., 2000.
MAHRP1	MC	involved in MC stability and PfEMP1 trafficking; deletion decreases cytoadhesion	Spycher et al., 2003; Spycher et al., 2006; Spycher et al., 2008.
PfSBP1	RBC skeleton, MC	involved in MC morphology and PfEMP1 trafficking; deletion decreases cytoadhesion	Blisnick et al., 2000; Cooke et al., 2006; Maier et al., 2007; Saridaki et al., 2009.
RIFIN/ STEVOR	RBC cytoplasm, RBC surface	may be surface-exposed in late stages; possibly involved in antigenic variation; impact deformability of the erythrocyte membrane (STEVOR)	Kaviratne et al., 2002; Przyborski et al., 2005; Niang et al., 2009; Sanyal et al., 2012.
Pf332	MC, RBC skeleton	involved in MC morphology and PfEMP1 trafficking; deletion increases RBC rigidity and decreases cytoadhesion	Glenister et al., 2009; Hodder et al., 2009.
PFB0106c	RBC cytoplasm, MC	involved in PfEMP1 trafficking	Maier et al., 2008.
PF14_0758	RBC cytoplasm	involved in PfEMP1 trafficking; deletion increases RBC rigidity	Maier et al., 2008.
PF13_0076	unknown	involved in PfEMP1 trafficking	Maier et al., 2008.
PFD0225w	unknown	deletion decreases RBC rigidity	Maier et al., 2008.
PfMC-2TM	MC	subfamily of STEVOR	Sam-Yellowe et al., 2004; Maier et al., 2008.
Cys repeat modular proteins	MC	may mediate host-parasite interactions at different stages of the life cycle	Thompson et al., 2007
ETRAMP/SEP	PVM/TVN, MC	unknown	Spielmann et al., 2003; Maier et al., 2008.
REX2	MC	unknown	Haase et al., 2009
PFD0495c	TVN	promotes TVN-mediated lipid import	van Ooij et al., 2008; Tamez et al., 2008.

Table II. 3: **Characteristics of some membrane-bound exported proteins of *Plasmodium falciparum*** (from Maier et al., 2009). PfMC-2TM family: PFB0985c, PFA0680c, PFC1080c, PF11_0014, MAL7P1.5, PF11_0025, MAL6P1.15, PF10_0390, PFA0065w, PFB0960c, MAL7P1.58. ETRAMP/SEP family: PFB0120w, PFD1120c, PFE1590w, MAL8P1.6, PF10_0019, PF10_0323, PF10_0164, PF11_0039, PF11_0040, PFL1945c, PF13_0012, PF14_0016, PF14_0729.
KAHRP, knob-associated His-rich protein; MC, Maurer's cleft; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; PVM, parasitophorous vacuole membrane; RBC, red blood cell; STEVOR, subtelomeric variable open reading frame proteins; TVN, tubulovesicular network.

KAHRP has been reported to be critically important for both knob formation and the strengthening of the adhesive interactions mediated by PfEMP1 (Rug et al., 2006). KAHRP binding with the membrane skeleton (repeat 4 of the spectrin α-chain) leads to an increased rigidity, blockage of blood vessels and resistance to flow (Pei et al., 2005), thus make this protein likely to be an important virulence factor *in vivo*. Furthermore, the interactions of knob components with RBC skeletal proteins probably alter the architecture of the sub-membrane skeleton and its interactions with membrane proteins (reviewed in Mohandas and An, 2006), and result in increased rigidity and adhesiveness of the RBC membrane.

Another described protein, expressed in trophozoites and schizonts, MESA (also known as PfEMP2) has been shown to interact with band 4.1 and thus play an important role for intraerythrocytic growth of the parasite (Magowan et al., 1995).

Several other characterized proteins, as stated, are listed in Table II. 2 and Table II. 3. Indeed, e.g. *P. falciparum* skeleton binding protein 1 (PfSBP1), a Maurer's cleft-resident integral membrane protein, has been shown to interact with the cytoskeleton in mature-stage of infected RBC (Blisnick et al., 2000). Deletion of PfSBP1 prevents export of PfEMP1 to the surface of the erythrocyte. This indicates that PfSBP1 plays an important role in parasite virulence (Cooke et al., 2006; Maier et al., 2007).

PfEMP3, protein associated with the cytoplasmic surface of the RBC membrane in trophozoite stage interacts with the membrane skeleton (Waller et al., 2007), by binding to spectrin. This connection disrupts the spectrin–actin–4.1 interaction and probably contributes to the loss of membrane deformability in mature-stage-infected RBCs.

Pf332, (*P. falciparum* antigen 332), the largest (~ 1 MDa) known malaria protein, involved in Maurer's cleft morphology and exported to the RBC membrane has been described to modulates the level of rigidity. It has been also shown that RBCs infected with Pf332-deletion-mutant parasites had decreased levels of PfEMP1 on the surface and were significantly less adhesive (Glenister et al., 2009).

Taken together, infection of the human red blood cells by the malaria parasite *Plasmodium falciparum* results in a variety of changes in the host erythrocytes (Cooke et al., 2001), which have been extensively studied and advanced considera-

bly over the past few years since the completion of *P. falciparum* genome (Gardner et al., 2002). Indeed, these include modifications of physical and biological red cell characteristics, for example: i) loss of normal discoid shape, ii) establishment an elaborate membrane system in the RBC cytosol (Hanssen et al., 2008), such as PV, PVM, TVN, Maurer's cleft or vesicles, iii) alteration of the infected erythrocyte surface such that it possesses electron dense knob-like protrusions (Kilejian, 1979; Crabb et al., 1997). Furthermore, parasite increased rigidity of the host membrane, capacity to adhere to the walls of vascular endothelial cells and ability to vary the antigenic coat of the infected RBC to avoid protective antibodies (Cooke et al., 2004; Rowe et al., 2009). Moreover, the human malaria parasite increases red blood cell membrane permeability to allow for import of nutrients and other solutes. All these modifications are based on parasite proteins exported to the erythrocyte membrane thus facilitating survival of the parasite within the host cell (escape its immune response) and tend to increase the virulence of malaria disease. The latter instance, adhesion to the vascular endothelium is essential for virulence of *P. falciparum* and underlies much of the associated pathology, including life-threatening complications such as cerebral and placental malaria (Scherf et al., 2008).

Although the best documented changes in infected RBC occur as *P. falciparum* matures to the trophozoite and schizont stages, it is likely that altered deformability of ring-stage, with for example the export of the parasite RESA protein to the red blood cell membrane, plays a crucial role for further supporting of the malaria infectivity. Moreover, red blood cell remodelling by the *Plasmodium falciparum* including new permeability pathways (anionic channels) and modifications caused by parasite's proteins require precise characterization, since they could be targeted by specific drugs or antibodies thus being important for future therapies, but also essential in the understanding of RBC membrane transporters, particularly channels. Pathological conditions of malaria disease were used during this PhD thesis as a crucial model for new insights into characteristics and physiological role of anionic conductance in the red cell membrane. On the other hand, it should be noticed, that the above-described parasite exported proteins have been chosen deliberately, since all of them interact with host membrane cytoskeleton. Due to

the primary function of RBCs, as being able to transport high amounts of oxygen and at the same time withstand the enormous pressure of being repeatedly squeezed through the small capillaries of the body, the RBC has a strong but very deformable sub-membrane cytoskeleton. What the remodelling of the host erythrocyte membrane including its skeleton means therefore for both, red blood cell and the parasite? An objective of the present work is to integrate these informations into a model of red cell membrane anionic channels in health and disease.

II. 4. Electrophysiological studies on anionic channels in human RBCs.

For a long time, the question of whether NPPs represent endogenous channels up-regulated and/or modified by the parasite or either they are parasite engendered has been around and still remains unanswered. The origin of the NPPs was quite controversial and they have been suggested to be both parasite and host cell derived (reviewed by Kirk, 2001). On the other hand, the techniques available to study these conductive pathways were poor. For instance, taking into account how little protein represent a few hundred channels the standard proteomic protocols for detection of these channels were very limiting. However, since parasite-induced transport pathways for anions and other solutes in infected RBCs showed importance in transport studies and since NPPs demonstrated many similarities to anion-selective channels, the patch-clamp electrophysiological technique represented the best method available to investigate channel-mediated transport of charged solutes through the infected red blood cell membrane (description of this technique: section III. 7, *Materials and Methods*).

Using the patch-clamp electrophysiological technique, S. Desai and co-workers in Bethesda demonstrated that the membrane conductance of infected RBCs is 150 times greater than that measured in non-parasitized RBCs and that this increased conductance results from activation of small anionic channels showing functional and pharmacological properties of NPPs (Desai et al., 2000). In addition, they reported that these channels had better open probability at negative potentials

which correspond to inwardly rectified conductance. Moreover, identified channels have shown anion selectivity $I^- > Br^- > Cl^- >$ lactate$^-$. Furthermore, they named these channels PSAC (Plasmodial Surface Anionic Channels) and suggested that NPPs have a parasite origin and are channels formed from parasite derived proteins (Alkhalil et al., 2004). In recent study (Nguitragool et al., 2011) they have identified protein which is exported after *P. falciparum* infection to the host erythrocyte and plays a role in PSAC activation. They gave evidence for this parasite-encoded CLAG3 protein as a key component contributing to solute uptake in infected RBCs.

Another group of electrophysiologists, the group of Roscoff in France, used the *cell-attached* configuration of the patch-clamp technique to characterize conductance of infected red blood cell membrane. In this condition they found spontaneous channel activity observed as bursts of channel openings separated by short closures (Egee et al., 2002). This channel exhibited mean unit conductance of ~ 18 pS and showed voltage-dependent gating, with the preference for opening at negative potentials. Moreover, the unitary conductance, substrate selectivity and pharmacology observed in their experiments indicated that parasite-induced channel in infected RBCs were identical to those of the endogenous linear anionic channel conductance measured by this group in non-infected erythrocytes. Thomas and coworkers detected also that it is possible to induce a *whole-cell* current in uninfected RBCs (notice: in physiological conditions non-infected RBCs did not exhibit current in *whole-cell* configuration) by the addition of PKA and ATP, which mimics the membrane current observed in infected cells (Egee et al., 2002). Another study showed that the membrane conductance of malaria RBCs was totally inhibited by alkaline phosphatase (0.1 U/ml) (Decherf et al., 2004).

Taken together, the electrophysiological studies on NPPs by the group of Thomas suggested that these anionic channels have endogenous nature and after *P. falciparum* invasion could be up-regulated, for example by the phosphorylation. Recent works gave more evidences for this proposed model (Bouyer et al., 2006; Decherf et al., 2007; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009).

A study in a *whole-cell* configuration of the patch-clamp by the F. Lang group from Germany confirmed an inwardly rectifying anionic conductance in *P. falciparum* in-

fected RBCs (Huber et al., 2002). Inhibitory experiments with the use of NPPs blockers NPPB, DIDS, furosemide and glybenclamide indicated probability of existence at least two anion-selective channel types induced by the parasite. This group presented also evidence that anionic conductance, identical to those that they observed in infected RBCs, could be induced in non-infected RBCs by oxidation (Huber et al., 2002; Duranton et al., 2002). This result has been correlated with haemolysis experiments performed on non-infected and infected red cells which link with NPPs. Furthermore, they have reported inwardly rectifying conductance permeability to different organic compounds (Duranton et al., 2004) making it possible candidate for NPPs. In addition, they have also reported that a combination of the use of a negative holding potential and serum factors alters recorded conductance of the infected red cell membrane (Staines et al., 2003).
To summarize, the group of F. Lang concluded that the parasite induces an endogenous anionic pathways to form NPPs after red blood cell invasion.

In year 2004 the group of H. DeJonge from Rotterdam published their studies on electrophysiological characteristic of membrane conductance in red blood cells infected by *P. falciparum* and non-infected from the patients with cystic fibrosis (CF) (Verloo et al., 2004). *Whole-cell* observations indicated two types of anionic channels activated by malaria parasite: CFTR channel induced by ATP and according to these authors playing a role of regulator of the second channel, and the CFTR-like inwardly rectifying and stress osmotic-activated channel. These two types of channel have been not present in red blood cells from the patient with CF (absence of the functional CFTR channel). Only CFTR-like channel displayed characteristic identical to malaria-induced channels and was identical like PKA-sensitive channel described by the Thomas group.

Taken together, electrophysiological studies on the characterization of new permeability pathways induced by the *Plasmodium falciparum* after red blood cell infection indicated variations in the scope of the same subject. Different groups of electrophysiologists proposed their own point of view and the problem of whether

anionic conductance is endogenous host channel or parasite-derived still remains under study.

II. 5. Molecular nature of anionic channels.

As well as the origin of the above-described anionic channels, their number and especially molecular identity are yet unknown. It remains possible that the *P. falciparum* exports its own PSAC channel to the host membrane, as suggested by Desai and co-workers (Alkhalil et al., 2004; Desai, 2004) and modified its properties by the applying of parasite proteins (Nguitragool et al., 2011). However, since export of parasite-protein to the host erythrocytes seems to be complex process, especially due to the diversity of available range of proteins, it is likely that this effect could be synergistic. In this case, the presence of an up-regulated endogenous channel offers an interesting alternative to parasite-encoded channel proteins.

It has been postulated that identified anionic channels could be related to already known from other cell types. For instance, it has been proposed that ATP binding cassette family of chloride channels (including cystic fibrosis transmembrane regulator protein, CFTR), the volume-regulated anionic channels, and the chloride channel family (ClC) could be good candidate for these conductive pathways. In addition, it has been shown that malaria parasite modifies band-3 (Crandall and Sherman, 1991), and even if this anion exchanger is not a channel, it has been suggested that it could possess some channel-like properties.

Our group from Roscoff has showed evidence that CFTR-like channels may be involved in the formation of the NPPs (Egee et al., 2002). Moreover, conductance, pharmacology and mode of activation of the small, linear Cl^- channel (SCC) identified in non-infected and malaria-infected RBCs were similar to those of CFTR (Tabcharani and Hanrahan, 1991). Biochemical observations of Abraham and co workers (2001) indicating different ATP binding cassette proteins in the RBC membrane supported the presence of CFTR in the human plasma erythrocyte membrane. Another very important fact is, that the linear conductance channel observed by the group of S. Thomas displays mechanosensitivity which may be related to the ATP transport pathway in human RBCs. ATP release from the cell has

been reported (Sprague et al., 1998; Sprague et al., 2001) to be activated by mechanical deformation and reduced in red cells from patients with CF.
The group of DeJonge suggested that CFTR acts as a regulator, and the CFTR-like channel is suitable for anionic membrane conductance in human red blood cells.
The Lang group demonstrated evidence for expression of ClC-2 channels in RBC membrane and showed that these channels are activated by oxidation and by infection with *Plasmodium* (Huber et al., 2004). These anion-selective channels were shown to participate in the maintenance of the cell volume of *Plasmodium*-infected erythrocytes.

In conclusion, the molecular nature of regular anionic channels in the human red cell membrane is not yet identified and remains confusing subject under investigations. For instance, there are now indications that PSAC, SCC and ClC-2 are different names for the same channel demonstrated under varied conditions (Bouyer et al., 2007).

II. 6. Physiological role of human red cell membrane channels in health and disease.

The red blood cell membrane is naturally equipped with a variety of membrane transporters, mainly generated to optimize the respiratory function and to maintain cell homeostasis at minimal metabolic costs. Although, the background information of physiological role played by ionic channels is still unclear, it has been noted that under normal physiological conditions channels are dormant, but they can be activated experimentally. The spontaneous activation of cationic and anionic channels in nucleated erythrocytes has been reported from patch-clamp electrophysiological studies (Egee et al., 1998; Thomas et al., 2001; Lapaix et al., 2002) and was attributed to an involvement in volume regulation. The role of channels in anucleated erythrocytes (human) is not clearly defined yet.

Different reports suggested physiological role of Ca^{2+}-activated K^+ channels (Gardos), as pointed below:

- they play a key role in the regulation of cell volume (Gardos, 1958; Lew et al., 1982; Brugnara et al., 1983);
- they can participate in plasma K^+ buffering concentration (Hamill, 1983);
- they can participate in blood clot formation (Kaestner and Bernhardt, 2002);
- they can play a role in proliferation and differentiation process of red blood cells (hematopoiesis) (Maher and Kuchel, 2003);
- they can play a role in erythrocytes apoptosis (eryptosis) (Romero and Rojas, 1992; Lang et al., 2005).

In malaria-infected red blood cells Gardos channel does not seem to be involved in volume regulation or K^+ homeostasis (Kirk et al., 1992), even at physiologically relevant external Ca^{2+} concentrations (1.0 to 1.3 mM) and seemed only activatable in the presence of ionophore A23187. In normal physiological conditions without any treatment by ionophore, the presence of this channel has never been reported in the literature of *P. falciparum* infected RBCs.

In sickle cells they have been shown to play an important role in cell dehydration (Bookchin et al., 1991; Lew et al., 1991) and in protection against immune haemolysis (Halperin et al., 1989). Their potentially protective duty in damaged cytoskeleton conditions has been reported also in hereditary spherocytosis (*note:* loss of membrane surface area relative to intracellular volume due to abnormalities in cytoskeleton leads to spherocytic shape, which further increased osmotic and mechanical fragility resulting in haemolytic anaemia).
However, no clearly demonstrated role for the Gardos channel is available up to-date. A recent work performed in our laboratory in Roscoff (Dyrda et al., 2010) indicated that experimental membrane deformation triggers Gardos channel activity with immediate consequences on blood viscosity, suggesting possible involvement of Gardos channel in shear stress.

The non-selective cationic channels, activated either by oxidation or energy depletion (Duranton et al., 2002) have been shown to be involved in haemolysis and finally in apoptosis of human erythrocytes (Lang et al., 2004).

The physiological role of anionic channels remains also totally unclear. In the red blood cell membrane quiescent anionic channels have been indicated (Egee et al., 2002). We could suspect, that this could correspond to low metabolism of mature anucleated erythrocytes, since such a dormant (but activatable after stimulation) channels are probably linked to the physiology of premature nucleated stages during differentiation and maturation process of RBCs.
On the other hand, anionic channels could be involved in the transport of other anions *e.g.* ATP, since ATP release from RBCs is realized by membrane proteins like ATP binding cassette such as CFTR. Small conductance chloride channel may be also a candidate for ATP pathway, due to its mechanosensitivity properties. Concerning these facts, anionic channels would play an important role in regulation of vascular resistance *in vivo* by the activation of purinergic receptors and synthesis of nitric oxide (Sprague et al., 1998; Bao et al., 2004; Locovei et al., 2006; Jiang et al., 2007).

The possibility that ionic channels play a role in volume regulation of human red blood cells has been hypothesised by our group. Another recent study of our lab (Cueff et al., 2010) has demonstrated that dynamic changes occurring in the red cell homeostasis and membrane permeability when exposed to elevated intracellular calcium are not due to the cell hydration, rather are caused to a large extent by exovesiculation-induced membrane area loss. This opens a question of a role of vesicles in the red cell membrane.

In brief, it was still unclear when this PhD work was initiated, if channels play a specific role in erythrocyte physiology or if they are just residues of a lost previous function during evolution or their maturation process.

CHAPTER III

MATERIALS AND METHODS

III. MATERIALS AND METHODS.

In order to address the four above presented objectives we carried out two types of techniques. First, electrophysiological technique of patch-clamp was used for: i) description of the activity of anionic membrane conductance in human erythrocyte (objective 1); ii) characterisation of channel activity after *P. falciparum* invasion (objective 2); iii) determination of RESA1 protein involvement in the post-invasion activity (objective 3); iv) description of changes in Gardos channel activity related to aging of RBCs and after malaria-parasite infection (objective 4). Secondly, different biochemical assays, western blotting (WB), immunofluorescence (IF), sorbitol haemolysis (H), parasite growth (G) or Percoll-gradient separation (P) have been performed in order to: i) identify the molecular nature of anionic channels in red cell membrane; showing their up-regulation in pathophysiological conditions of malaria infection (WB, IF, H, G; objective 2); ii) confirm the presence of RESA1 protein in the strains used in our experiments (WB, IF); suggest the role played by RESA1 in the activation of NPPs (H) (objective 3); iii) separate erythrocytes according to their age (objective 4).

In this work both types of human red blood cells healthy and infected by *P. falciparum* have been used for experimental design with the special intention to answer the question: what is the physiological role of channels in the red cell membrane?
The 'Materials and Methods' section is presented briefly and generally, because some of the aspects are already detailed in respective publications included in this thesis. This chapter contains some of the additional informations.

Table III. 1

Thawing solutions		Freezing solution
12 % NaCl	(A)	3 % sorbitol
1,6 % NaCl	(B)	28 % glycerol
0,9 % NaCl	(C)	0,65 % NaCl
+ 0,2 % D-glucose		

Table III. 1: **Thawing and freezing solution(s) for malaria *P. falciparum.*** All reagents were obtained from Sigma. Components were dissolved in MiliQ water, filtered sterile with syringe and 0.2 µM millipore cellulose disks and stored at 4°C. The solutions were warmed up to 37°C before use.

Table III. 2

Product	Furnisher	Final concentration	Volume
RPMI 1640	Gibco		79,45 g
HEPES (1M)	Lonza	35 mM	50 ml
NaHCO3	Gibco	0,21 %	140 ml
Gentamicine	Sigma	20 mg/l	10 ml
Glucose	Sigma	2 g/l	10 g
Hypoxanthine	Sigma	50 mg/l	250 mg
Albumax	Gibco	5 g/l	25 g

Table III. 2: **Composition of malaria *P. falciparum* 3D7 strain complete culture medium** (for 5L of medium). After equilibration of products at 4°C, pH was adjusted to 7.3 - 7.35, then medium was filtered sterile with 0.22 µM filter (Milipore Express) and stored at 4°C no longer than 2 weeks, otherwise stored at -20°C in plastic bottles (Stericup and Steritop).

Table III. 3

Product	Furnisher	Final concentration	Volume
RPMI 1640	Gibco		500 ml
Hypoxanthine	Sigma	200 µM	10 ml
Gentamicine	Sigma	20 mg/ml	200 µl
Human AB$^+$serum	Hospital/Rennes	10 %	50 ml
Pyrimethamine*	Sigma	128 ng/ml	6,4 µl

Table III. 3: **Composition of malaria *P. falciparum* RESA1 WT, KI, KO* strains complete culture medium** (for 500 mL of medium). After equilibration of products at 4°C, pH was adjusted to 7.3 - 7.35, then medium was filtered sterile through 0.22 µM filter (Milipore Express) and stored at 4°C no longer than 2 weeks, otherwise stored at -20°C in plastic bottles (Stericup and Steritop). Human serum was heat-inactivated with 1 h incubation in 56°C and stored as stock in 50 ml aliquots at -20°C. *medium for RESA1-KO was supplemented with pyrimethamine.

III. 1. Red blood cells.

Venous blood from healthy volunteers, after written informed consent, was collected into heparinized vacutainers. RBCs were washed thrice by centrifugation (2.8 rpm, 5 min) and resuspended in large volumes of RPMI 1640 medium containing 25 mM HEPES and α-Glutamine (Gibco). The buffy coat containing platelets and white cells was removed by aspiration after each wash. After the last wash, the cells were suspended at 50% hematocrit in RPMI 1640 and kept at 4°C, during maximum one week.

III. 2. Malaria infected red blood cells.

Continuous culturing of erythrocytic asexual stages of *P. falciparum* was based on the technique developed by Trager and Jensen (Trager and Jensen, 1976). The malaria *in vitro* adapted parasites used in this study were: 3D7 Roscoff's laboratory strain; FUP/CB C32 wild type expressing *resa1* gene (RESA1-WT); A_3F_8 (RESA1-KO) pyrimethamine-resistant mutant; A_3F_8ocGCV (RESA1-KI revertant; gancilovir drug pressure was used to induce *resa1* gene reversion into RESA1-KO). All RESA strains were provided by the Pasteur Institute, Paris (Dr Serge Bonnefoy). Strains, when not cultured, freezed and stored in liquid nitrogen tank, were thawed according to the standard protocols of 'Methods in Malaria Research' (www.malaria.mr4.org/publication.html). Thawing and freezing solutions are shown in Table III. 1). Malaria clones were maintained with erythrocytes (III.1.) at hematocrit 5% in a suitable culture medium (Table III. 2 and Table III. 3), cultivated in 25 cm^3 flasks and incubated in the 37°C under 5% of CO_2. Culture growth was monitored every 24 h by changing the medium and estimating the percentage of parasitemia on Giemsa colored smear (Fig. III. 1) according to a standard procedure described in 'Methods in Malaria Research'. The parasitemia never exceed 10%. Only for RESA1 western blotting experiment *P. falciparum* was grown to higher parasitemias (10 – 20%). Parasites were successively synchronized to ring-stage with 5% sorbitol (w/v) for 10 min (according to Lambros and Vanderberg, 1979) and kept in static conditions for all described experiments.

Figure III. 1

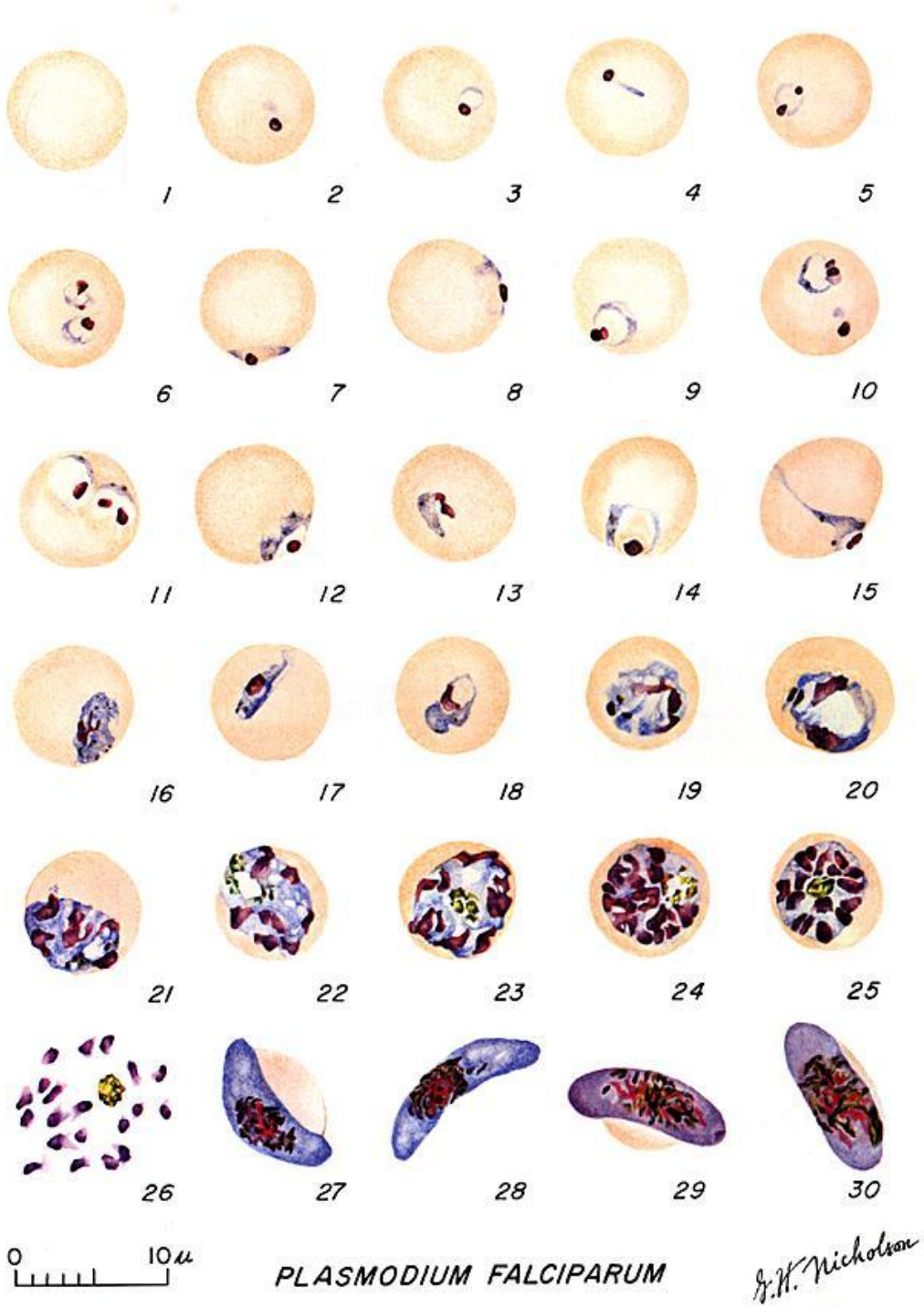


Figure III. 1: ***P. falciparum*** **blood stages thin blood smears.** 1: Normal red cell; 2 - 18: Trophozoites (among these, 2 - 10 correspond to ring-stage trophozoites); 19 - 26: Schizonts (26 is a ruptured schizont); 27, 28: Mature macrogametocytes (female); 29, 30: Mature microgametocytes (male). Illustration from Center for Disease Control and prevention, http://www.dpd.cdc.gov/dpdx/HTML/Frames/MR/Malaria/falciparum/body_malariadffalcipar.htm.

III. 3. Magnetic separation of malaria infected red blood cells.

III. 3. 1. Principle.

This technique is mainly used for enrichment and purification of *Plasmodium* parasitized red blood cells which led to synchronization of *in vitro* cultures. It was based on the fact that malaria parasites digest RBC haemoglobin, producing insoluble high-spin oxidized haem products, which polymerize into a pigmented substance, called haemozoin (Uhlemann et al., 2000; Ribaut et al., 2008). The paramagnetic properties of haemozoin (Fe (III) complex), in contrast to the diamagnetic low-spin haemoglobin (Fe (II) complex) led to use high-gradient magnetic separation method already in 1981 (Paul et al., 1981).

III. 3. 2. Protocol.

To enrich the parasite infected RBCs culture in trophozoites and schizonts stages, magnetic separation was used. For that purpose, LD separation column was mounted on high-gradient magnetic cell separator MidiMACS™ (Miltenyi Biotec, Bergisch Gladbach, Germany) with the flow velocity 0.2- 0.25 mL/min. Column was filled with warmed (37°C) RPMI 1640. Parasite culture was spun down, washed three times in RPMI 1640 (1.7 rpm, 5 min) and resuspended at 25% hematocrit in PBS + 1% BSA + 2 mM EDTA. The RBCs suspension was then loaded on the top of the column, typically 1 ml. The flow-through contained uninfected RBCs or ring-stage infected RBCs, whereas trophozoites and schizonts infected RBCs were retained in the column by the magnetic field. The column was subsequently washed with warmed (37°C) PBS + 1% BSA + 2 mM EDTA until the flow-out was cleared from RBCs (usually ten times, 1 mL) then removed from the magnetic field and further eluted in PBS + 1% BSA + 2 mM EDTA (three times, 1 mL). The eluent was then washed thrice in RPMI 1640 (1.6 rpm, 4 min). The supernatant was discarded and the pellet was used to prepare thin blood film stained after in Giemsa. The assessed parasitemia was over 90 - 95%. The purification was performed at room temperature (RT).

III. 4. Haemolysis of RESA1 *P.falciparum*-infected human erythrocytes in isosmotic sorbitol solution.

III. 4. 1. Principle.

This technique is based on the membrane properties of the red blood cell infected by malaria-parasite, permeable (*via* NPPs) to a wide variety of solutes including sorbitol, for which the membrane of the non-infected cell is completely impermeant. The placement of RBCs in isosmotic sorbitol solution creates huge gradient on both sides of the membrane. If the permeability of erythrocyte membrane is higher for sorbitol than for the cytoplasmic solutes, the influx of sorbitol exceeds much more the solute efflux from the cell, leading to a large influx of sorbitol accompanied with osmotic obliged water entry into the cell. This causes cell swelling and finally its lysis, releasing haemoglobin which can be then measured spectrophotometrically, representing so a semiquantitative measurement of haemolysis.

III. 4. 2. Protocol.

One of the sorbitol haemolysis method of 3D7-infected human red blood cells used for our experiments was already described in Bouyer et al., 2011 (added article). For RESA1 strains we used a modified protocol described below. Pelleted trophozoite/schizont-stage-enriched by the magnetic separation method infected RBCs were resuspended in RPMI 1640 (hematocrit 4%). Isosmotic lysis was initiated by addition of 50 µL of the RBCs suspension (10 s intervals between strains) to 1 mL of pre-warmed sorbitol lysis solution (300 mM sorbitol, 10 mM HEPES, 5 mM glucose, pH 7.4) in a cuvette. Haemolysis was monitored by measuring 700 nm light transmittance through the sample (Ultrospec 2000, UV/Visible Spectrophotometer, Pharmacia Biotech). Experiments were performed in RT and lasted 1 h (predetermined intevals: 0' 1' 2' … - 20' - 22' - … - 30' - 35' - … - 60'). Each 5 min, RBCs were gently mixed with a pipettman to maintain a homogeneous suspension. Control cuvette contained sorbitol solution with 50 µL of RPMI 1640. Data analyses were carried out according to Krugliak and Ginsburg (Krugliak and Ginsburg, 2006). The % of lysis values at different times were fitted by nonlinear regression using SigmaPlot® equation for a sigmoid dependence of y on x. $y = a/(1+\exp(-(x-x_0)/b))$ where 'y' is the % of lysis, 'a' is the maximal lysis, 'x' is the

sampling time, 'x_0' is the $t_{1/2}$ of lysis, and 'b' is the variability of cells in the population.

III. 5. Western blotting of RESA1 *P.falciparum*-infected human erythrocytes.

III. 5. 1. Cells preparation.

In order to confirm that RESA1 protein can be visualized in western blot in the ring stage (in the erythrocte membrane)[1] or in the late schizont stage (in the parasite)[2] two different ways of cells preparation were used. In the first case[1], RESA1 WT, KI, KO were cultured in RPMI 1640 medium supplemented with 10% human serum according to above described method, with parasitemia between 10 and 20%. At very high ring-stage, cultures were synchronized by exposure to a 5% (w/v) sorbitol solution. Thereafter membrane ghosts were obtained (see below). In the second case[2], strains were again cultured in RPMI 1640 medium supplemented with 10% human serum according to above described method, with parasitemia between 10 and 20%. For the schizont purification Percoll-sorbitol gradient was performed (according to Deitsch Lab, Cornell University, Plasmodium Gene Expression, Standard Protocols, 2003). Two different Percoll-sorbitol gradients were first prepared and placed in layers in 15 mL falcon tube: 3 mL of the 70% solution on the bottom of the tube (prepared from the 90% Percoll / 6% sorbitol stock) and 3 mL of the 40% solution (from the same stock as above) on the top. Cultures were spun down, resuspended with fresh culture media to 50% hematocrit and layered on the top of prepared gradient (~ 2 mL of total volume). Tube was centrifuged for 20 min at 10 000 rpm, at RT (Sorvall SS-34 rotor). Schizonts were recovered from 40/70 solutions interface, transferred to a 50 mL tube and washed thrice with 10 volumes PBS (PBS was added gently drop by drop and suspension was shaking after each addition). After last wash, cells were spun down, supernatant removed and effectiveness of schizonts purification was examined on blood smear stained with Giemsa.

III. 5. 2. Samples preparation.

For the ring-stages, ghost membranes were obtained. For that purpose, synchronized cultures were washed twice in PBS on ice (1.7 rpm, 15 min) and lysed on cold diluted RPMI 1640 (dilution 1/5) in the presence of protease inhibitor cocktail (Roche Complete®). Probes were then microcentrifuged at 15 000 rpm for 15 min at 4°C. After that, the supernatant was removed and the membrane fraction (white/pink) above the dark pellet was taken to another tube. This pellet was washed in cold diluted RPMI 1640 (dilution 1/5) in the presence of protease inhibitor cocktail at least 3 times.

Percoll-sorbitol purified schizonts were lysed in cold hypotonic medium by treating with 0.1% saponin in PBS for 2 min (Fontaine et al., 2010). Free parasites were discarded by centrifugation 9 300 rpm for 4 min and recovered from the pellet.

III. 5. 3. Extraction and denaturation.

The proteins have to be brought into solution by breaking their membrane bindings. To achieve this, extraction in the presence of detergents which prevent proteins aggregation related to their hydrophobic regions was used.

In both cases, ring-stage ghost membranes and schizont saponin extracts, pellets were resuspended in reduction Laemmli buffer (1 volume of pellet in 4 volumes of buffer) and denaturated immediately at 100°C for 5 min prior to load on acrylamide gel.

III. 5. 4. Electrophoresis and blot.

Proteins were resolved via SDS-PAGE on a polyacrylamide gel. Experiments were performed twice, once on the standard 10% gel (BioRad) suitable for all proteins, and once on the self-prepared gels: stacking 4% and separating 7.5%. Wells were loaded with 5 - 10 µl of prestained proteins and the migration was runned during 1h30min at 100V. They were then blotted onto nitrocellulose membrane and let transferred during 1h at 100V. After that, nitrocellulose membrane was incubated in a blocking solution consisting of non-fat milk in Tris buffered saline Tween20 (TBST) to avoid nonspecific binding. Furthermore they were washed in TBST buffer and primary antibody labelling was done, with the RESA-mouse antibody

Table III. 4

Membrane denaturation buffer (for PBR/VDAC)
SDS 4,5%
NaPi 150 mM
EDTA 3 mM
DTT 1 mM
pH adjusted to 7.6

Loading buffer 4X, Laemmli buffer (8 mL)

Tris 0,5M pH 6.8	1 mL	β-mercaptoethanol	0,4 mL
SDS 10%	1,6 mL	Bromophenolblue 1%	0,4 mL
Glycerol	0,8 mL	Distilled water	3,8 mL

Run buffer 5X		Blot buffer		
Tris	9 g	Tris 25 mM	3 g	
Glycine	43,2 g	Glycine 192 mM	14,4 g	
SDS	3 g	SDS 0,1%	10 mL of 10% SDS	
Distilled water	up to 600 mL	Methanol 10%	100 mL	Distilled water 1L

TBS 10X		TBST		Blocking buffer
Tris-HCl	12,1 g	TBS 10X	100 mL	TBST
NaCl	43,55 g	Tween20 0,05%	1 mL	5% milk
Distilled water	500 mL	Distilled water	1 mL	

pH adjusted to 7.4

Gel 7.5%		Stacking gel 4%	
Acrylamide 30%	2 mL	Acrylamide 30%	1,33 mL
Tris-HCl pH 8.8	2,1 mL	Tris-HCl pH 6.8	2,5 mL
H_2O MiliQ	3,7 mL	H_2O MiliQ	6,1 mL
SDS 10%	0,2 mL	SDS 10%	100 µL
APS 10%	30 µL	APS 10%	50 µL
Temed	30 µL	Temed	10 µL

Acrylamide 30%

Acrylamide	30 g
Bisacrylamide	0,8 g
Distilled water	100 mL

Table III. 4: **Principal compounds of buffers used for western blotting.** Shown membrane denaturation buffer were used for PBR/VDAC experiments. The rest solutions were the same for PBR/VDAC and RESA1. NaPi, sodium dihydrogen phosphate $NaH_2PO_4 \cdot 2H_2O$; DTT, dithiothreitol; APS, ammonium persulfate; TEMED, tetramethylethylenediamine; TBS, tris buffered saline; TBST, tris buffered saline tween20.

mAB 1F1 (diluted 1/1000), overnight at 4°C. The next day, membranes were washed in TBST, labelled with anti-mouse secondary antibody (1/2000) conjugated with horseradish peroxidase (Santa Cruz) and let 1 h in RT. After, they were incubated 5 min with the substrate ECL Plus (Amershan). In the last step chemiluminescence detection (Typhoon, GE Health Care Life Sciences) was performed. Table III. 4 contains solutions/ingredients used for western blotting experiments (RESA1 and PBR/VDAC).

III. 6. Immunofluorescence staining and confocal microscopy of RESA1 *P.falciparum*-infected human erythrocyte.

Analyses were carried out on intact RBCs according to Campanella and co-workers (Campanella et al., 2005). The main solutions are shown in Table III. 5. Briefly, cells were washed twice in PBS containing 5 mM glucose (2.5 rpm, 4 min) and then fixed for 5 min in 0.5% acrolein in PBS. They were rinsed three times with solution 1 (2.5 rpm, 4 min), then permeabilized in PBS containing 0.1 M glycine (rinsing buffer) plus 0.2% Tween20 for 5 min and again rinsed 3X in rinsing buffer (including a 30 min incubation at room temperature). Then all non specific binding was blocked by incubation for more than 60 min in saturation buffer (PBS containing 0.05 mM glycine, 0.2% fish skin gelatin (GE-Healthcare)). Staining of fixed, permeabilized RBCs was performed by using antibodies diluted in saturation buffer. Primary antibody used was mAB 1F1 (diluted 1/25). The secondary antibody was anti-mouse (diluted 1/500) coupled to AlexaFluor 568 nm (In Vitrogen). After labelling, resuspended red blood cells were allowed to attach to slides coated with polylysine and mounted using a PBS/glycerol (50/50) solution. Images were acquired on a LEICA SP5 confocal microscope equipped with a 63x1.40 oil immersion objective, at the plate-forme d'imagerie, Station Biologique, Roscoff, France.

III. 7. Patch-clamp.

III. 7. 1. Principle.

The patch-clamp technique, developed by Sakman and Neher in 1976 (Neher et al., 1978; Sakman and Neher, 1984; Neher and Sakman, 1992), allows to measure

Table III. 5

1 washing	2 fixation	3 permeabilisation	4 incubation	5 saturation
PBS + 5 mM Glucose	PBS + 0,5% Acrolein	PBS + 0,1 M Glycine + 0,2% Tween20	PBS + 0,1 M Glycine	PBS + 0,05 M Glycine + 0,2% Fish skin gelatin

Table III. 5: **Solutions used for immunostaining.** Reagents were purchased from Sigma. PBS from tablet was dissolved in MilliQ water and adjusted to pH 7.4. All solutions were prepared at the room temperature (solution 2 under the hood), filtered through 0.2 µM millipore cellulose disks and used for experiments, otherwise stored at 4°C.

Table III. 6

Compound [mM] Solution	NaCl	NaSCN	KCl	$MgCl_2$	$CaCl_2$	Glucose	HEPES
RnD	115		5	10	5	10	10
RnD-10SCN	135	10	3	0,15	0,05		10
RnD-69SCN	66	69	3	0,15	0,05		10
$KCl_{\ pipette}$			150	1	1,4	10	10

Table III. 6: **Composition of the standard bath and pipette solutions used in patch-clamp experiments.** All solutions were prepared in MilliQ water from stock solutions (in mol/l): NaCl (1), KCl (0,5), $MgCl_2$ (1), $CaCl_2$ (1). The osmolarities were always controlled and equilibrated to 300± 5 mOsm (kg $H_2O)^{-1}$. pH were adjusted to 7.40 with NaOH or KOH. All solutions were filtered through 0.2 µM millipore cellulose disks, equilibrated in air (oxygen partial pressure, pO_2 = 155 mmHg; carbon dioxide partial pressure, pCO_2 = 0.3 mmHg) and stored at 4°C till experimental use. The total free calcium concentration was adjusted to 10^{-3} M and 10^{-7} M (pCa3, -log[Ca^{2+}]=3 and pCa7, - log[Ca^{2+}]=7 using Calcium version 1.1 software; Chang et al., 1988) respectively for extracellular and intracellular solutions.

transmembrane currents or voltages of cell ionic channels by the use of a single glass microelectrode (micropipette). This part of the present work discusses the principles of this electrophysiological method, since the technical details can be found in the original literature (Hamill et al. 1981; Sakmann and Neher, 1984). In addition, it will focus on 'voltage-clamp' in order to describe the process of current measurements of a piece of membrane imposed with defined voltage.

Briefly, the mechanical aspect of this technique is simply, under a microscope, to brought in close contact a small glass tube, so-called patch pipette thinned to a cone-shaped end (usually with area ~ 1 μm^2 and tip resistance ~ 10 MΩ) and filled with proper solution, with the surface of a cell (patch cell). This contact has to be very tight in order to generate highly resistant, in the range above 10 GΩ, electrical seal and thus prevent artefacts resulting from leakage. Once the seal is established, two electrode system (reference, Ag/AgCl, in the bath and second one in the pipette) enable detection and measurements of electrical currents (in picoAmpere, pA) through channels in the cell membrane. The membrane potential across the patch or whole-cell is fully under experimental control and may be held (holding potential) or varied depending on need. Currents across the membrane are measured by applying voltage-steps defined in pre-set sequences (ramps).

In our experimental conditions, patch pipettes (tip resistance 10 - 20 MΩ) were prepared from borosilicate glass capillaries (GC 150F Clark Electromedical), pulled and heat-polished on a programmable puller (DMZ, Werner Zeitz, Augsburg, Germany). Patch-clamp seals (4 to 20 GΩ) were obtained by a suction pulse of 10 - 20 mmHg applied for less than 5 s, using a syringe connected to the patch pipette. The main solutions used for the pipette and bath are shown in Table III. 6.

III. 7. 2. Current recordings.

The patch-clamp current recordings may be performed on red cells in three different configurations (Fig. III. 2):

A: *Cell-attached (C-A)*, when a small piece of membrane is isolated of the rest of the cell and current going through is recorded. The exchange of ions between the inside of the patch pipette and the cell can occur only when ionic channels exist in

Figure III. 2

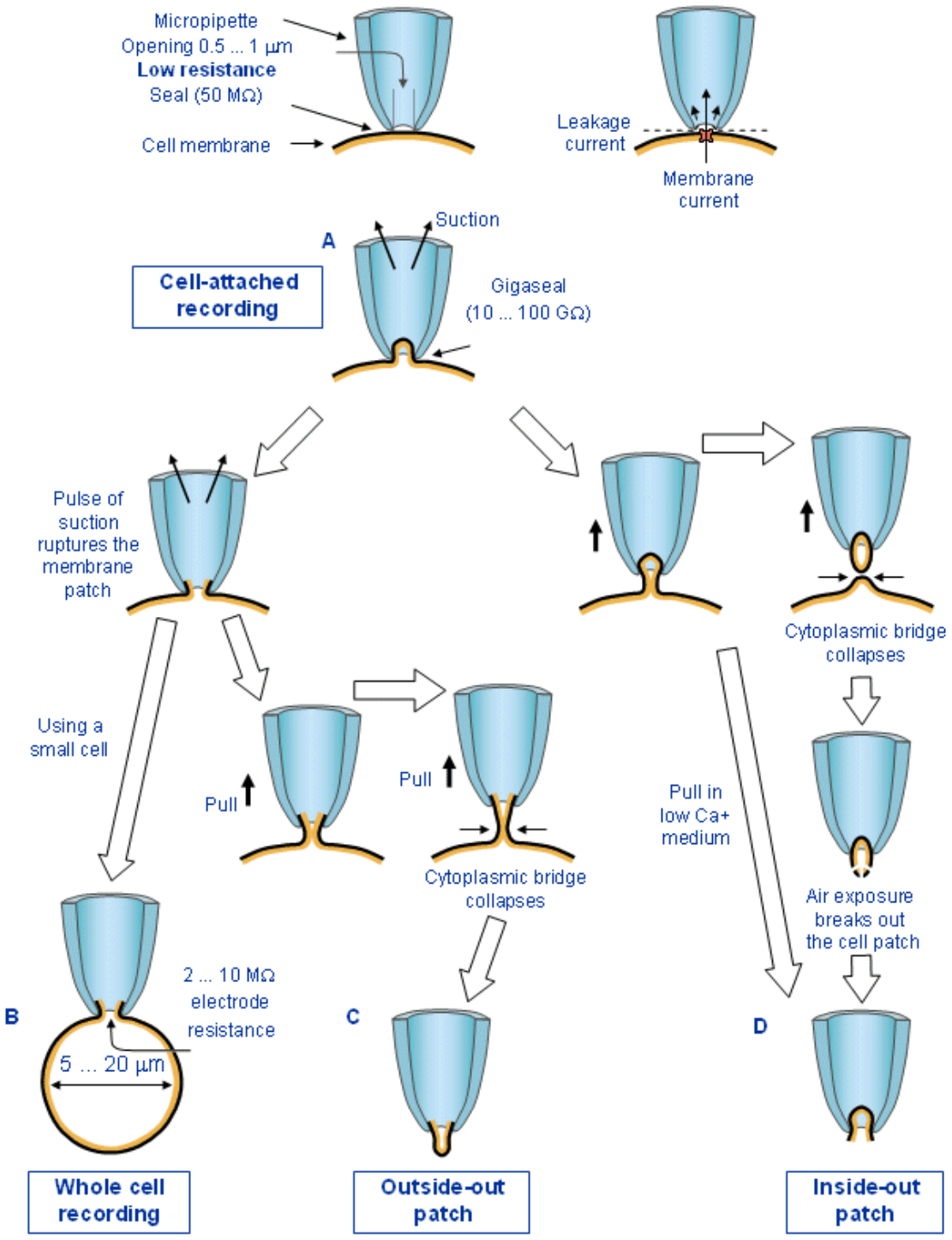


Figure III. 2: **Schematic illustrations of four configurations of patch-clamp electrophysiological technique**. (**A**) cell-attached, (**B**) whole-cell, (**C**) excised outside-out, (**D**) excised inside-out. Figure modified from Hamill et al., 1981.

patched fragment. In view of the small size, only very few channels may be present in the patch of membrane. However, when ionic channel opens, the movement of charged particles (ions) generates electrical current. The recordings provide important information on how single channel conductances and open-state probabilities vary with voltage. They enable also biophysical characterization of all channel types present in the membrane under study. Although this configuration is the most physiological, it does not provide an access to the cytosolic side of the membrane. The membrane potential V_m of the cell is defined by the equation (i):

$$V_m = E_m - V_p \quad \text{(i)}$$

where E_m is the resting membrane potential of the cell and V_p is the holding potential applied to the pipette.

B: *Whole-cell (W-C)*, obtained from C-A configuration when the patch of membrane within the tip is ruptured either by brief electric shock pulse or suction. In this situation, the micropipette, connected directly to the inside of the cell, measures currents of all the ionic channels present and active in the whole cell. The cell content changes dramatically as the cell cytosol equilibrates with the solution within the patch pipette. The membrane potential is described as (ii):

$$V_m = + V_p \quad \text{(ii)}$$

In this configuration, the conductance through the whole membrane area measures the number of active channels per cell and it is used mainly for pharmacological tests. For that purpose different drugs have been tested in the present work and they are listed in Table III. 7.

C: Excised, which consists two separate modes:

- *inside-out (I-O)*, pulled from C-A situation without rupturing the membrane. After withdrawal of the patch pipette a small cytoplasmic bridge surrounded by the membrane is isolated forming a closed structure inside the pipette. Short exposure to the air break part of the membrane outside the pipette and thus finally the cytosolic side becomes accessible. *I-O* patches can also be obtained directly without air exposure if the withdrawal is performed in Ca^{2+}-free medium. With this configuration, by changing the ionic concentrations in the bathing solution, one can examine the effect of a quick change in concentration on the cytoplasmic side of the mem-

Table III. 7

Product	Effect	Concentration	Solvent	Furnisher	References (e.g.)
DIDS	inhibitor of Cl^- channels, band 3 and cotransporter K^+-Cl^-	100 μM	DMSO	Sigma	Lepke et al., 1976; Soltoff et al., 1993; Freedman et al., 1994; Freedman et al., 1997; Ginsburg & Kirk, 1998; Huber et al., 2002.
Furosemide	inhibitor of band 3 and cotransporter K^+-Cl^-	1 - 1000 μM	DMSO	Sigma	Kirk et al., 1994; Ginsburg & Kirk, 1998; Huber et al., 2002.
Glybenclamide	(CFTR)Cl^- and K^+-ATP channel blocker	100 μM	DMSO	Sigma	Kirk et al., 1994; Ginsburg & Kirk, 1998; Lee et al., 2005; Huber et al., 2002.
Human serum	activator of anionic channels	0,50%	Rn-Desai	Sigma	Staines et al., 2003; Staines et al., 2006; Huber et al., 2008; Glogowska et al., 2010.
NPPB	inhibitor of Cl^- channels	1 - 100 μM	DMSO	Sigma	Tilmann et al., 1991; Egee et al., 2000; Huber et al., 2002; Duranton et al., 2004.

Table III. 7: **The basic chemical compounds used mainly in *whole-cell* configuration of patch-clamp**. Solutions of the appropriate final concentration were always fresh prepared in MilliQ water from the stocks, filtered through 0.2 μM millipore cellulose disks and used for experiments.

brane. It can therefore be used to investigate the cytoplasmic regulation of ionic channels for selectivity, substitution and pharmacological assays. The membrane potential here is only a function of the holding potential according to the equation (iii):

$$V_m = - V_p \qquad \text{(iii)}$$

This configuration was not used in this work.

-*outside-out (O-O)*, complementary to the *I-O* with the respect to the orientation of the membrane surface and microversion of the *W-C* configuration. After the rupturing of the cell membrane, the micropipette is pulled away from the cell. During withdrawal, a cytoplasmic bridge surrounded by membrane becomes collapsed, leaving behind an intact cell and a small piece of membrane, which is isolated and attached to the end of the micropipette. The result is an attached patch membrane with cell exterior in contact with the bath solution and the former cell interior faces with the inside of the micropipette. With this method the outside of the cell membrane may be exposed to different bathing solutions, therefore leading to investigate the behaviour of single ionic channels activated by extracellular receptors. Although, this configuration appears to be very delicate and less stable. The *O-O* was not used in this work.

In our laboratory conditions cells (non-infected or malaria-infected) were in the first step washed three times and resuspended (50% hematocrit) with the solution used after as a bath in patch-clamp (Table III. 6). A few microliters of cell suspension were placed into 35 mm Petri dish, adapted with a perfusion chamber made from neutral material. Next, Petri dish was placed on the stage plate of an inversed microscope (Diaphot 300, Nikon) equipped with micromanipulator (Narishige). Single channel currents were recorded using a RK400 patch-clamp amplifier (Bio-logic, Claix, France), filtered at 0.3 or 1 kHz, digitized (CED 1401, Cambridge Electronic design, UK), and stored. *Whole-cell* currents were recorded using the same RK400 amplifier, with voltage command protocols generated by evoking a series of test potentials from -100 to +100 mV in 10 mV steps for 500 ms from a holding potential (V_H) of 0 mV. The oscilloscope connected to amplifier allows visualization of observed currents.

Figure III. 3

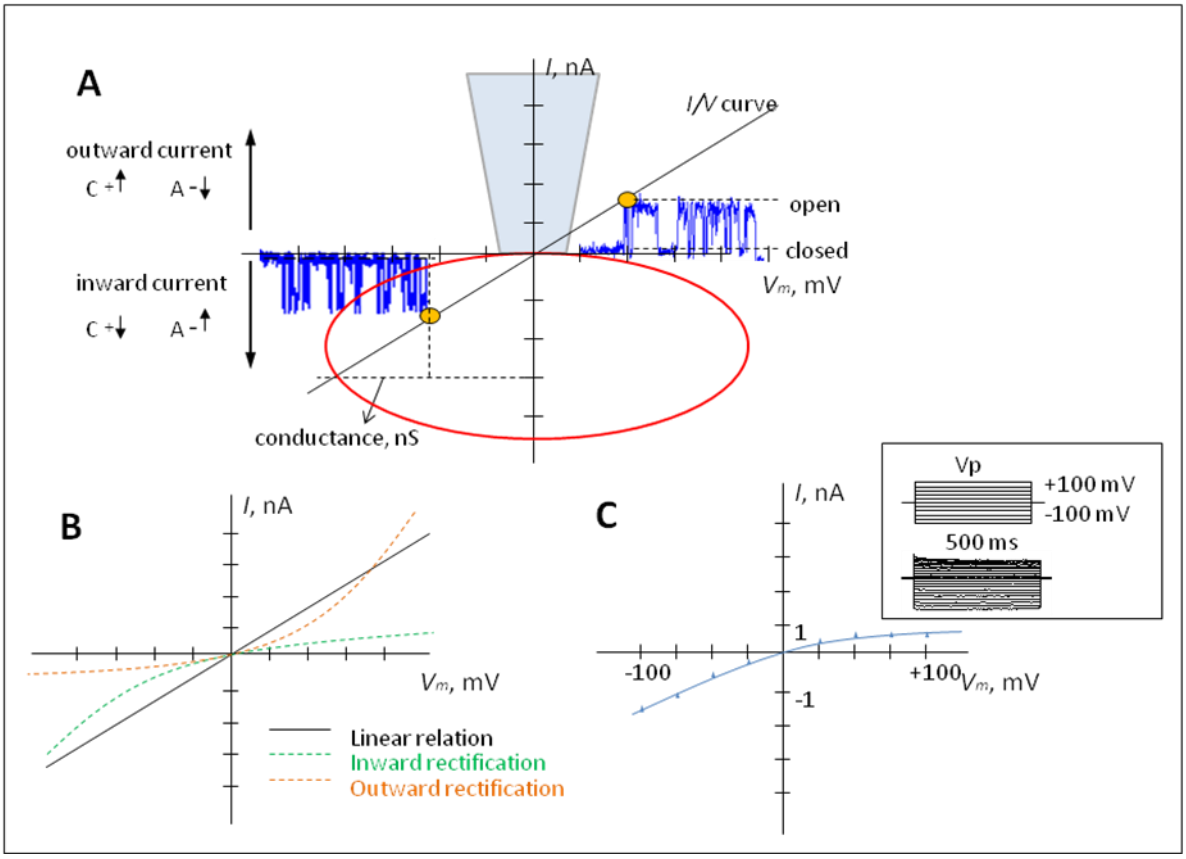


Figure III. 3: **Principles of analysis of current recorded in patch-clamp electrophysiological technique**. (**A**) I/V curve constructed from single-channel recording in *cell-attached* configuration. Directions of current are shown: outward current, cations (C^+) leaving the cell (↑), anions (A^-) entering the cell (↓); inward current, opposite direction of ions movement. Open and closed states of single channel are marked. (**B**) Determination of different I/V curves options. Black solid, linear relation; green dotted, inward rectify; orange dotted, outward rectify. (**C**) I/V relationship of analysed current in *whole-cell* configuration. Indeed, test ramp (up) of imposed potential from -100 to + 100 mV in 10 mV steps during 500 ms; corresponding membrane current measured (down).

III. 7. 3. Current analysis.

For analysis of single channel recordings of patch-clamp WinEDR V2.7.6 computer program (Dempster, Strathclyde Electrophysiology Software, Scotland) was used. As reported above, because the imposed potential is an important driving force for movement of ions, from the data we can describe ionic channel selectivity (i.e. which ions are able to permeate), gating (i.e. how ionic channels open and close), and transport rates. For these purposes, to calculate the conductance of the channel present under the patch pipette, the current/voltage relationship (I/V curves) were constructed by plotting the mean amplitude for each clamped potential (Fig. III. 3A). In addition to recordings, I/V plots provide information on the collective kinetics of channels (for single channels, the kinetics of the molecular components controlling the conductive pathway). In general, usual ohmic behaviour of channels results in linear I/V curve, whereas when deviated they are described as rectified: outwardly if the current is reduced at negative membrane voltages, and inwardly if reduction takes place at positive voltages. Thus an inward current can be attributed to an entry of cations into the cell or an efflux of anions leaving the cell (Fig. III. 3B). We depict thereby the unitary conductance. For description of channels gating, open probability (P_o) was determined as the fraction of digitized points above a threshold set midway between the closed and open peaks of current-amplitude histograms. P_o was determined from 60 seconds stable recordings. In these conditions, P_o was defined as the ratio of the total time spent in the open state to the total time of the complete record. Transitions (known as gating) between an open and closed state of the channel are important in order to determine existence of different subconductance levels of the same channel. Replacement of Cl^- ions by SCN^- ions was used for characterization of channel selectivity.

Whole-cell currents of patch-clamp were recorded in stimulation protocol with a series of test potentials from -100 to +100 mV in 10 mV steps for 500 ms from a holding potential of 0 mV and analyzed using the WCP Software (WCP V3.3.3. Software, Strathclyde, UK). Data for the construction of I–V curves were the mean current measured between 200 and 400 ms (Fig. III. 3C). For statistical analyses, significance was assessed using the Fisher *F* test and Student's *t* test or ANNOVA

Table III. 8

Compound	Stock	Volume
Percoll		59,78 g
NaCl	3 M	2,66 mL
Phosphat	0,2 M	3,50 mL
EDTA	0,5 M	70 µL
Glucose	40 g/L	1,75 mL
H_2O		up to 70 mL
PMSF	50 mg/mL	21 µL

Table III. 8: **The Percoll buffer compounds** (for 70 mL). All reagents were obtained from Sigma. Buffer was prepared with gently mixing of all compounds; pH was adjusted to 7.40. Phosphat: Na_2HPO_4 (0,2M) + KH_2PO_4 (0,2M). PMSF, protease inhibitor (inhibits serine, cysteine, mammalian acetylocholinesterase proteases), was dissolved in ethanol.

(Two Way Analysis of Variance). Data are given as mean values ± S.E.M. Differences were considered to be significant when $P \leq 0.05$.

III. 8. Percoll-gradient separation of human red blood cells.

III. 8. 1. Principle.

In a population of RBCs, cells are different from each other depending on their age, with altered density. Young cells have lower density in a comparison to old cells. This makes it possible to separate them into fractions with different densities according to cells age, by self-forming Percoll gradient centrifugation (Lutz et al., 1992).

Percoll™, a low viscosity density gradient medium, non-toxic and almost chemically inert for biological systems, consists of colloidal silica particles coated with polyvinylpyrrolidone (15 - 30 nm diameter). It forms a gradient under centrifugation force in which the different dense RBCs were distributed.

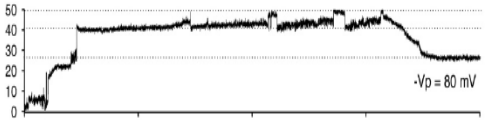
III. 8. 2. Protocol.

Fresh blood was drawn into heparinized vacutainers. RBCs were washed thrice by centrifugation (2.5 rpm, 4 min) and resuspended in large volumes of standard laboratory physiological solution RnD (Table III. 6). The buffy coat containing platelets and white cells was removed by aspiration after each wash. 30 ml of Percoll buffer (Table III. 8), prepared on ice, were mixed with 5 - 6 ml of washed RBCs and centrifuged at 18 000 rpm for 35 min at 4°C. The cell suspension in the centrifuge tubes was separated into 5 layers. Each layer was individually taken and collected by washing three times with physiological RnD buffer at 2.5 rpm for 4 min at 4°C to remove remaining Percoll. Finally, the RBCs were resuspended in physiological RnD solution at 50% hematocrit and used for experiments, otherwise kept at 4°C.

CHAPTER IV

RESULTS

IV. 1.



Further clues on electrophysiological characterization of anionic channels in human red cell membrane.

First objective:

IV. 1. Further clues on electrophysiological characterization of anionic channels in human red cell membrane.

IV. 1. 1. Introduction.

Except an early reports by Schwarz and co-workers (Schwarz et al., 1989), anion conductance through the human red cell membrane attracted attention only recently compared with a large number of studies on the other transporters, and historically has been attributed to band-3 dominating the total membrane conductance. In addition, for a long time informations about anionic pathways in erythrocyte membrane were based mainly on cells in suspension with measurements of net fluxes or radiotracer fluxes. Patch-clamp electrophysiological technique gave revolutionary informations, showing a basic membrane conductance of less than 100 pS (Desai et al., 2000; Egee et al., 2002) which was in fair agreement with the value calculated from experiments on cell suspensions (Hoffman et al., 1980; Bennekou 1984). Thereafter different groups of electrophysiologists reported their own point of view. The team 'Comparative Physiology of Erythrocytes' from Station Biologique in Roscoff (France) have identified three types of anionic channels in uninfected and infected RBCs: a small conductance channel, a medium inwardly rectifying linear conductance channel, and an outwardly rectifying channel (Egee et al., 2002; Decherf et al., 2004; Bouyer et al., 2006; Decherf et al., 2007; Bouyer et al., 2007; Merckx et al., 2008; Merckx et al., 2009) and has given evidence for anionic channel activity generated after Gardos channels activation upon membrane deformation (Dyrda et al., 2010).

In spite of these indications, the molecular nature of such anionic channels remained unknown, even if tentative was done by several groups based on pharmacological and selectivity profiles. However, most results were acquired on malaria infected cells, compared to non-infected cells. Among the channels described, the linear conductance Cl^- channel identified by single channel recordings in human RBCs has been attributed originally to cystic fibrosis transmembrane regulator (CFTR), since it displays some of the similar functional characteristics: small con-

ductance, pharmacology, and mode of activation (PKA-dependence), but it differs by its selectivity sequence $I^->Br^->Cl^-$, instead of $Cl^->Br^->I^-$. At the same time, Verloo and co-workers (Verloo et al., 2004) according to their *whole-cell* observations suggested that the CFTR protein would only (if any) play a role as regulator. The presence of CFTR protein has never been clearly demonstrated electrophysiologically. Moreover, transcripts for CFTR protein were not found in the cDNAs prepared from human erythroid progenitor cells and CFTR protein could not be detected by use of different specific antibodies in RBCs ghosts (Hoffman et al., 2004). Outwardly rectifying anionic channels have been described in epithelial and non-epithelial cell membranes. They are supposed to share the major role in chloride secretion with the CFTR playing a role of regulator (Tabcharani et al., 1991; Schwiebert et al., 1994; Schwiebert et al., 1995). On the other hand, the study on ORCC is rather difficult, because their occurrence is usually extremely low. Using the *whole-cell* configuration the group of Lang from Tuebingen (Germany) has shown activation of ORCC and chloride channels ClC2 by oxidation process of normal cells (Duranton et al., 2002; Huber et al., 2004).

It appears from all the above-presented facts, that informations obtained by different groups on anionic channel activity in red cell membrane are rather confusing. In 2003, Staines and co-workers (Staines et al., 2003) have published a *whole-cell* study confronting the effects of the different experimental conditions used by these groups on malaria infected cells, regarding the mode of cells preparation prior experiments and the conditions used during membrane currents recordings. They indicated that anionic conductance was increased in the presence of extracellular serum left behind from *in vitro* culturing or cell preparation. In all cases, human plasma (0.5%) or serum (0.5%), as well as Albumax but in a lesser extent, added to bathing solution during recordings, induced immediate increase in the *whole-cell* outward and inward currents. Furthermore, the current activated by serum was carried by a conductive pathway presenting a different sensitivity to NPPB (compared to control without serum) since addition of NPPB at the concentration of 1 μM to serum-treated cells resumed the control I/V curve, which itself was suppressed at the concentration of 10 μM NPPB.

Taking into account all these confused informations and still uncomplete knowledge of anionic channels present in the red cell membrane it was necessary to go further with these studies, with the specific intention for the first step of my PhD thesis to give electrophysiological characterization of anionic conductive pathways in normal healthy erythrocytes and thus rationalizes and explains earlier observations and results.

Considering that previously indicated diversity of activity could correspond to different manifestations of a unique channel type, we made the hypothesis that anionic conductance above the ground leak of the human red cell is mediated by anionic channel displaying varied kinetic modalities, gating properties, and pharmacology, depending on conditions. We therefore designed a series of experiments using the *cell-attached* configuration aimed at clarifying at the single channel level the effect of serum addition already well documented in *P. falciparum*-infected erythrocytes in the *whole-cell* configuration (Staines et al., 2003; Duranton et al., 2008) but never addressed in non-infected cells at the single channel level.

Moreover, earlier net fluxes experiments using cells in suspension have shown that after ionophores e.g. gramicidin or valinomycin treatment of RBCs membrane, which selectively increase the cationic permeability, the net salt efflux from red cells is anion restricted (Chapell and Crofts, 1966; Harris and Pressman, 1967; Pressman and Heeb, 1971; Scarpa et al., 1968; Scarpa et al., 1970). Because of this marked dependence on the nature of the anion present in the suspending medium where SCN^- ions frequently were used to bypass any rate-limiting effects of the anion permeability (Parker, 1983; Tiffert et al., 2001), we tested the possible preference of the anionic pathway(s) for this member of the lyotropic series. This set of experiment was also supported by the observation that a $SCN^->I^->Br^->Cl^->acetate^->lactate^->glutamate^-$ permeability sequence corresponding to Eisenman 1 sequence for anions was found for permeation pathways activated in *P. falciparum*-infected RBCs.

Figure IV. 1. 1

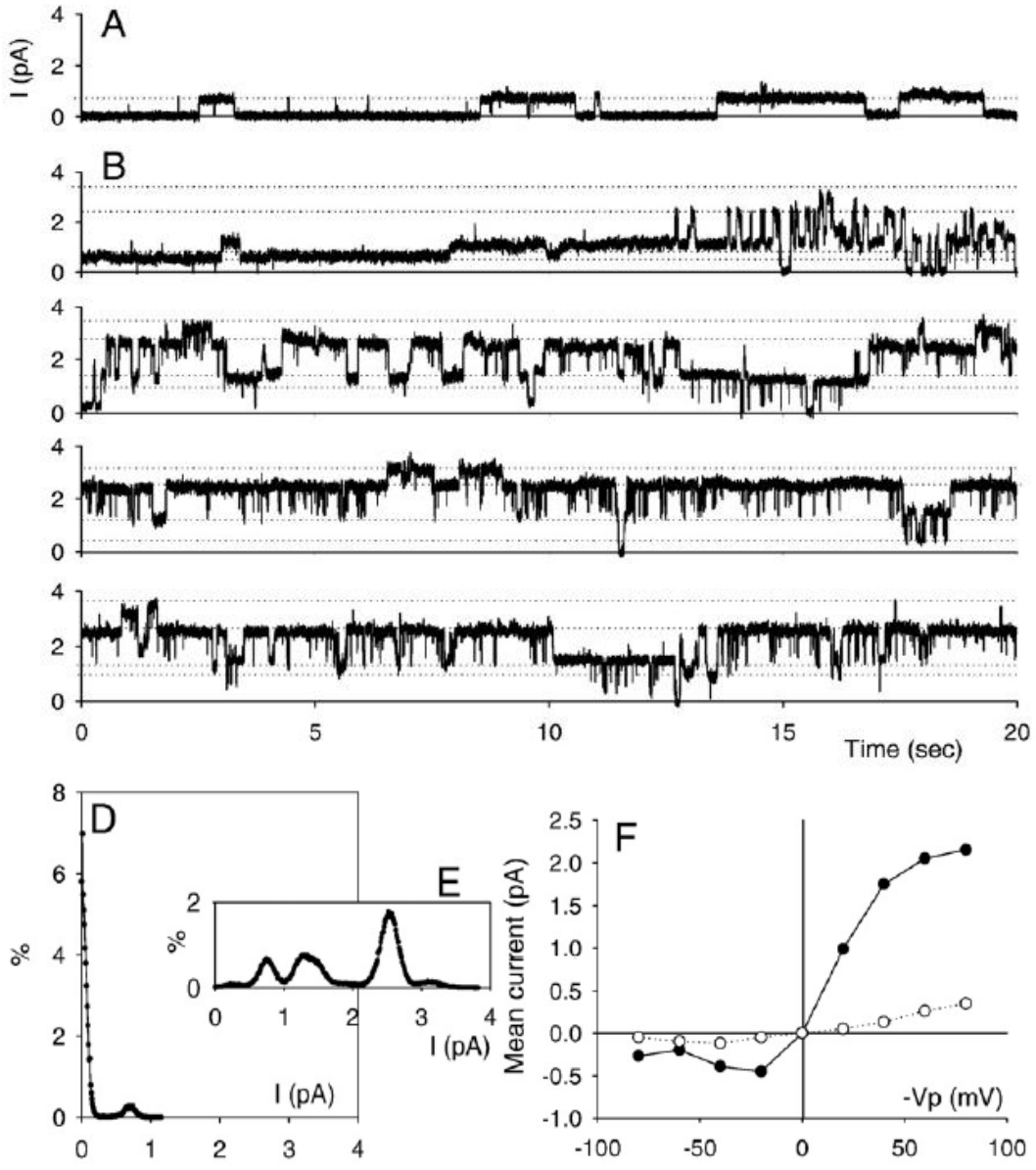


Figure IV. 1. 1: **Increased channel activity in *cell-attached* patches after serum addition (pattern A).** (**A**) Electrical activity recorded after seal formation and before the addition of serum at the imposed membrane potential (-Vp) of +70 mV in a RBC suspended in physiological NaCl solution. (**B**) Rapid changes in the observed current recordings (at –Vp = +70 mV) during 80s following addition of 0.5% (v/v, final nominal concentration) human serum to the bath solution. (**D** and **E**) Histograms of distribution of current amplitudes showing (at +70 mV) the recruitment of 3 new conductance levels (1.30, 2.60, and 3.25 pA, panel **E**) in addition to the initial 0.80 pA (**D**). (**F**) Mean current amplitude measured in apparent stationarity (60-s recordings) in the range from -80 to +80 mV showing 5 - 20 times increase at positive potential and 2 - 10 times increase at negative potentials after addition of serum (●) compared to respective controls (○).

IV. 1. 2. Results.

The results from this part of my PhD thesis have been published in *Blood Cells, Molecules and Diseases (2010)*, article added at the end of discussion, and are presented here briefly.

In the first series of experiments *cell-attached* configuration of patch-clamp was used to test an effect of serum on current recordings of RBCs suspended in physiological 150 mM NaCl solution. It should be noticed, that this configuration has been seldom used before by electrophysiologists, due to above-mentioned technical difficulties, but now is routinely used in our lab.

First, stable channel activity was recorded with the use of 150 mM NaCl in the bath and pipette. Short periods of activity separated by long periods of inactivity at hyperpolarizing voltages, and long bursts of activity separated by long periods of inactivity at positive potentials were observed. These channels were inhibited by 10 μM of NPPB. Above facts, together with linear I/V curves over ± 100 mV range and 12 ± 1.1 pS unit conductance indicated anionic nature of observed channels. These data are in agreement with previously published results. Figure IV. 1. 1A shows a typical example, representative of 54 similar recordings.

Next, another series of test were performed, in which 0.5% of human serum was added to the bathing solution and recordings were made at different holding potentials. In 49 of 54 experiments serum addition changed drastically the observed current, as shown in Fig. IV. 1. 1B, during the first 2 min after serum supplementation and could be detected as long as the seal remained intact. Histogram of observed current amplitudes (Fig. IV. 1. 1D-E) showed the recruitment of 3 new conductance levels at 1.30, 2.60 and 3.25 pA, respectively. Moreover, Patlak mean variance analysis indicated that these multiple conductance levels correspond to a single channel, with multiple level of subconductance rather than recruitment of new channel under the patch. Figure IV. 1. 1F presents mean current amplitude and confirms that serum increases it 5 - 20 times at positive and 2 - 10 times at negative potentials (closed circles) compared to the control (open circles). Because the effect of serum on channel activity was not always the same, as it will be discu-

Figure IV. 1. 2: **Increased channel activity in *cell-attached* patches after serum addition (pattern B).** (**A, B, C**) Three representative types of current recordings corresponding to pattern B obtained at –Vp = +90 mV, +50 mV, +90 mV, respectively. (**A**) Shift from medium velocity gating to very fast gating between two states during 360 s following addition of 0.5% (v/v, final nominal concentration) human serum to the bath solution. (**B**) An example of residence in a predominant substate interrupted either by frequent and brief closures or by fast gating between two states. (**C**) An example of oscillatory evolution between three levels of fast gating. (**D**) A typical example (representative of six experiments) of progressive inhibition by the anionic channel blocker NPPB (1 µM, -Vp = +90 mV) of the different subconductance levels activated by addition of serum.

Figure IV. 1. 2

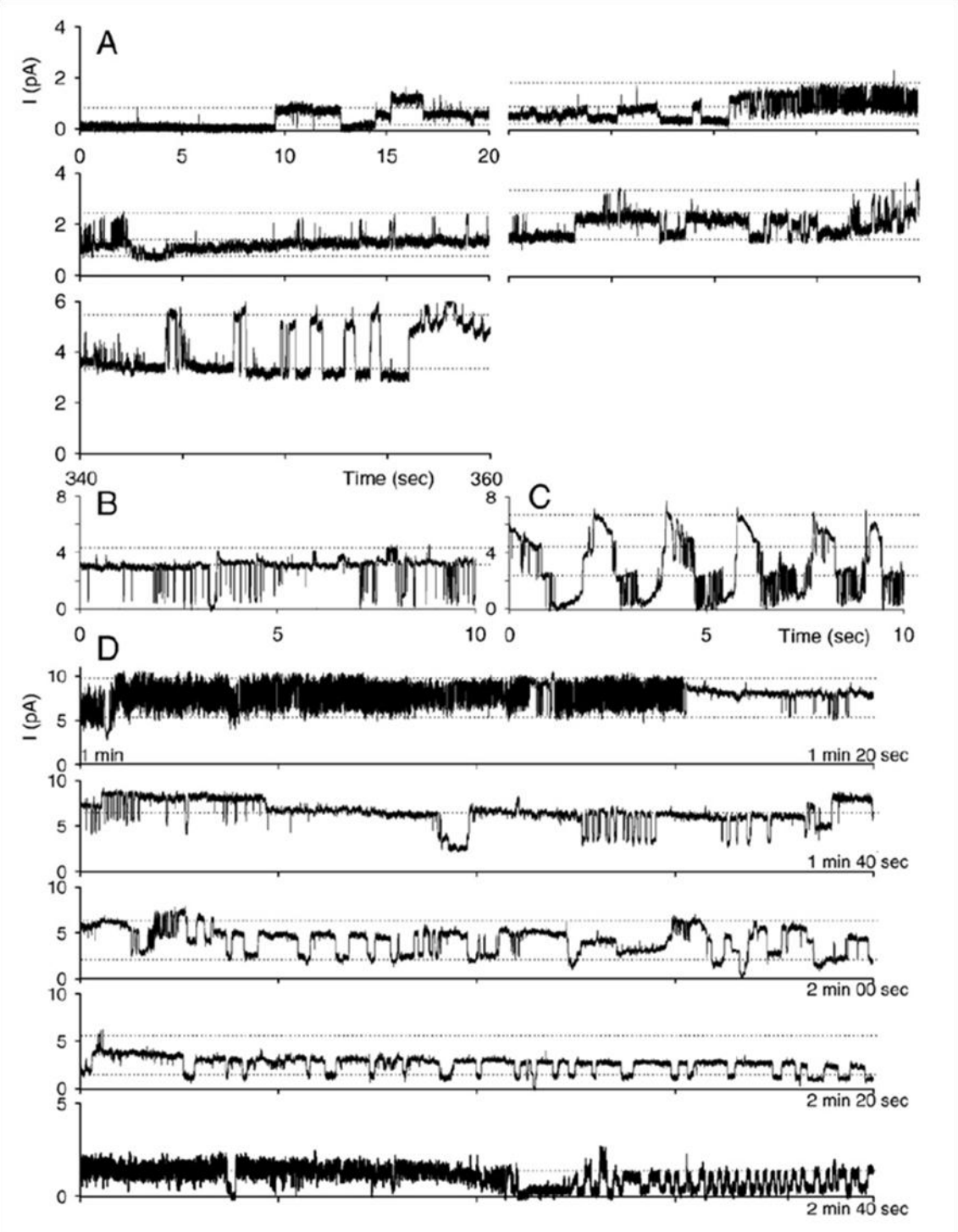

A
I (pA)
Time (sec)
340
360
B
C
D
1 min
1 min 20 sec
1 min 40 sec
2 min 00 sec
2 min 20 sec
2 min 40 sec

Figure IV. 1. 3

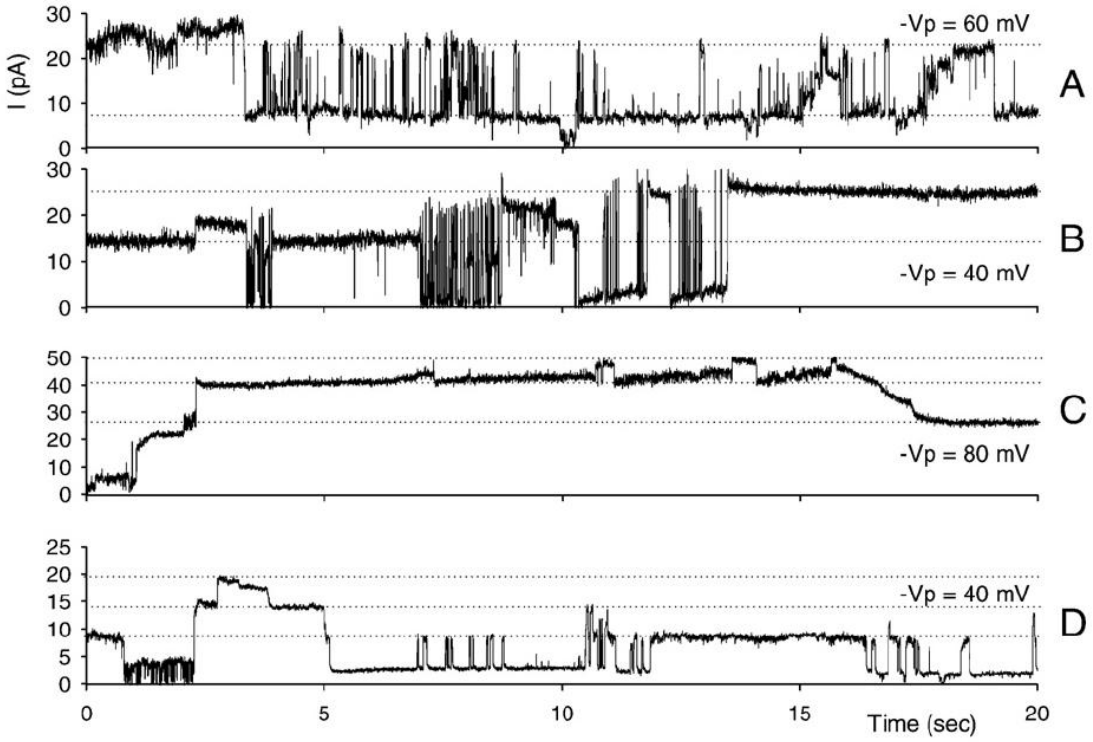


Figure IV. 1. 3: **Increased channel activity in *cell-attached* patches after serum addition (pattern C).** (**A, B**, **C**, **D**) Examples of the most often encountered types of behaviour corresponding to pattern C characterized by the presence of high subconductance states in the range 100 - 600 pS. (**A**) Residence in a predominant substate (8 pA) interrupted either by brief closures or by fast gating between two states (8 and 23 pA). (**B**) Residence in 2 - 3 predominant substates (15, 20, 25 pA) interrupted by frequent and brief closures. (**C**) Long-lasting residence in high conductance levels (27, 40, 50 pA). (**D**) Long-lasting residence in high conductance levels (8, 14, 20 pA) interrupted either by brief closures or by fast gating between two states (2.5 and 8 pA).

ssed further, we classified this new multiple conductance levels observed in our experimental conditions as pattern A.

In 22 patches made in the presence of serum we observed another type of conductance levels, corresponding to pattern B. As illustrated in Fig. IV. 1. 2A, the 1 - 2 pA current (well-established after serum addition) could suddenly give new subconductance levels in the range between 2 and 10 pS depending on imposed voltage. In addition, another representative type of current recordings is shown in Fig. IV. 1. 2B - C presenting complex open-closed kinetics. Pattern B of activity was mostly observed at positive potentials and displayed similarities with a previous study (Decherf et al., 2007) describing outwardly rectifying type of chloride channels. As presented in Fig. IV. 1. 2D, highest subconductance levels were inhibited by 1 μM of anionic channel blocker NPPB, indicating their higher sensitivity relative to the lower subconductances, totally inhibited at the concentration 10 μM.

Thereafter, 6 of our tested cells showed the presence of high sub-conductance states in the range of 100 - 600 pS (pattern C) at depolarizing voltages. Fig. IV. 1. 3 A - D illustrates this typical behaviour. Among them, in 3 cases inhibition by 1 μM of NPPB was observed.

Taken together, we demonstrated in the present study the effects of serum on currents recorded in *cell-attached* configuration and we indicated several current levels for channel activity, which may be classified into three different patterns: A, B and C. I/V plots presented in Fig. IV. 1. 4 summarize our observations. Pattern A provides I/V pairs located between the 'x' axis and the 50 pS I/V curve (line a), symmetrically distributed between -100 and +100 mV, with high P_o for positive voltages and low P_o for negative voltages. The channel activity in these cases can be qualified as being of medium velocity gating with mean residence times in each level in the order of several tens of milliseconds (according to arbitrary classification of Bathori et al., 1998). Pattern B provides additional I/V pairs located between the 50 pS I/V curve (line a) and the 100 pS I/V curve (line b). This activity can be characterized as residence in predominant substates interrupted either by frequent and very brief closures or by very fast gating between two states. Pattern

Figure IV. 1. 4

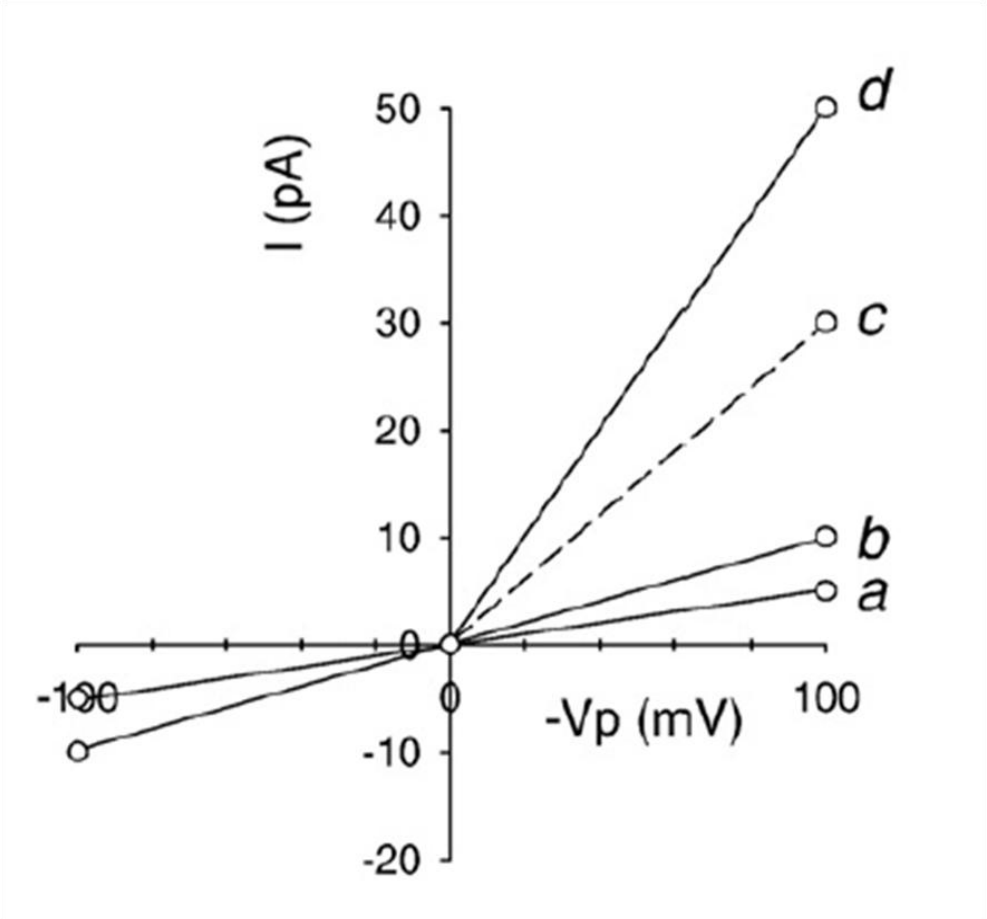


Figure IV. 1. 4: **I/V plots of different patterns of electrical activity observed in *cell-attached* configuration of patch-clamp technique.** Pattern A (line a), pattern B (line b), pattern C (line d). Line c represents a subconductance level frequently encountered in pattern C.

C provides additional conductance levels repeating, albeit at a higher level (between lines b and d), the same type of activity. Predominant level corresponding to 300 pS (line c) was also frequently observed.
These data and the percentage of occurence for each pattern were confirmed in a large number of experiments following this publication.

In a second series of experiments, in order to test the effect of SCN^- ions on *cell-attached* current recordings of RBCs, part of NaCl was replaced by NaSCN (Table III. 6, *Materials and Methods*) for having a final concentration of 10 mM SCN^- ions and used in bath and pipette solution in absence of serum. Representative record from 65 experiments is shown in Fig. IV. 1. 5A. In these experimental conditions we observed increased anionic channel activity relative to control and this multiple conductance levels were present at positive potentials. This activity did not differ significantly from pattern A described above. Interestingly, the channel activity totally changed when instead of 10 mM SCN^- we used 69 mM SCN^-, as illustrated in Fig. IV. 1. 5B. In these conditions, channel activity was consistently observed as bursts of short channel openings separated by brief closings at negative potentials and exhibited voltage-dependent gating, with low P_o between +50 and +100 mV, and increasing P_o between +50 and –100 mV. For a given membrane patch the number of substates was dependent on the membrane potential, with the maximum number activated at negative potentials.

In addition to above-described patterns of anionic channel activity we observed seldom, only in patches where the three patterns were not present, channel exhibiting long periods of opening separated by long intervals of closing. This electrical activity (~ 5 pS) corresponded to small conductance chloride channels and showed the same characteristics as the SCC described in previous works (Bouyer et al., 2006; Bouyer et al., 2007) in *P. falciparum*-infected cells.

Figure IV. 1. 5

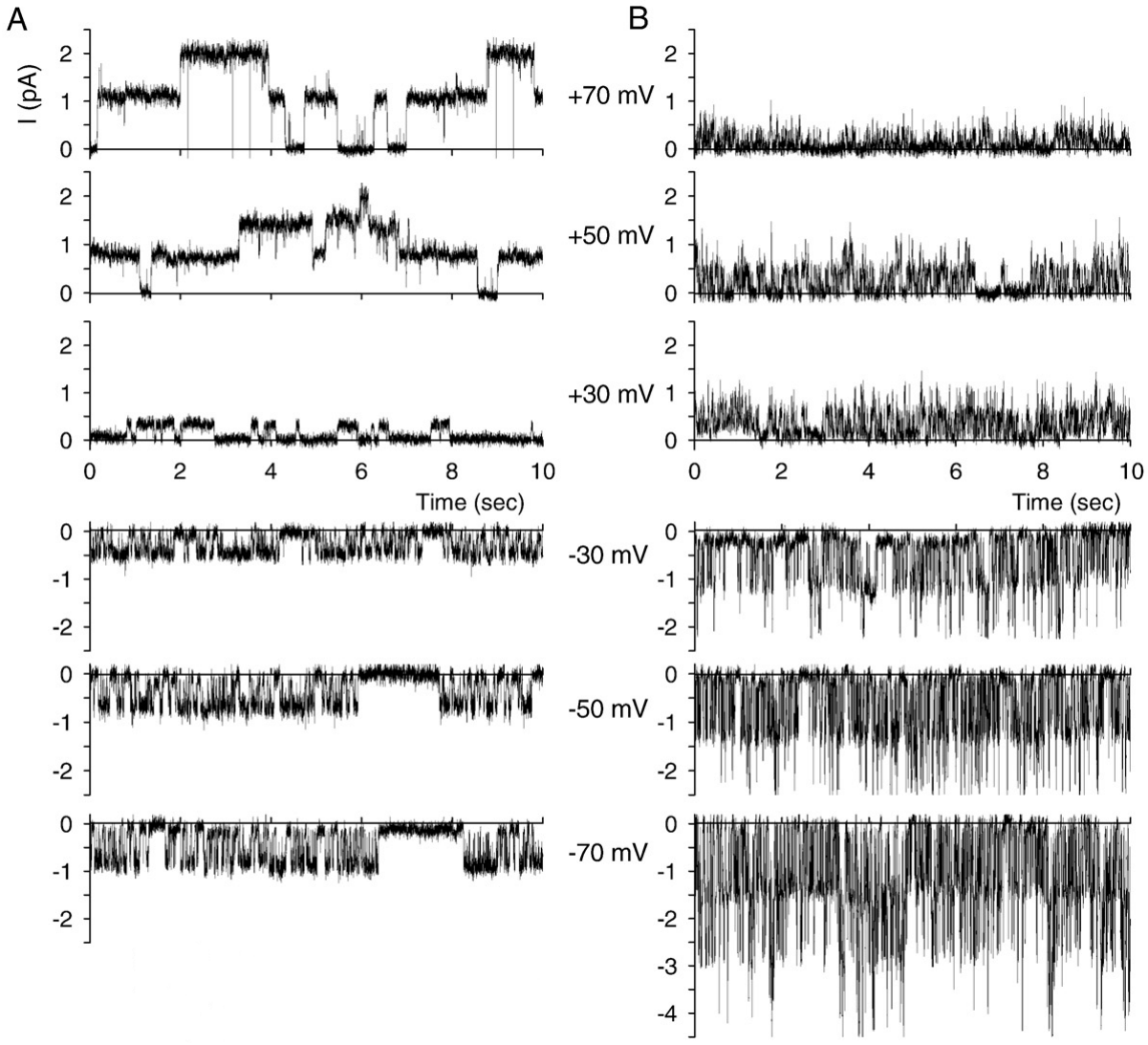


Figure IV. 1. 5: **The effect of SCN^- ions on *cell-attached* current recordings in the absence of serum.** Two typical examples of electrical activity obtained by evoking a series of command potentials from −70 to +70 mV when RBCs were incubated 1 h prior experiments in bath solution where 10 mM (**A**) or 69 mM (**B**) of Cl^- ions were replaced by SCN^- ions.

IV. 1. 3. Discussion.

Patch-clamp electrophysiological technique allowed confirming that the overall anionic conductance of red cell suspension does not exceed the 100 pS. It also allowed to show that 'dormant' channels, in normal physiological conditions, are potentially activatable and after up-regulation could give very high membrane conductance under the effect of yet unidentified activators. As said, the first published results about anionic channels in red blood cells were often difficult to link together, or even contradictory. This work demonstrates that the diversity of recorded activities comes from the activity of *maxi-anion channels*, 600 pS of maximum conductance, with multiple conductance levels, kinetics, selectivities and highly variable pharmacologies. These serum-activated channels are mostly visible at positive potentials and indicate an increased efflux of cations or influx of anions. It should be kept in mind that this phenomenon was observed by using *cell-attached* configuration of patch-clamp, which respects the integrity of the cell, but does not provide full control of the ionic composition of the cytosolic side of erythrocyte membrane. Pharmacological tests and analysis of kinetics and gating properties of such anionic channels should be carried out with excised *inside-out* (*I-O*) configuration in further studies.

Despite these limitations, this work explains, clarifies and rationalizes previous observations from the literature. For instance, three different patterns of activity observed in our experimental conditions were linked together with previously described channel behaviours (Decherf et al., 2007) which were up to this time attributed to separate channels characterized as 'linear conductance anionic channel' (IRC) and 'outwardly rectifying chloride channel'(ORCC). We report here, that pattern A from this work, with the low levels of conductance (between 5 and 15 pS), visible in more than 80% of the records, corresponds to the linear anionic conductance, activated by forskolin and protein kinase A and inhibited by DIDS and NPPB (µm). Next, the ORCC corresponds to pattern B visible in less than 10% cases. 300 pS anionic channel described in 2003 by Decherf and co-workers (Decherf et al., 2003) corresponds to pattern C of the present study.

Furthermore, the fact that high sub-conductance levels were inhibited by NPPB at the concentration 1 µM while the lower sub-conductances were inhibited at 10 µM

strongly suggests that the frequent observations by the Lang group in Tuebingen (Duranton et al., 2002; Huber et al., 2004) of large outwardly rectifying anionic conductance, inhibited by 1 μM NPPB in *whole-cell* configuration, was probably attributable to a large activation of high conductance levels under their experimental conditions.

In addition, we know that small conductances are activated by one or more elements of human serum but the activators of large conductances are not known yet. It is most likely that oxidative stress as claimed by the Lang group, as well as acid–base and redox states, play a major role and will provide important clues for further studies. For such studies, it is interesting to note that large conductances (ORCC, pattern B) spontaneously appear in more than 30% of recordings made on red blood cells from patients with cystic fibrosis (Decherf et al., 2007). This observation should also provide some useful indications with regard to the intracellular activators and should open new area of research for the determination of the pathophysiological importance of such channels in cystic fibrosis.

Whole-cell previous experiments showed a membrane conductance under 100 pS, which is in fair agreement with the value calculated from experiments on cell suspensions from normal donors (in range 40 to 50 pS). As reported, channel activity was negligible in cells suspended in physiological saline solution 10 to 20 min following seal formation. Although the question can not be finally answered, it seems probable, as mentioned above, that the observed channels can be activated experimentally and likely become so under pathological conditions, but under normal experimental conditions, they are dormant. They can potentially give a very high single cell conductance, up to 50 to 100 times the normal conductance. The normal cell has only a limited ability to loose salt or volume. At maximum Gardos channel activation, the cell can loose only about 0.3 mmol/s of KCl, corresponding to a volume loss of only 0.2%/s. However, the sudden parallel opening of large anionic conductance (Dyrda et al., 2010) could help the cell to loose volume rapidly.

Although it was not the aim of this study to determine what component of serum is responsible for the observed activity effect, it must be kept in mind that this component is obviously abundant in the circulating blood and it will be important to determine its contribution to the 'physiological' or 'resting cell' anionic conductance.

The obvious preference of anionic channels for SCN^- ions could explain how substitution of these ions to chloride permitted to bypass any rate-limiting effects of the anion permeability in experiments using selective increase of the cation permeability of RBC membrane by ionophores (Parker et al., 1983; Tiffert et al., 2001). The explanation for the different data obtained at high SCN^- concentration is probably a shift of the isoelectric point for intracellular charges as well documented by Payne and co-workers (Payne et al., 1990). It should also be taken once more into consideration, especially for the better understanding of further objectives of this study, that the pattern obtained with 69 SCN^- fits exactly with the channel activity described in *cell-attached* configuration in *P. falciparum*-infected RBCs.

In conclusion, a major task for the future will be to understand the dynamical aspects of red cell electrophysiology for cells in circulation. One of the hypothetical view is that most of the time, the 'resting cell' is the valid regime, but transiently, the need could arise for a very high, but transient anionic conductance, caused, for example, by full activation of the Gardos channel, the NSC channel, or channels yet to be found, for example, normally dormant sodium channels.

Next part of this PhD thesis focuses on the molecular nature of such maxi-anion channel (see section IV. 2).

IV. 1. 4. Article.

'Anion conductance of the human red cell is carried by a maxi-anion channel'.

Blood Cells, Molecules and Diseases (2010)

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Anion conductance of the human red cell is carried by a maxi-anion channel

Edyta Glogowska [a], Agnieszka Dyrda [a], Anne Cueff [a], Guillaume Bouyer [a], Stéphane Egée [a], Poul Bennekou [b], Serge L.Y. Thomas [a,*]

[a] Centre National de la Recherche Scientifique, Université Pierre et Marie Curie Paris6, UMR 7150, Station Biologique, B. P. 74, 29682 Roscoff cedex, France
[b] Institute of Molecular Biology and Physiology, University of Copenhagen, Denmark

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ABSTRACT

Historically, the anion transport through the human red cell membrane has been perceived to be mediated by Band 3, in the two-component concept with the large electroneutral anion exchange accompanied by the conductance proper, which dominated the total membrane conductance. The status of anion channels proper has never been clarified, and the informations obtained by different groups of electrophysiologists are rather badly matched. This study, using the cell-attached configuration of the patch-clamp technique, rationalizes and explains earlier confusing results by demonstrating that the diversity of anionic channel activities recorded in human erythrocytes corresponds to different kinetic modalities of a unique type of maxi-anion channel with multiple conductance levels and probably multiple gating properties and pharmacology, depending on conditions. It demonstrates the role of activator played by serum in the recruitment of multiple new conductance levels showing very complex kinetics and gating properties upon serum addition. These channels, which seem to be dormant under normal physiological conditions, are potentially activable and could confer a far higher anion conductance to the red cell than the ground leak mediated by Band 3.

Introduction

The present day picture of the human red blood cell (RBC) chloride transport emerged 50 years ago, when Dan Tosteson [1] calculated the anion self-exchange rate and raised the possibility that the chloride translocation across the RBC membrane could be a mediated process contrary to free electrodiffusion.

The experimental tools at hand at the time were isolated red cells in suspension with measurements of net fluxes or radiotracer fluxes, whereas determination of the RBC membrane potential was out of reach. However, many early studies indicated that diffusible anions are distributed in accordance with a Donnan equilibrium [2–4] and that the RBC membrane was relatively impermeable to cations compared to anions. Consequently, it was generally agreed that the membrane potential was identical to the Nernst potential for chloride, about −10 to −12 mV [5,6]. This assumption was supported by estimates of human RBC membrane resistance [7,8] and subsequently confirmed by direct measurements on *Amphiuma* cells with micro-electrodes [9,10] and with the fluorescence technique [11,12] on both *Amphiuma* and human red cells.

Because the red cell membrane is much less permeable to cations and because of electroneutrality restriction, it is not normally possible to measure the true anion permeability. With the availability of compounds such as gramicidin and valinomycin, which selectively increase the cation permeability of the RBC membrane, Chapell and Crofts [13], Harris and Presman [14], Pressman and Heeb [15], and Scarpa and co-workers [16,17] showed that the net salt efflux from the red cells could be anion restricted, which led to the concept of a two-component anion transport: a large exchange component fundamental to the CO_2-carrying capacity of the blood [18,19] and a much smaller electrogenic component that normally determines the RBC resting potential [20] and could be important as the rate-limiting step for electrolyte and water movements through the RBC membrane.

Hunter in 1971 [21] estimated the net permeability to be 4 orders of magnitude less than the tracer permeability, strongly suggesting the exchange concept, and, in 1977 [22], came up with an estimate of the human RBC anion permeability proper (conductance) of about 10 μS/cm². With an independent technique, membrane potential-dependent fluorescence, to determine the membrane potential, and with the electrogenic fraction of the Na-K-pump flux, Hoffman and co-workers [23] arrived at a similar value.

Furthermore, using radiolabeled covalent inhibitors, a 100-kDa membrane protein was identified as the pathway for anion transport [24], and in spite of differences with regard to pH dependence, activation energy, selectivity sequence, etc., it was inferred that both components (the electroneutral anion exchange and the conductance proper) were mediated by the same protein, called Band 3 (or AE1 or, nowadays, SLC4A1). This statement was based on the concomitant inhibition of total and net flux [25,26], although at full DIDS inhibition

* Corresponding author. CNRS-UPMC, UMR 7150, Station biologique, B. P. 74, 29682 Roscoff cedex, France. Fax: +33 298 292310.
E-mail address: thomas@sb-roscoff.fr (S.L.Y. Thomas).

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IV. 2.

The molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells.

Second objective:

IV. 2. The molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells.

IV. 2. 2. Introduction.

We have described above the presence of anionic channels in healthy human red cell membrane, obviously dormant in steady state, but the question of their molecular identity and regulation is still open. As mentioned, many studies have been done trying to resolve this problem, but at the same time most of them were confusing. To continue our considerations on anionic channels we have to switch now to the pathological conditions, due to the fact, that activation of these conductive pathways is strongly associated with sickle cell anemia or malaria. As said, NPPs induced after *P. falciparum* infection in the erythrocyte membrane, and allowing the parasite to ensure the transport of nutrients and waste products, have been shown to be at least partly constituted by anionic channels (Kirk et al., 1994; Kirk, 2001; Ginsburg and Stein, 2004). On the other hand, many studies have been reported trying to answer the question of whether this activity is host- (up-regulated and/or modified) endogenous RBC protein or parasite-derived. For instance, in year 2000, Desai and co-workers (Desai et al., 2000) reported that anionic channels have parasite origin and are formed from the proteins exported by the parasite to the membrane. They called this channel Plasmodial Surface Anion Channel, PSAC. Other groups proposed another model for anionic channel activity in infected cells. Among them, the group of S. Thomas from Roscoff suggested its endogenous nature (Egee et al., 2002) and gave evidence to confirm this theory, showing that after infection these channels are up-regulated and display complex gating and kinetics similar to those of ligand-gated channels (Bouyer et al., 2006). Studies made by the group of F. Lang from Tuebingen (Huber et al., 2002; Staines et al., 2003; Huber et al., 2004; Duranton et al., 2004) proposed that NPPs represent at least two different type of anionic channels, from which one of them belong to the ClC-2 type of channels, activated by the oxydation process.

Taken together, it seems that there are different proposed possibilities leading to recognize the nature of anionic channels in the red cell membrane and this problem remains under debate. However, anionic current recorded in our previous experiments displays similarities with ligand-gated channels (Bouyer et al., 2007; Glogowska et al., 2010). Among them, GABA, glycine and glutamate receptors, and peripheral-benzodiazepine receptor PBR (Jentsch et al., 2002; Shoshan-Barmatz, 2010): i) correspond to Eisenman 1 type of selectivity reported by Desai and coworkers (Desai et al., 2000) as an anionic permeability in infected red blood cells, ii) have comparable with our observations gating properties and iii) pharmacology. Nevertheless, except PBR none has been found in human erythrocytes (Shimizu et al., 2001; Sugiyama et al., 2002). With regard to these facts we made the hypothesis that anionic conductance of red blood cell is a voltage dependent anion channel VDAC. Originally such a channel is characterized as mitochondrial porins, (Schein, 1976; Colombini, 1979) but it can also be expressed in plasma membranes alone, (Thinnes, 1989; Woods and Williams, 1996) or as a component of PBR complex, (Veenman and Gavish, 2006). The PBR complex consists of at least three components (Veenman and Gavish, 2006): a 32-kDa VDAC, a 18-kDa translocator protein TSPO (also called isoquinoline-binding protein IBP), and a 33-kDa adenine nucleotide transporter (ANT).

Literature reports made PBR/VDAC good candidate for the anionic pathway in the RBC membrane. For instance, weak anion selectivity, permeability to big anions like glutamate or ATP and cations like acetylcholine (Rostovtseva and Colombini, 1997; Gincel et al., 2000), susceptibility to anion inhibitors (Basile et al., 1988) could fit with some properities of NPPs. Furthermore, some ligands of PBR have been shown to have strong anti-malarial activity *in vitro* (McEnery et al., 1992; Dzierszinski, 2002; Bah, 2007).

In addition, VDAC channel has been shown to have strong voltage dependence and give multiple subconductance levels at high positive and negative potentials (Shoshan-Barmatz and Gincel, 2003). VDAC has been also reported to exist in multiple functional states with variable conductivities, including 'closed' states (Colombini, 1987; Rostovtseva et al., 2005). Interesting, to keep in mind, that at high conductance levels currents are carried by small ions (Na^+, K^+, Cl^-,…) as well as large ani-

Figure IV. 2. 1

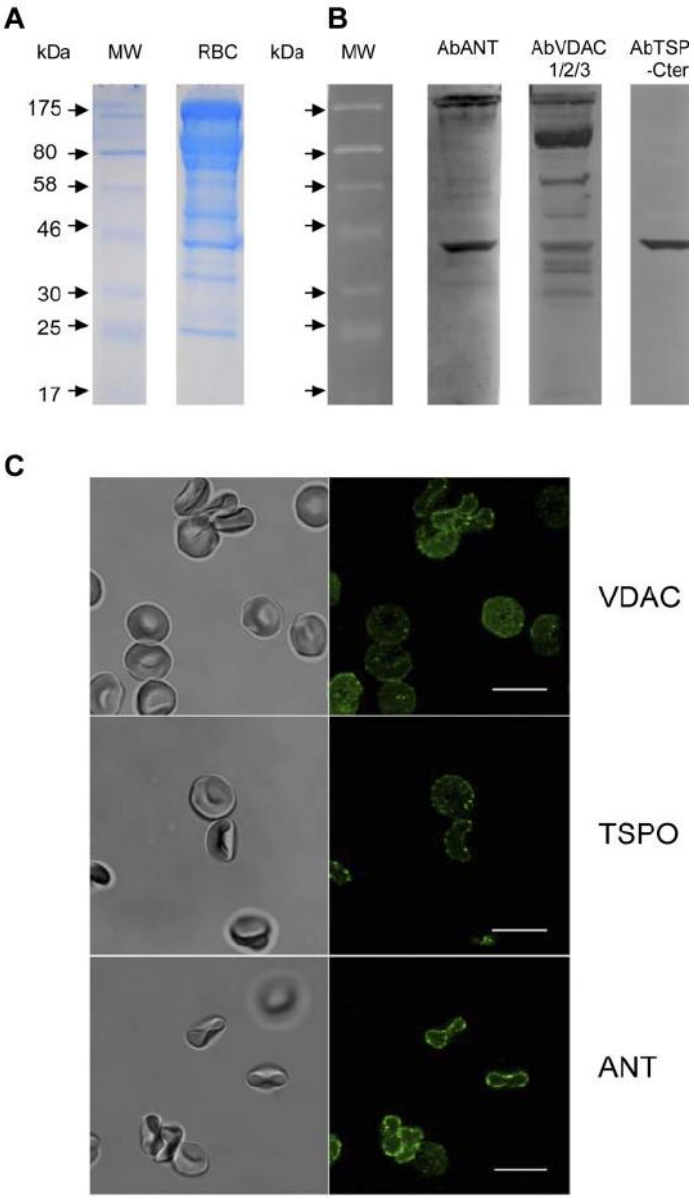


Figure IV. 2. 1: **VDAC, ANT and TSPO detection in human red blood cell ghosts.** Samples (15 g of protein) of whole lysates were subjected to SDS-PAGE (10% acrylamide) and stained with Coomassie blue (**A**) or analyzed by western blotting using polyclonal anti-ANT (1:1000 dilution), rabbit polyclonal anti VDAC1 -2, -3 (1:100 dilution) or polyclonal goat anti-TSPO raised against the C terminus of human TSPO (1:1000). The positions of molecular weight (kDa) protein standards are indicated by arrows. (**B**) 4 bands appearing between 29 - 30 kDa and 36 kDa correspond to different isoforms of VDAC and marked band at 58 kDa correspond to dimers of VDAC1 isoform (29-30 kDa). Multiple bands at higher molecular weights suggest oligomerization of VDAC proteins. ANT and TSPO proteins are also clearly visible. TSPO protein could not be expected at 18 kDa but rather at 36 kDa. These blots are representative of 12 replicates. Immunofluorescence experiments are presented in (**C**). Dilutions were 1/5 for primary and 1/20 for secondary antibodies. Scale bars represent 10 μm.

ons (glutamate, ATP, …) and large cations (acetylcholine, dopamine, Tris, …) with a global preference for anions. At the opposite, at low conductance levels (closed-states) currents are carried exclusively by small ions with a marked preference for cations and especially for Ca^{2+} (Benz et al., 1990; Rostovtseva and Colombini, 1997; Gincel et al., 2000; Bathori et al., 2006).

In the framework of this PhD thesis, I was involved in a common effort to test the above-mentioned hypothesis concerning the molecular identity and regulation of anionic channels in the red cell membrane. We performed biochemical experiments leading to confirm the presence and functioning of the three principal components of PBR complex in the human erythrocyte membrane. Electrophysiological tests were made in order to confirm that PBR is responsible for the previously described erythrocyte membrane-associated maxi-anion current. Pathological conditions of *P. falciparum* infection of red blood cells were used with the purpose of finding the regulation mechanism of anionic channels underlying the new permeability pathways.

IV. 2. 2. Results.

The results presented in this chapter have been published in *Blood (2011)*, and are shown here briefly.

In the first series of experiments we used biochemical assays: western blotting and immunofluorescence to demonstrate that the three components of the peripheral-type benzodiazepine receptor (PBR), including the voltage dependent anion channel (VDAC) are present in the human RBC membrane (Fig. IV. 2. 1A - C). The evidence for VDAC being present in the membrane, among the other proteins (Fig. IV. 2. 1A), is given by 4 bands appearing between 29 - 30 kDa and 36 kDa on western blots (Fig. IV. 2. 1B). VDAC isoforms correspond to molecular weights ranging between 30 and 36 kDa. The 58 kDa band could correspond to VDAC1 dimers. The large band appearing between 90 and 120 - 130 kDa could be interpreted as oli-

Figure IV. 2. 2

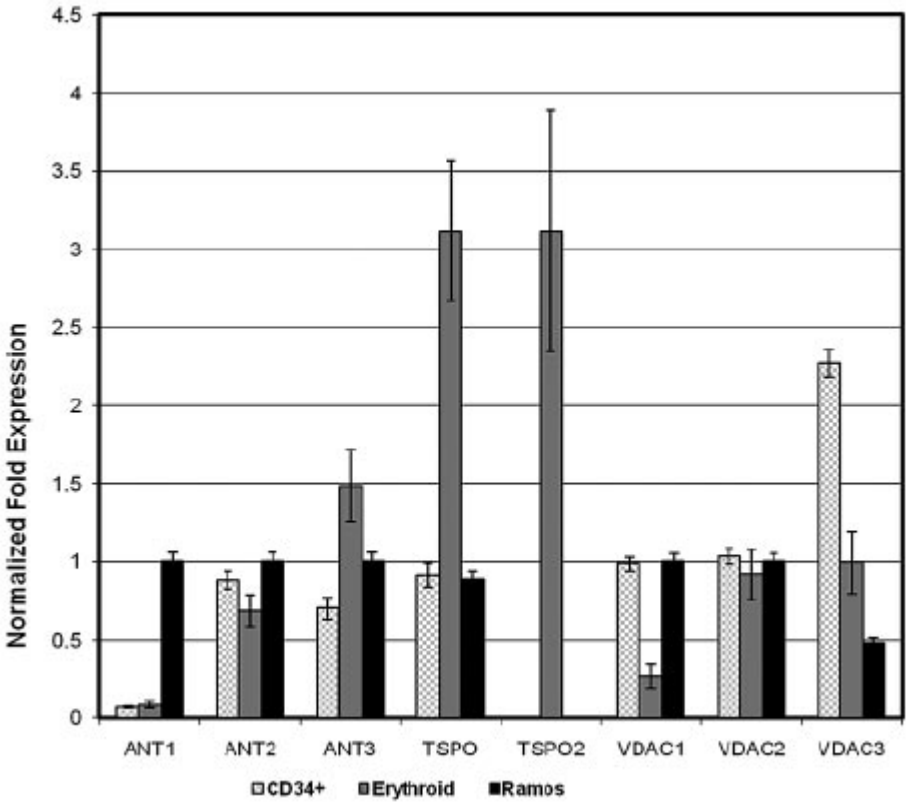


Figure IV. 2. 2: **Quantitative RT-PCR of mRNA isolated from cultured human primary erythroid cells.** Q-PCR was performed using iQ SYBR Green Supermix (BioRad). Relative expression was normalized to geometric mean of unvarying, ubiquitously genes, ornithine decarboxylase antizyme 1 (OAZ1), hypoxanthine phosphoribosyltransferase 1(HPRT1), ubiquitin-fold modifier 1 (UFM1), TATA box binding protein (TBP), pumilio homolog 1 (PUM1), and ribosomal protein S13 (RPS13) as controls. The changes in specific mRNA levels were calculated using the $\Delta\Delta C_T$ method (where C_T is threshold cycle), with results presented as means ± SEM. Results were normalized to the gene with the highest expression level in each group. Triplicate analyses were performed for each target gene.

gomerization of VDAC proteins or as monomers associated with the band 3 protein. Figure IV. 2. 1B also demonstrates the presence of the 2 other components of PBR complex. Polyclonal anti-TSPO antibody raised against the C terminus of human TSPO and polyclonal anti-ANT antibody show that our membrane preparation was positive for both markers.

Immunostaining used for localization of the three PBR proteins demonstrates the presence of VDAC, TSPO and ANT in the RBC membrane (Fig. IV. 2. 1C).

Quantitative RT-PCR of mRNA isolated from cultured human primary erythroid cells, CD34+ hematopoietic stem and Ramos cells (experiment made by our collaborator in Yale, and presented in Fig. IV. 2. 2) confirmed that all three VDAC genes were expressed in erythroid cells, predominantly VDAC2 and 3. Both TSPO genes were highly expressed. There were low level of ANT1 expression and higher levels of ANT2 and ANT3 expression in erythroid cells.

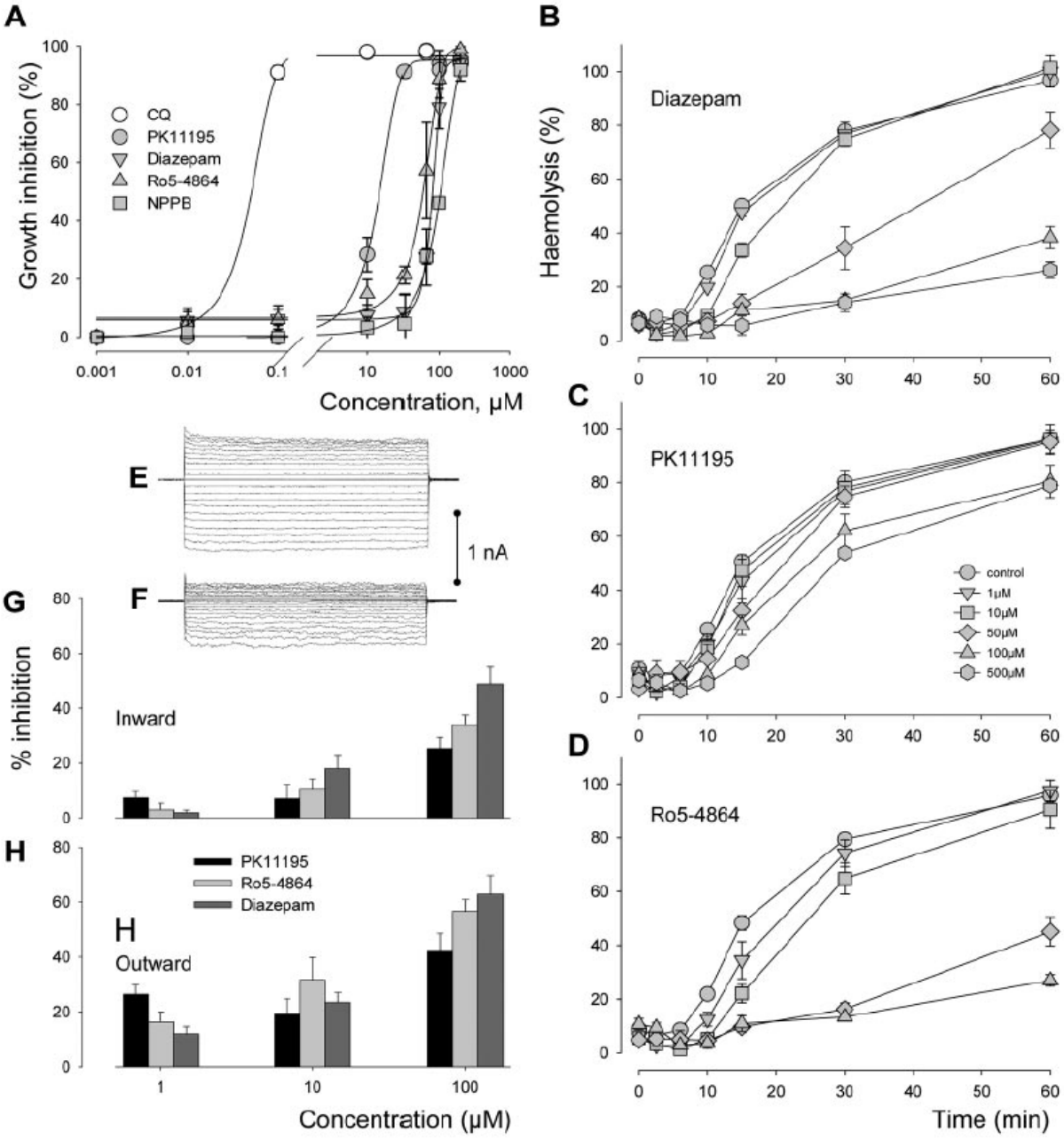
The above data give strong evidences for the existing of endogenous PBR/VDAC in the human RBCs.

In order to study the regulation mechanism of PBR/VDAC activity we used the cells infected by *P. falciparum*. Because NPPs displays many common characteristic features with the PBR/VDAC (Ginsburg and Kirk, 1998), and because the ligands of PBR are known as potent inhibitors of *P. falciparum* growth in infected RBCs *in vitro* (Dzierszinski et al, 2002; Bah et al., 2007), we next considered if the antiplasmodial effects of PBR ligands could correspond to an interaction of the parasite with this native PBR complex through up-regulation of PBR/VDAC activity.

We used growth assays, sorbitol haemolysis and electrophysiology to test the functionality of PBR ligands: PK11195, Ro5-4864 and diazepam. The results are shown in Fig. IV. 2. 3A - H.

Figure IV. 2. 3: **Antiplasmodial effects of PBR ligands.** (**A**) Cultures of the 3D7 strain synchronized at ring stage (in 96-well plates at 1.5% parasitemia and 2% hematocrit) were exposed to different concentrations of PBR ligands PK11195, Ro5-4864 and diazepam during 72 hours at 37°C. Their effects were compared with effects of antimalarial drug chloroquine (CQ) and anionic channels blocker NPPB. Inhibition of parasite growth was evaluated by comparison of the total parasitemia to the negative control where cultures were treated with the solvent (DMSO) only. The lines connecting the experimental points were drawn according to nonlinear regression analysis of the experimental results converted into percent values. Each count was made in triplicate and each point on the curves corresponds to the mean (± SEM) of 3 separate experiments. (**B, C, D**) Sorbitol lysis experiments. The effects of diazepam (**B**), PK11195 (**C**) and Ro5-4864 (**D**) added at t = 0 minutes of lysis experiments at concentrations below and above their IC_{50s} were tested by comparison to nontreated cells when haemolysis was at maximum at t = 60 minutes. Each count was made in triplicate and each point on the curves corresponds to the mean (± SEM) of 3 separate experiments. Note that Ro5-4864 was not tested at 500 μM because of solubility limitation. (**E - H**) The *whole-cell* membrane conductance of infected RBCs was calculated by measurement of the amplitude of currents obtained by evoking a series of test potentials (*V*T) from + 100 to -100 mV in 10 mV steps for 500 ms from a holding potential (*V*H) of 0 mV in the *whole-cell* configuration of the patch-clamp technique before and 15 minutes after addition of a ligand. The examples of panels **E** and **F** were obtained before and 15 minutes after addition of 100 μM PK11195 to the bathing solution containing (in mmol/l) 115 NaCl, 5 KCl, 10 $MgCl_2$, 5 $CaCl_2$, 10 Hepes, 10 glucose, 1% human serum, pH 7.4. The pipette solution contained 155 NMDG-Cl, 1 $MgCl_2$, 10 HEPES (pH 7.4). The calcium concentration was adjusted to pCa 3 in the bathing solution and to pCa 7 in the pipette solution. The impacts of the 3 different ligands were assessed by calculating the percentage reduction of inward conductance (**G**), (cord conductance between + 100 mV and 0 mV) and outward conductance (**H**), (cord conductance between 0 mV and - 100 mV). Bars are means ± SEM from 6 experiments.

Figure IV. 2. 3


A
Growth inhibition (%)
CQ
PK11195
Diazepam
Ro5-4864
NPPB
Concentration, μM
B
Haemolysis (%)
Diazepam
C
PK11195
control
1μM
10μM
50μM
100μM
500μM
D
Ro5-4864
Time (min)
E
F
1 nA
G
% inhibition
Inward
H
PK11195
Ro5-4864
Diazepam
Outward
Concentration (μM)

Growth inhibition curves are presented in Fig. IV. 2. 3A. The effects of ligands were compared with those of the reference antimalarial drug chloroquine (CQ) and inhibitor of anionic channels NPPB. Although the inhibitory effects of the ligands remained far below the CQ effect, the IC_{50} for PK11195 was ~ 10 times lower than for NPPB while Ro5-4864 and diazepam displayed similar IC_{50} as NPPB, in the range 100 μM.

If this interaction was via any inhibitory effect of channel activity, we could expect a reduction of cell swelling and haemolysis occurring when infected cells containing the new permeability pathways are exposed to isotonic solutions containing sorbitol. Fig. IV. 2. 3B - D shows that the percentage of lysis in cells exposed simultaneously to isotonic solutions containing sorbitol and to various concentrations of the 3 ligands is considerably decreased. The half-times of lysis ($t_{1/2}$) plotted against the ligand concentration shows that the membrane permeability was significantly decreased ($P < 0.01$) when concentration reached 50 μM, 1 μM, and 10 μM for PK11195, Ro5-4864, and diazepam, respectively.
Electrophysiologic tests confirmed that this loss of permeability occurred via the inhibition of a conductive pathway. In a representative experiment shown in Fig. IV. 2. 3E and F, an infected RBC displays a reduction in *whole-cell* membrane conductance at both positive and negative potentials over the first 15 minutes after addition of PK11195 at the concentration of 100 μM. The dose-dependence of currents inhibition for 3 ligands is presented in Fig. IV. 2. 3G and H, for inward (G) and outward (H). These figures confirm that the immediate reduction in the membrane permeability of *Plasmodium*-infected RBCs occurs to a large extent through inhibition of conductive pathways. This effect was more pronounced for diazepam and Ro5-4864 than for PK11195.

Taken together, this set of experiments indicates that the 3 principal ligands of PBR/VDAC inhibit of *in vitro* intraerythrocytic parasite growth, reduce RBC membrane transports and conductance.

IV. 2. 3. Discussion.

The main aim for the present part oh this work was to link together all the informations and observations made in studies on anionic channels. In this quest for clues of the molecular identity of the RBC native maxi-anion channel, for the first time we link together the presence of PBR proteins in the RBC membrane and the NPP up-regulated in infected RBC. In this study we confirm experimentally the hypothesis that dormant, endogenous PBR/VDAC become the so-called "new permeability pathways" in infected erythrocytes after up-regulation by *P. falciparum*. Since the present work demonstrates that ligands PK11195, Ro5-4864 and diazepam block parasite growth, induce rapid reductions in permeation and conductance, we suggest that the new permeability pathways are largely carried by PBR/VDAC. Moreover, the fact that NPPs properties resemble those of VDAC supports the conclusion that the two channels are at least partly one and the same protein.

Although the gating mechanism of this channel is still unclear, we suggest that activation could take place by voltage changes as is the case for VDAC. This situation could happen for example by transient activation of the Gardos channel upon membrane deformation as recently shown (Dyrda et al., 2010). Once activated, VDAC possesses multiple sub-conductance levels similar to those displayed by RBC's maxi-anion channels (in the range 350-450 pS for large openings; Shoshan-Barmatz and Gincel, 2003). On the other hand, it appears that NPPs are not parasite-derived channels as previously suggested (Desai et al., 2000), but it is very possible that some proteins exported by the parasite to the host RBC membrane contribute to the up-regulation of this native pathway. Next part of this PhD thesis focuses on regulation of such anionic conductance (see section IV. 3).

Concerning the role played by PBR/VDAC in RBC physiology, we know very little, but according to the identified properties of its three components (we need to keep in mind that VDAC forms a PBR complex with TSPO and ANT molecules), we can predict a major role in RBC membrane transports, volume, electrolyte, redox and acid–base regulations. For instance, we could suspect that this complex controls the fluxes of ions between the RBC cytosol and the extracellular milieu in

health and in diseases. It could also be involved in red cell homeostasis and membrane permeability (Shoshan-Barmatz, 2010).

IV. 2. 4. Article.

'Erythrocyte Peripheral Type Benzodiazepine Receptor/Voltage-Dependent Anion Channels are up-regulated by *Plasmodium falciparum*.'
Blood (2011)

Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are upregulated by *Plasmodium falciparum*

*Guillaume Bouyer,[1] *Anne Cueff,[1] Stéphane Egée,[1] Justyna Kmiecik,[1] Yelena Maksimova,[2] Edyta Glogowska,[1] Patrick G. Gallagher,[2] and Serge L. Y. Thomas[1]

[1]Centre National de la Recherche Scientifique, Université Pierre et Marie Curie Paris, Roscoff, France; and [2]Departments of Pediatrics and Genetics, Yale University School of Medicine, New Haven, CT

Plasmodium falciparum **relies on anion channels activated in the erythrocyte membrane to ensure the transport of nutrients and waste products necessary for its replication and survival after invasion. The molecular identity of these anion channels, termed "new permeability pathways" is unknown, but their currents correspond to up-regulation of endogenous channels displaying complex gating and kinetics similar to those of ligand-gated channels. This report demonstrates that a peripheral-type benzodiazepine receptor, including the voltage dependent anion channel, is present in the human erythrocyte membrane. This receptor mediates the maxi-anion currents previously described in the erythrocyte membrane. Ligands that block this peripheral-type benzodiazepine receptor reduce membrane transport and conductance in *P falciparum*-infected erythrocytes. These ligands also inhibit in vitro intraerythrocytic growth of *P falciparum*. These data support the hypothesis that dormant peripheral-type benzodiazepine receptors become the "new permeability pathways" in infected erythrocytes after up-regulation by *P falciparum*. These channels are obvious targets for selective inhibition in anti-malarial therapies, as well as potential routes for drug delivery in pharmacologic applications. (*Blood*. 2011; 118(8):2305-2312)**

Introduction

The most severe form of malaria in humans is caused by parasite *Plasmodium falciparum*, infecting 225 million people and causing 781 000 deaths in 2009 (World Health Organization, 2010). Erythrocyte invasion by *P falciparum* provides the parasite access to a plentiful source of nutrients in a locale that is largely shielded from host immune defenses. After invasion, the invading parasite uses a variety of strategies to adapt to the intraerythrocytic environment. To ensure the transport of nutrients and waste products necessary for its replication and survival, *P falciparum* relies on broad specificity anion channels activated in the erythrocyte membrane after invasion.[1] Initially, this transport was attributed to "new" permeability pathways (NPPs)[2] exported by the parasite to the host membrane.[3] However, later studies revealed that the current is because of up-regulation of endogenous channels[4] and that the diversity of anion channel activities recorded in these studies correspond to different kinetic modalities of a unique type of maxi-anion channel.[5] This channel displays complex gating and kinetics similar to those of ligand-gated channels.[5]

Anions are transported through the human erythrocyte membrane by a 2-component system: a large electroneutral exchanger mediated by band 3 and a 4 orders of magnitude smaller electrogenic component estimated at approximately 10 $\mu S/cm^2$ corresponding presumably to a small number of channels.[6] Remarkably, the molecular identification and characterization of this conductive pathway has not yet been achieved. Neither genomic nor proteomic studies have provided meaningful clues to the composition of this pathway.[7] Considering the small amount of protein a few hundred channels represent, it is most likely that they remain below the detection limit in current, standard proteomic protocols. However, there is a growing body of information on the electrophysiologic characteristics of this pathway.[5]

Our previous work using the cell-attached configuration of the patch-clamp technique to this issue demonstrated that a unique type of maxi-anion channel with multiple conductance levels mediates band 3-independent anion conductance across the erythrocyte membrane.[5] These channels are dormant under normal physiologic conditions, yet on activation confer a far higher anion conductance to the erythrocyte membrane than the ground leak mediated by band 3. We hypothesized that this anion conductance is mediated by a voltage dependent anion channel (VDAC). VDACs, originally characterized as mitochondrial porins,[8] can be expressed in plasma membranes alone,[9] or as a component of the peripheral-type benzodiazepine receptor (PBR) complex.[10] The PBR complex consists of at least 3 components: a 32-kDa VDAC, a 18-kDa translocator protein (TSPO, also called isoquinoline-binding protein IBP), and a 30-kDa adenine nucleotide transporter (ANT).[10] The PBR is characterized by a primary distribution in tissues outside the central nervous system and by nanomolar affinity for the ligands PK 11 195 > Ro5-4864 > diazepam. The TSPO component is considered to be primarily responsible for binding to PK 11 195, while Ro5 4864 and other benzodiazepines may bind to all components of the PBR complex.[11] Based on the entropy and enthalpy driven nature of ligand–receptor interactions, PK11195 was classified as an antagonist, whereas Ro5-4864 was classified as an agonist.[12]

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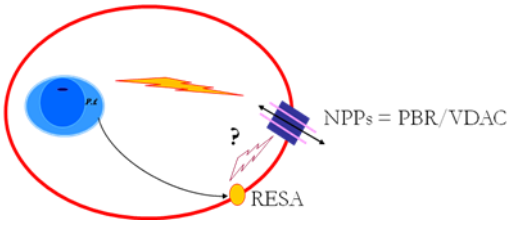
*G.B. and A.C. contributed equally to this article.

IV. 3.



The activation of anionic channels by *Plasmodium falciparum* and possible involvement of RESA1 protein in this process.

Third objective:

IV. 3. The activation of anionic channels by *Plasmodium falciparum* and possible involvement of RESA1 protein in this process.

IV. 3. 1. Introduction.

Upon entering the red blood cell, *Plasmodium falciparum* escapes the host immune system, and enters suddenly in a new physical and chemical environment, unfavourable for malaria parasite. The intraerythrocytic parasite growth and survival depends strongly on its ability to remodel host cell in order to create an adequate environment. These changes induced at the red blood cell membrane and sub-membrane skeleton structure and morphology following invasion by *P. falciparum* have several functions for the parasite. The most necessary for its replication and life inside erythrocyte is the activation of anionic channels, poorly selective, to ensure the transport of nutrients and metabolic waste disposal (Kirk, 2001). These channels are activated from 16 to 20 hours post invasion (hpi). As previously-mentioned, this transport was initially attributed to new permeability pathways NPPs, thought to be anionic channels exported by the parasite to the host membrane (Ginsburg et al., 1983; Ginsburg and Kirk 1998; Desai et al., 2000; Kirk 2001). Since then, numerous studies, including those presented in the previous chapters, showed that NPPs are actually endogenous channels of red cell membrane (Bouyer et al., 2011), but the gating mechanism and triggering factors of these channels still remain unknown. There is numerous of possibilities for their activation: i) a change in membrane potential induced by the activity of Gardos channel following a deformation of erythrocyte membrane, ii) a change in the oxydo-reduction state of the internal medium, iii) interaction with haemoglobin, band 3 or with any other component of the cytoskeleton, and it is likely that several could be synergistic.

In addition, an increasing number of exported parasite proteins to the host erythrocyte have been identified that might be essential for the parasite control of channel activity. It has been reported that these proteins, at least altered adhesive and mechanical properties of host RBCs (Nash et al., 1989; Paulitschke and Nash,

1993; Glenister et al., 2002; Marti et al., 2004; Rug et al., 2006; Maier et al., 2008; Maier et al., 2009; Sanyal et al., 2012). Experimental assays and bio-informatic analysis indicate that over 400 proteins are produced and exported by the parasite to the erythrocyte cytoplasm and a number of these proteins interact with membrane skeleton (Hiller et al., 2003; Marti et al., 2004; Sargeant et al., 2006).

Taken together, various red cell-parasite protein interactions are responsible for striking structural and morphological changes in erythrocyte membrane. Furthermore, one of the direct consequence of erythrocyte remodelling after *P. falciparum* infection is up-regulation of the endogenous dormant PBR/VDAC anionic channel allowing malaria parasite growth, replication and survival inside erythrocyte.

Seeking clues for activators/modulators of NPPs in this thesis we made the hypothesis that export of parasite proteins to the erythrocyte membrane during the first twelve hours after the invasion could be related to activation of endogenous anionic channels. We have chosen the Ring infected Erythrocyte Surface Antigen (RESA) family proteins, since these proteins associate with the red cell membrane cytoskeleton and play crucial role in erythrocyte remodelling already at young, ring stages of parasite. Most studies aiming at identifying parasite virulence factors have focussed on mature parasite sequestered in deep organ vasculature, their cytoadhesion, immunological disorders and host genetic susceptibility. However, the most important for malaria pathogenesis is probably a dramatic expansion of the parasitic load, leading to excessive local parasite burden. The parasite, which reproduces by a 10^{12} factor in a few weeks, has evolved pathogenicity and virulence factors that determine infection outcome. Variations in virulence may result from an increased parasite capacity to uptake nutrients and/or a more efficient efflux of waste products, high multiplication rate, optimal remodelling of the host infected erythrocytes and resistance to host defences such as fever and adaptative immune reply. In this regard, young, ring stages of malaria parasite have received little attention, despite being a critical step in parasite installation into its host cell.

Figure IV. 3. 1

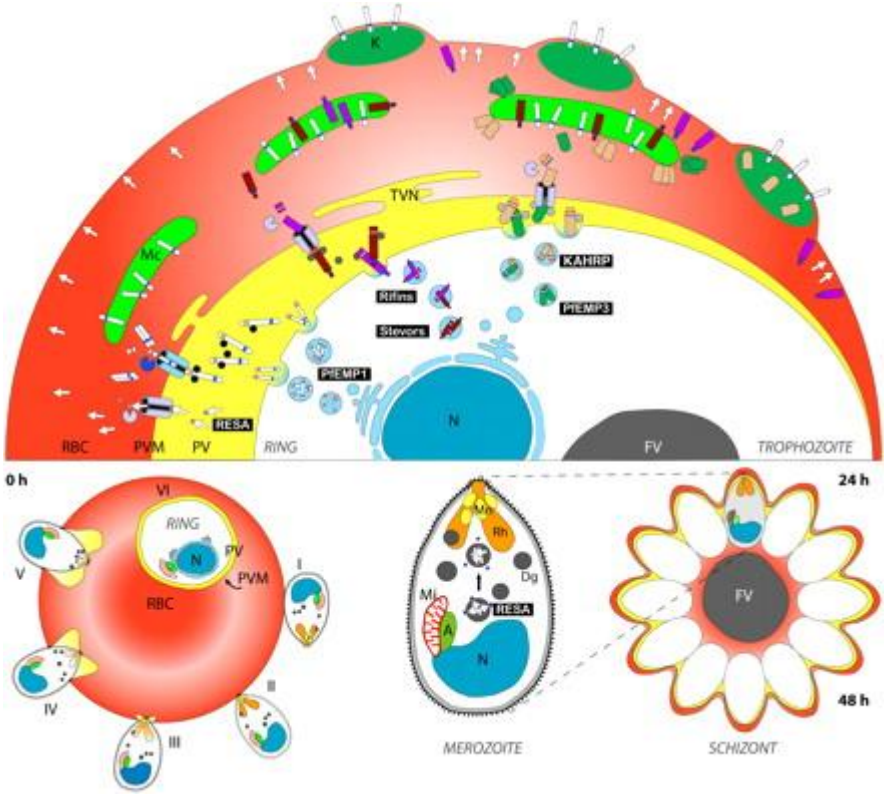


Figure IV. 3. 1: **Protein export during the asexual life cycle of malaria parasite.** *Plasmodium* prepares for the invasion and remodelling process of a new host cell in late asexual stages (schizont stage) by transporting crucial molecules to the three prototypic apicomplexan organelles (the microneme [Mn], the rhoptry [Rh], and the dense granule [Dg]) located close to the apical end of the parasite. Microneme proteins are mainly involved in initiating invasion (stages I–III), rhoptry and dense granule proteins are implicated in establishment of the parasite in the newly invaded host cell (stages III–VI). RESA (arrows) is targeted to the dense granules in late schizogony, released into the parasitophorous vacuole (PV) after invasion, and subsequently exported into the red blood cell (RBC), where it associates with the red cell cytoskeleton to stabilize the newly invaded RBC membrane. The presence of a PEXEL motif in RESA implies the establishment of the corresponding translocation machinery very early after formation of the parasitophorous vacuole membrane (PVM). Other exported proteins are shown: PfEMP1, PfEMP3, KAHRP, Rifin and Stevor. Mi, mitochondrion; A, apicoplast; MC, Maurer's cleft; K, knob; TVN, tubulovesicular network; FV, food vacuole; N, nucleus (Marti et. al., 2005).

Figure IV. 3. 2

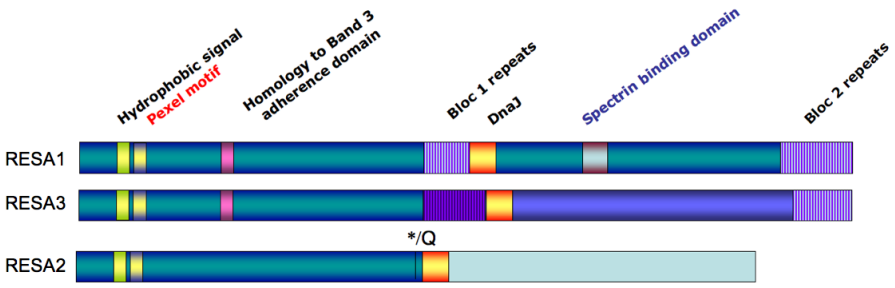


Figure IV. 3. 2: **Schematic map of the RESA family proteins:** resa1 (PFA0110w), resa2 (PF11_0512), resa3 PF11_0509). * : internal stop codon (from S. Bonnefoy).

IV. 3. 1. 1. Ring infected Erythrocyte Surface Antigen (RESA).

One such protein, that modifies structural components and the machinery of its host cell in order to create an adequate environment and to overcome host responses is Ring infected Erythrocyte Surface Antigen (RESA), also called Pf155 (according to its molecular weigh 155 kDa). This protein is synthesized in mature-stage parasites (schizonts) and stored in forms known as dense granules (Culvenor et al., 1991). After invasion, it is released into the host erythrocyte cytosol, phosphorylated (Foley et al., 1991) and transported to the cell membrane, where it binds to repeat 16 of β-spectrin (βR16) and becomes associated with the membrane of newly invaded cell (Pei et al., 2007). This RESA-spectrin connection has been shown to be very important in further role played by RESA in infected erythrocyte (see below). The model of protein export including RESA targeting is shown in Fig. IV. 3. 1. RESA remains detectable in the infected erythrocytes until about 18 to 24 hpi (the known peak of activity of the NPPs).

IV. 3. 1. 1. 1. Structure.

RESA belongs to a small multigene family of three members that share extensive homology. The structure of these proteins is shown in Fig. IV. 3. 2. The rest of this work will focus on the most known, up to-date, RESA1. This protein has a well conserved N-terminal domain, with a typical Pexel motif following a hydrophobic sequence, a signature of export signal outside the parasitophorous vacuole (Marti et al., 2004; Sargeant et al., 2006). It contains a band 3-related adherence domain (Iqbal et al., 1995) and a two immunogenic blocks of repetitive sequence, called the 5' and 3' repeats (Favaloro et al., 1986). Between the two repeat regions is a segment of 70 residues with similarity to the J domain of *E. coli* and human DnaJ chaperon proteins, suggesting that RESA1 may have some chaperone-like properties. Additionally, RESA1 contains a domain shown to interact with the β-16 repeat of spectrin (Pei et al., 2007). Figure IV. 3. 3 presents localisation of this RESA1 protein in red cell membrane.

Figure IV. 3. 3

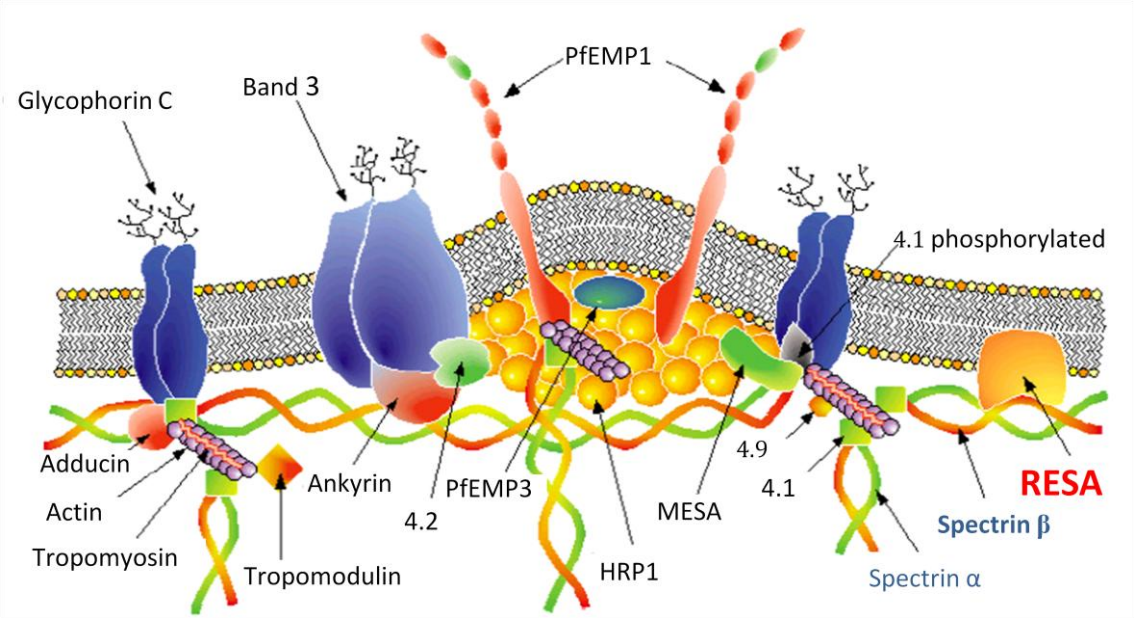


Figure IV. 3. 3: **Localisation of RESA protein in red blood cell membrane cytoskeleton.** RESA protein binds to repeat 16 of β-spectrin and becomes associated with the erythrocyte membrane cytoskeleton. PfEMP1, *P.falciparum* erythrocyte membrane protein 1; PfEMP3, *P.falciparum* erythrocyte membrane protein 3; MESA, mature parasite-infected erythrocyte surface antigen; HRP1, histidine-rich protein 1 (modified from R. Durand, http://www.pasteur.mg/Atelier-Palu/r0308.html).

Figure IV. 3. 4

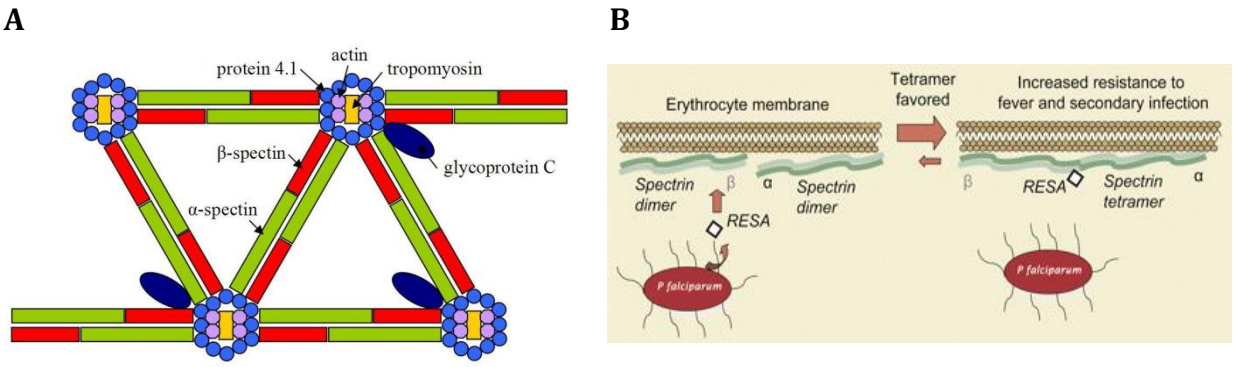


Figure IV. 3. 4: **Spectrin and its interaction with RESA.** (**A**) A schematic diagram of spectrin and other cytoskeletal molecules. Dimeric spectrin is formed by the lateral association of α and β monomers to form a dimer. Dimers then associate in a head-to-head formation to produce the tetramer. End-to-end association of these tetramers with short actin filaments produces the hexagonal complexes observed. This complex is associated with other cytoskeletal molecules, protein 4.1, actin, glycoprotein C. (**B**) Stabilization of spectrin tetramers under the influence of RESA binding to β-spectrin chain. Spectrin binds to a 108-amino acid fragment (residues 663-770) of RESA, and this RESA fragment binds to repeat 16 of the β-chain, close to the labile dimer-dimer self-association site. This connection stabilizes the spectrin tetramer against dissociation into its constituent dimers (A: adapted from http://en.wikipedia.org/wiki/Spectrin; B: from Johnson and Discher, 2007).

IV. 3. 1. 1. 2. Link with spectrin.

The RBC membrane owes its remarkable deformability and durability to the membrane skeleton, composed of a regular hexagonal array of proteins that form a two-dimensional meshwork at the cytoplasmic surface of the cell (Luna and Hitt, 1992; Mohandas and Chasis, 1993). Deformability, a very important property from the physiological point of view, is caused by the interaction of structural flexibility of cytoskeleton's proteins, such as spectrin, protein 4.1, tropomyosin, adducin, dematin (protein 4.9) and tropomodulin (Fig. IV. 3. 3) (Shen et al., 1986; Derick et al., 1992). Furthermore, the membrane skeleton is connected to the overlying plasma membrane via a series of interactions with different membrane proteins including ankyrin, band 3, protein 4.2 (also known as pallidin), glycophorin C (Fig. IV. 3. 3).
As mentioned, after malaria invasion different parasite's proteins are exported to the host red blood cell membrane. One such protein, RESA1 interacts with spectrin and this connection plays very important role for infected erythrocyte.
Spectrin is formed as a long, flexible rod with a contour length of 200 nm, and exists in the cell predominantly as an $\alpha_2\beta_2$ tetramer (Fig. IV. 3. 4A). The protein is characterized by repeated units (21⅓ in the α-spectrin chain, and 16⅔ in the β-chain) containing 106 residues each, folded into a left-handed, antiparallel triple helical coiled-coil structure (Speicher and Marchesi, 1984; Yan et al., 1993; Grum et al., 1999). The tetramers can undergo transient dissociation into their constituent dimers, particularly when the cells undergo deformation under shear (An et al., 2002). Spectrin has been identified as the primary attachment site for RESA in the infected erythrocytes (Foley et al., 1991). The protein (residues 663-770) binds to repeat 16 of β-spectrin and this interaction stabilizes the spectrin tetramer (Pei et al., 2007) relative to the dimer, both in solution and in the erythrocyte, as shown in Fig. IV. 3. 4B. In 2007, Pei and co-workers (Pei et al., 2007) have demonstrated that this RESA-induced stabilization of the tetramer played a crucial role in mechanical properties of red cell membrane, particularly in reduction of susceptibility to heat-induced vesiculation and also in increased resistance to further merozoite invasion (Dluzewski et al., 1983). This mechanical membrane stability is very strong, even despite the fact, that red cell membranes can be destabilized through altered spectrin interactions in a variety of ways.

At this point another note should be underlined for the reader. Well established connection between dimer-tetramer balance and mechanical stability, especially resistance of the infected erythrocyte membrane to shear stress is very important and interesting regarding this thesis. It is noteworthy, that changed mechanical properties of red cell membrane infected by malaria parasite could be the link to different channel activity induced by membrane deformation (Dyrda et al., 2010) and observed in different RESA1 strains. This could confirm the role played by RESA in membrane stabilization. This problem will be addressed in the later part of this work.

IV. 3. 1. 1. 3. Role in malaria-infected erythrocyte.

Ring stages of malaria parasite are exposed to fever, a hallmark of malaria disease, which is triggered by toxic materials released upon rupture of the infected erythrocyte. Febrile temperatures are detrimental to parasite development of late (Kwiatkowski et al., 1989) but also of young stages (Silva et al., 2005; Oakley et al., 2007). It has been shown that RESA1 contributes to parasite fitness at febrile temperatures. The protein, which is discharged during invasion of the erythrocyte and interacts with erythrocyte spectrin, is present throughout the ring stage development. In 2005, Silva and co-workers (Silva et al., 2005) reported that RESA1 allows ring stage parasites to resist red blood cell membrane fragilisation caused by exposure at febrile temperatures. RESA1 also contributes to increasing membrane rigidity of ring-infected erythrocytes (Mills et al., 2007). This is consistent with a role for RESA1 in stabilization of the erythrocyte cytoskeleton (Pei et al., 2007), a critical interface between intracellular parasites and the external environment, particularly at febrile temperatures. RESA1 has also been shown to be targeted by the adaptative immune response in populations living in endemic areas. Antibodies reacting with RESA1 inhibited erythrocyte invasion (Wählin et al., 1984; Wählin et al., 1992; Siddique et al., 1998; Siddique et al., 1999) and moreover were associated with protection against clinical malaria (Carlsson et al., 1990; Astagneau et al., 1994; Astagneau et al., 1995; Aucan et al., 2000). RESA1 is expossed to host immune system during invasion.

Figure IV. 3. 5

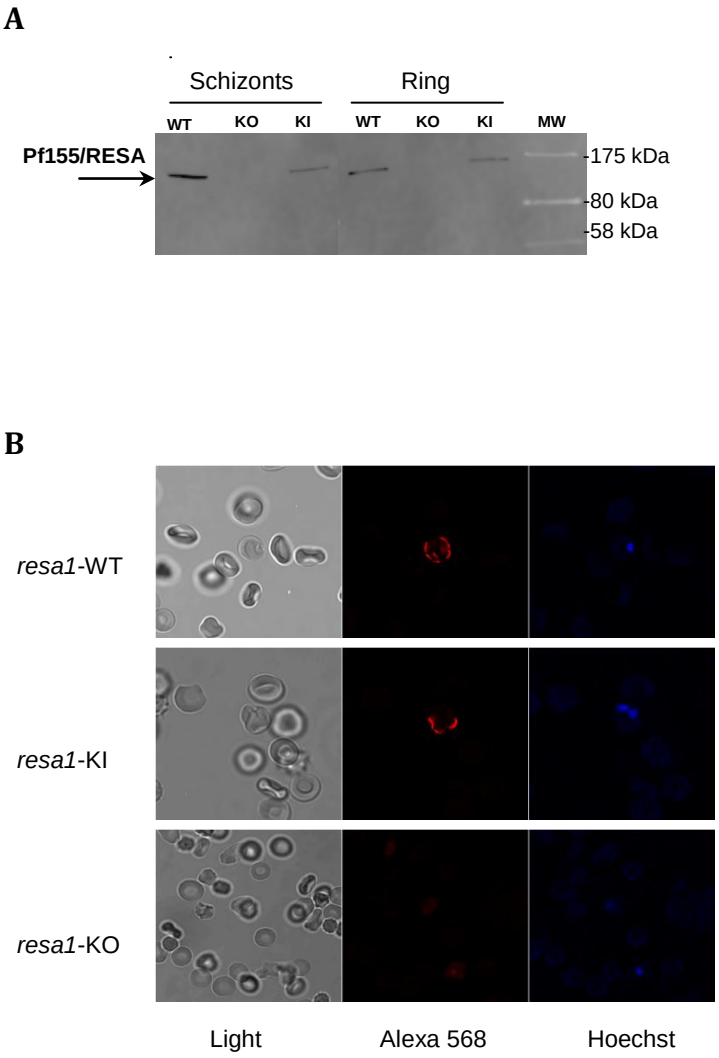


Figure IV. 3. 5: **RESA expression in *P. falciparum* infected human red blood cells.** The RESA protein is expressed in the parental FUP/CB C32 (*resa1* wild-type, WT) and A_3F_8ocGCV revertant (*resa1*-KI), both in the ring-stage and late schizont, but not in the A_3F_8 mutant clone (*resa1*-KO). (**A**) Proteins from extracts of ring-stage or late schizont parasites were separated by 7.5% SDS-PAGE and immunoblotted using mAb 1F1. Bound antibodies were detected using an anti-mouse HRP (horseradish peroxidase) conjugate. The 155 kDa RESA protein is indicated with an arrow. (**B**) Erythrocyte membrane immunofluorescence of *resa1*-WT, *resa1*-KI and *resa1*-KO using mAb 1F1. Parasite nucleus was indicated using Hoechst staining.

IV. 3. 2. Results.

As above-mentioned, we seek here for activators and regulators of endogenous PBR/VDAC up-regulation after *P. falciparum* infection, and test the link between physiological role played by RESA1 in red blood cell membrane remodelling with its possible involvement in activation of anionic channels. *Plasmodium falciparum* expressing RESA1 protein (WT), recombinant mutant KO and KI revertant strain with *resa1* gene reversion (see *Materials and Methods*) were used in this part of present work.

In the first step, we performed biochemical experiments: western blotting and immunofluorescence in order to check, if RESA1 is exported/or not in appropriate strains. It has been demonstrated previously that this protein is associated with the micronems and rhoptries of merozoites (Brown et al., 1985; Aikawa 1986; Uni et al., 1987), and with improved analysis it has been localized precisely to dense granules in the apical end of the merozoites (Aikawa et al., 1990). After invasion it becomes associated with the membrane of newly infected erythrocytes (Perlmann et al., 1984; Aikawa 1986; Uni et al., 1987) by interaction with the cytoskeleton (Brown et al., 1985; Foley et al., 1990). Taken together, RESA1 can be visualized either in early ring-stage in the membrane of infected erythrocyte or in the late schizonts in the parasite. For that purpose, expression of RESA1 protein in WT, KI and KO strains were tested in these two different stages on western blot. High parasitemia of ring-infected erythrocytes was obtained by sorbitol synchronization, and enrichment of late-stage-schizonts was accomplished by Percoll/sorbitol gradient (see *Materials and Methods* for details of cells preparation).

Immunoblotting with RESA reactive mouse antibody (mAB 1F1) confirmed that this protein is expressed at the expected size (155 kDa) in wild-type (WT) and revertant (KI), but not in mutant (KO), both in the ring stage and schizonts (Fig. IV. 3. 5A).

Next, we applied immunofluorescence by using mAb 1F1 to localize RESA1 in infected erythrocyte and check if protein is correctly addressed to the host membrane. In this set of experiment we used again cultures at high parasitemia (10 – 20%) but mostly at ring-stage. The association of RESA1 with erythrocyte mem-

Figure IV. 3. 6

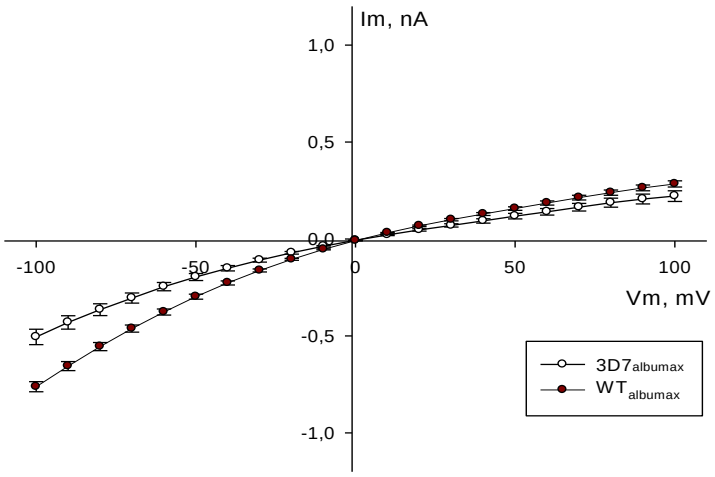


Figure IV. 3. 6: **I/V curves from *whole-cell* configuration of the patch-clamp technique obtained for 3D7 and RESA1-WT.**

brane was demonstrated in parental and revertant strains. No signal onto the red cell surface was detected in mutant, which confirmed the loss of protein expression in this strain (Fig. IV. 3. 5B, middle panel). Simultaneous Hoechst staining labelling was performed for identification of nucleus and confirmation that chosen cells were infected (Fig. IV. 3. 5B, right panel).

Once the level of RESA1 expression was assessed we used *whole-cell* configuration of patch-clamp electrophysiological technique to compare membrane conductances of the different strains, knowing that membrane currents in *Plasmodium* infected cells are thought to be the correlate of new permeability pathways (NPPs) as described by sorbitol haemolysis or fluxes experiments. Control tests were performed in physiological conditions using RnD in the bath and pipette. Ten minutes after seal formation *whole-cell* currents were recorded using ramps of voltage between +100 to -100 mV in 10 mV steps for 500 ms from a holding potential of 0 mV. Preliminary set of experiments were performed to compare RESA1-WT strain to internal laboratory standard strain 3D7. Indeed, the majority of data obtained in the laboratory during the last 10 years were obtained using this strain or its parental line NF54. Indeed, it is known that slight differences between *P. falciparum* strains can be observed in patch-clamp experiments at the *whole-cell* level or at the *single-channel* recordings level (Alkhalil et al., 2004). Since RESA1 strain is issued from the Uganda Palo Alto strain of *Plasmodium falciparum* (FUP) this point had to be checked before going further. In our experimental conditions 3D7 strain shows a slighter decreased inward current compared to FUP (RESA1-WT) strain ($P<0.05$, $n=38$ and $n=145$, respectively). Figure IV. 3. 6 presents I/V curves obtained for 3D7 and RESA1-WT. Nevertheless, no difference was observed for outward current. However, if slight difference between strains tested so far were already noticed, one explanation could be the time of cultivation (see discussion).

Since 3D7 was cultivated using Albumax medium (called after in short: Albumax) for replacement of human serum in the medium for cultivation, FUP strains (RESA1 WT, KO and KI) were acclimated to these conditions. This was only possible by using a step by step progressive removal of serum and replacement with Al-

Figure IV. 3. 7

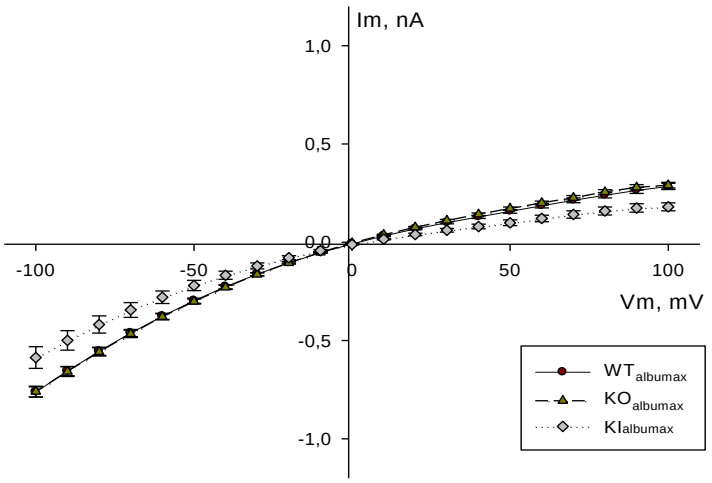


Figure IV. 3. 7: **I/V curves from *whole-cell* configuration of the patch-clamp technique obtained for RESA1-WT, RESA1-KO and RESA1-KI cultivated in Albumax medium.** No significant differences were obtained between strains when cultivated in Albumax.

Figure IV. 3. 8

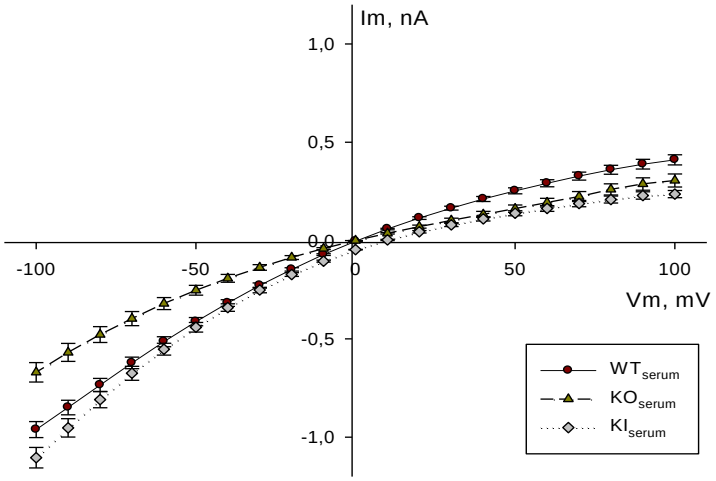


Figure IV. 3. 8: **I/V curves from *whole-cell* configuration of the patch-clamp technique obtained for RESA1-WT, RESA1-KO and RESA1-KI cultivated in serum medium.** RESA1-WT and RESA1-KO infected RBCs conductances display significant difference when cultivated in serum (see Fig. IV. 3. 10).

bumax. However, in this set of experiments (global conductances) we used RESA1 strains cultivated either in Albumax or serum (see Table 2, *Materials and Methods*). Interestingly, once acclimated to Albumax cultivation conditions, no significant difference was observed between RESA1-WT and RESA1-KO. However, currents measured in RESA1-KI were significantly smaller than in RESA1-WT. Figure IV. 3. 7 shows corresponding I/V curves. Differences between RESA1-WT and RESA1-KI could come from manipulation of strain at the molecular level, occurring during the process of revertant preparation.

When cultivated in serum, picture was radically different. Indeed, in these conditions, highly significant difference in membrane currents was observed between RESA1-WT and RESA1-KO strains. In KO strain both inward and outward currents are ablated leading to 30% and 50% decrease, for inward and outward currents respectively. Figure IV. 3. 8 shows I/V curves corresponding to RESA1 strains when cultivated in serum medium. The highest value of conductances correspond to the parental RESA1 (mean: 4.1±0.3 nS outward and 9.9±0.4 nS inward) and the lowest to the mutant (mean: 2.1±0.2 nS outward and 6.9±0.4 nS inward). More surprisingly, membrane currents recorded with both RESA1-WT and RESA1-KI strains were larger than those recorded when cultivation was made with Albumax only. This last result tends to indicate that culture conditions are a critical point for generation of fully activated NPPs notably in the perspective that *P. falciparum* proteins can participate to generation or modulation of such phenomenon. Furthermore, it is noteworthy that serum components are essential for correct addressing of *P. falciparum* proteins (such as PfEMP1) to the host membrane (Frankland et al., 2006).

To confirm this latest hypothesis, membrane currents were then recorded along the process of acclimatization of RESA1 cultures from 100% of Albumax to 100% serum (Fig. IV. 3. 9). Then again, in RESA1-WT there is a strict correlation between membrane currents and culture conditions. Indeed, membrane currents measured in 100% serum are larger than those recorded when cells were cultivated in 50% serum + 50% Albumax, which are themselves larger than those recorded on cells cultivated only with Albumax. However, whatever the culture conditions, in

Figure IV. 3. 9

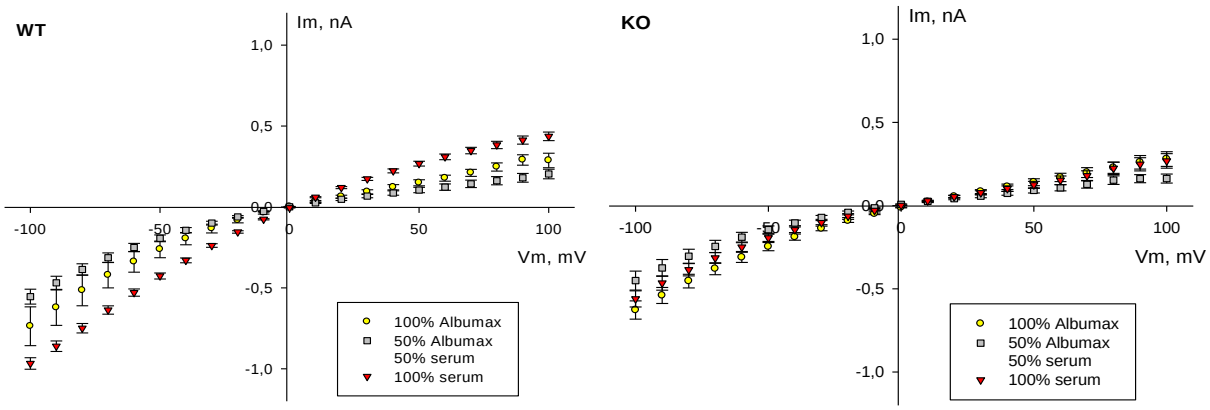


Figure IV. 3. 9: **Currents obtained in *whole-cell* configuration of the patch-clamp technique for RESA1-WT and RESA1-KO strains according to cultivation medium.** Currents were recorded either from strains cultivated in 100% Albumax (yellow circle), or 50% Albumax + 50% serum (gray square), or 100% serum (red triangle down). Adaptation process of RESA1-WT and RESA1-KO to different media demonstrates that cultivation conditions play very important role for RESA1 protein and indicates that a component of human serum is necessary for RESA1 activity.

RESA1-KO strain recorded currents were similar, suggesting that: i) at least correct exportation of *P. falciparum* proteins are needed for full activation of NPPs, and ii) that RESA protein, even if it is exported at ring stages into the host membrane, participate somehow to the modulation of membrane currents through the NPPs. Western blotting and immunofluorescence from RESA1 strains (especially wild type) cultivated only in Albumax are necessary to check if export of RESA1 protein and/or its presence in the host erythrocyte membrane is unsettled in the absence of serum factors in the cultivation medium.
Summary of inward/outward conductances obtained in this set of experiments for different strains according to cultivation conditions is shown in Fig. IV. 3. 10. Table IV. 3. 1 presents corresponding statistical comparison between all strains (ANNOVA, two way analysis of variance).

Former experimental data have shown that NPPs are pore channels with poor anionic selectivity, and we have demonstrated that PBR/VDAC is a good candidate. We performed second set of patch-clamp experiments in which the reported blockers of the NPPs (see Table 7, *Materials and Methods*): 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4'-diiothiocyano-stilbene-2,2'-disulfonic acid (DIDS), furosemide and glybenclamide (Kirk et al., 1994; Ginsburg and Kirk, 1998) were used. The aim was to examine the sensitivity of RESA1 infected erythrocytes for these blockers. Indeed, if these 4 inhibitors are known anionic transporters inhibitors, their exact targeting is not fully described at the molecular level. Then, if RESA1 protein participate to the modulation of the NPPs, pharmacological profiles may help to decipher and to understand what type of relationships exist between NPPs and RESA1 protein. Trophozoites-infected RESA1 erythrocytes (WT, KO, KI) were taken from cultures either in Albumax or serum medium. Cells, after three washing, were placed into physiological RnD solution, with the same RnD in the pipette, and *whole-cell* currents were recorded once ten minutes after seal formation (control), and secondly 10 minutes after addition of different concentration of drugs. Inhibitors were added to the bathing solutions to obtain final concentrations 1, 10, 100 µM for NPPB, 1, 10, 100, 1000 µM for furosemide, 100 µM for DIDS

Figure IV. 3. 10: **Summary of conductances obtained in control tests in *whole-cell* configuration of the patch-clamp from different strains of *P. falciparum* infected cells according to cultivation medium.** (**A**) Comparison of inward/outward conductances (G, nS) of *P. falciparum* infected human red blood cells (3D7, RESA1: WT, KO, KI) depending on cultivation media. Data are mean ± SEM of: 3D7, n=38; WTa, n=145; KOa, n=129; KIa, n=29; WTs, n=52; KOs, n=24; KIs, n=36; n, number of individual cells. Index: 'a' Albumax medium; 's' serum enriched medium, 'n.s.' no significant difference, *P<0.05.

Table IV. 3. 1: **Statistical analysis (ANNOVA, two way analysis of variance) of currents obtained in control tests in *whole-cell* configuration of the patch-clamp from different strains of *P. falciparum* infected cells according to cultivation medium**. Significant difference (*P<0.05) were obtained between RESA1 strains when cultivated in serum. Index: 'n.s.' no significant difference.

Figure IV. 3. 10

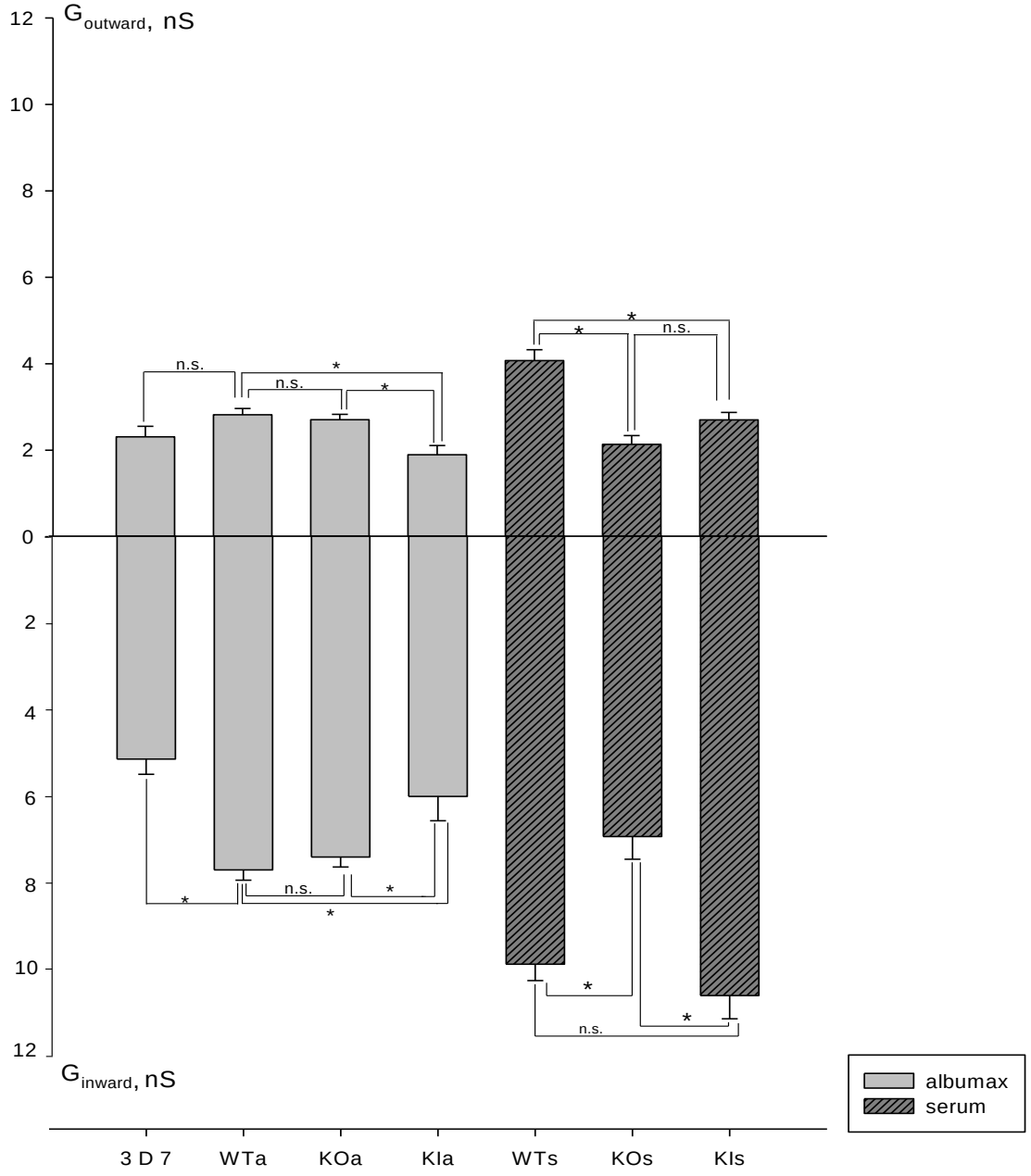


Table IV. 3. 1

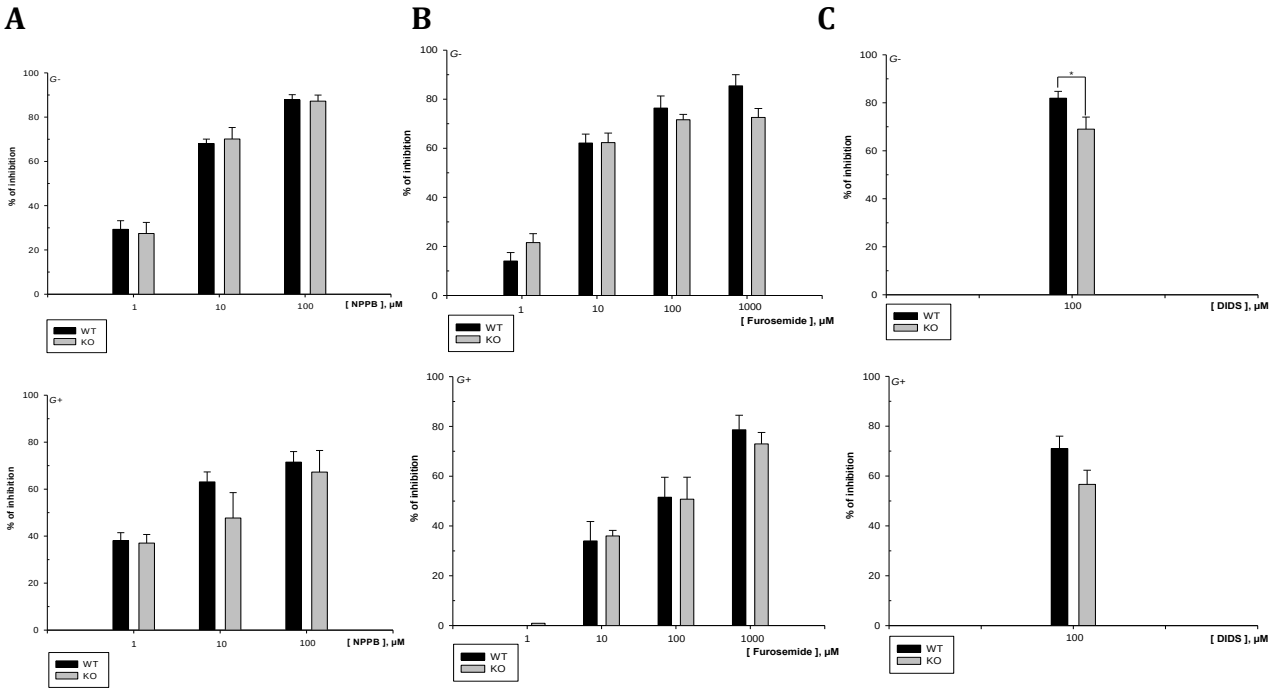
	3D7 vs WTa	3D7 vs KOa	3D7 vs KIa	3D7 vs WTs	3D7 vs KOs	3D7 vs KIs	WTa vs KOa	WTa vs KIa	WTa vs WTs	WTa vs KOs	WTa vs KIs	KOa vs KIa	KOa vs KOs	KOa vs KIs	WTs vs KOs	WTs vs KIs	WTs vs KOa	WTs vs KIa	KIa vs KIs	KIa vs KOs	KOs vs KIs
Inward	*	*	n.s.	*	n.s.	*	n.s.	*	*	*	*	*	*	*	*	n.s.	*	*	*	n.s.	*
Outward	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	*	n.s.	n.s.	*	*	*	*	*	n.s.	n.s.

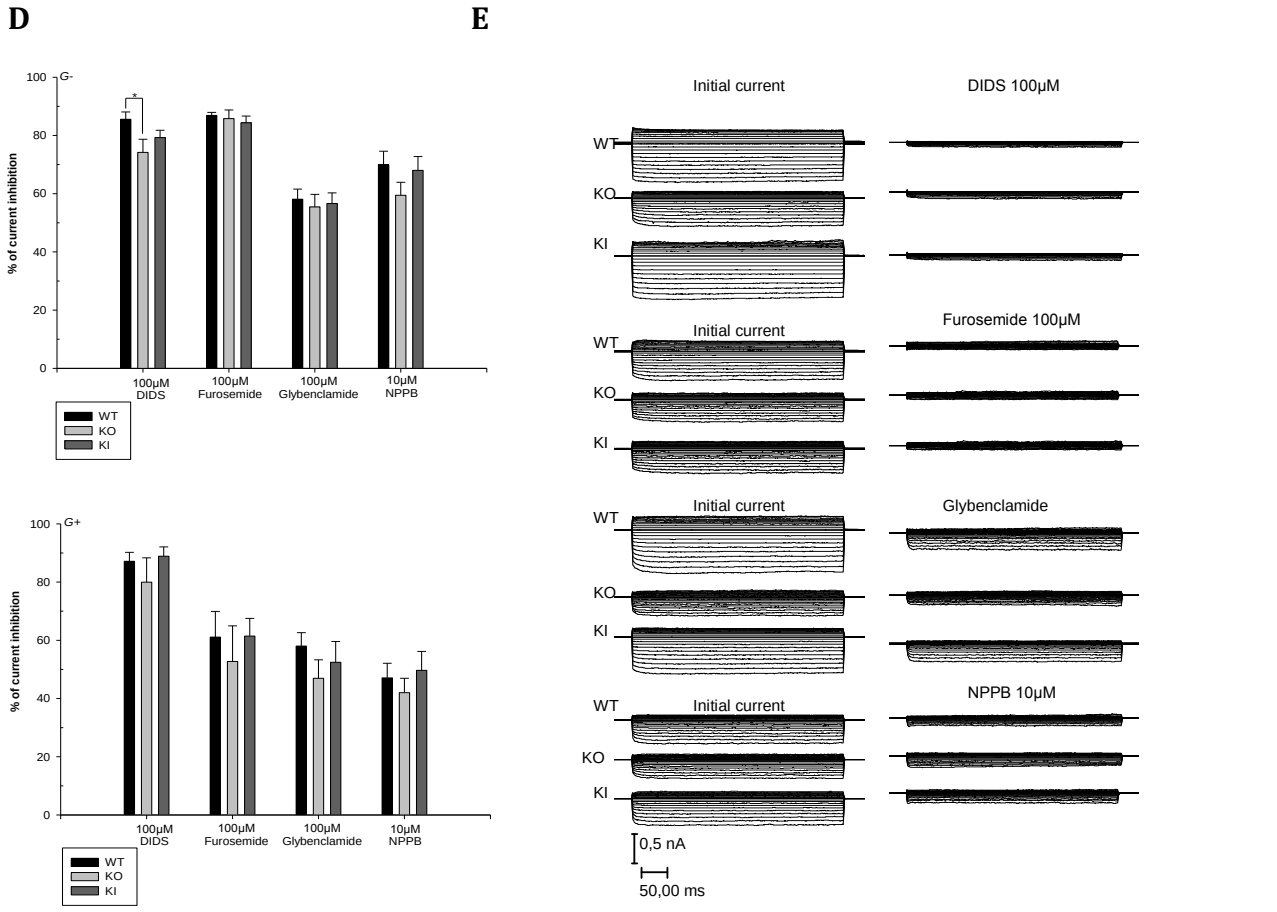
and 100 μM for glybenclamide. Figure IV. 3. 11A-E shows the results obtained in these series of experiments. When cultivated with Albumax no significant difference in IC_{50} was observed between WT and KO for NPPB and furosemide (Fig. IV. 3. 11A, B). However, KO strain presents less sensitivity to DIDS compared to WT (Fig. IV. 3. 11C). Nevertheless, if obviously both inward and outward currents are differentially affected by DIDS, only DIDS effects on inward currents are significant. The IC_{50s} found in this study for the 4 inhibitors tested are in good agreement with those already reported in the literature on other strains, both for WT and KO, albeit slight differences (Huber et al., 2002). The outward current at +100 mV was inhibited by: i) NPPB with IC_{50} in the range of 6 μM and 4 μM for RESA1-WT and RESA1-KO, respectively, ii) furosemide (8 μM), DIDS (< 100 μM) for both strains. In contrast, the IC_{50s} of the inward current at -100 mV were: 10 μM and 4 μM (NPPB) for RESA1-WT and RESA1-KO, respectively, ~ 100 μM (furosemide) and < 100 μM (DIDS) for both strains (curves not shown). Moreover, similar round of experiments performed with parasites cultivated with serum instead of Albumax gave similar results (Fig. IV. 3. 11D). Nevertheless, again inhibitions obtained with DIDS at 100 μM show differential sensitivity of membrane current to this compound. Indeed, inward currents are more sensitive to this drug in RESA1-WT compared to RESA1-KO strain. However, it should be noticed that efficiency of drugs inhibition was more pronounced for the serum-cultivated malaria cultures. For instance, the calculated percentage values for DIDS were: inward current inhibition 81.9% ± 2.8 and 85.6% ± 2.5 for RESA1-WT from Albumax and serum, respectively, 69.0% ± 5.0 and 74.2% ± 4.5 for RESA1-KO from Albumax and serum, respectively; outward current inhibition: 71.0% ± 4.9 and 87.1% ± 3.1 for RESA1-WT from Albumax and serum, respectively, 56.7% ± 5.7 and 80.0% ± 8.4 for RESA1-KO from Albumax and serum, respectively. Typical examples of registered currents, initial (control) and after addition of an appropriate concentration of drug, are presented in Fig. IV. 3. 11E.

It is noteworthy that inhibition whatever the drug used was always more pronounced while parasites were cultivated using serum instead of Albumax. However, it is hard to conclude regarding efficiency of the drug *per se*. Indeed, the percentage of inhibition calculated is based on the nominal current (before inhibition)

Figure IV. 3. 11: **NPPB, furosemide, DIDS and glybenclamide: blockers of new permeability pathways (NPPs). Testing the sensitivity for these drugs of RESA1-WT, RESA1-KO, RESA1-KI.** Results obtained for cells cultivated in Albumax (**A, B, C**) or serum (**D, E**). Figures show percentage of inward (G^-)/outward (G^+) inhibition of *whole-cell* currents by NPPB (**A, D, E**), furosemide (**B, D, E**), DIDS (**C, D, E**) and glybenclamide (**D, E**). Inhibitors were added to the bathing solutions to obtain final nominal concentrations 1, 10, 100 μM for NPPB, 1, 10, 100, 1000 μM for furosemide, 100 μM for DIDS and 100 μM for glybenclamide. Typical examples of registered currents are given in (**E**). *Whole-cell* patch-clamp recordings were obtained by evoking a series of test potentials from +100 to -100 mV in 10 mV steps for 500 ms from a holding potential of 0 mV. The percentage of inhibition was calculated on the cord conductance between -100 mV and -10 mV, and +10 mV and +100 mV for each drug concentration. Vertical bars correspond to SEM, and each calculated value was a mean of six or more individual experiments. Statistical significance between strains ($P<0.05$) was observed only for DIDS on inward current. Efficiency of drugs inhibition was more pronounced for the serum-cultivated malaria cultures.

Figure IV. 3. 11





and the final value (after addition of drug). Since nominal currents were larger in serum condition, this may account for the difference considering that final currents are composed by the remaining current after inhibition plus the residual leak though the patch. Nevertheless, considering that leak currents are constant throughout experiments or in between experiments, according the very high reproducibility of recordings and seal resistance, the differential effect of DIDS can reflect an effect of RESA1 expression. Furthermore, as already mentioned in the introduction part of this chapter, RESA1 is known to interact with the membrane cytoskeleton notably spectrin. However, spectrin belongs to the proteins that maintain the meshwork consisting of ankyrin, protein 4.1 and band 3. All of these proteins are intimately interconnected to give a highly structured junctional complex. Band 3, as the major protein in erythrocyte membrane is being increasingly appreciated as a critical regulator of red cell deformability, which in consequence is essential for red cell elasticity or rigidity (Mohandas et al., 1992). Then by interacting with band 3, DIDS may interfere with the natural flexibility of RBCs membrane proteins. Since RESA1 protein interact physically with spectrin (and thus participates to cytoskeleton stabilisation) we could suspect that the differential effect of DIDS between WT and KO is indirect through band 3.

Another particularity of membrane currents induced upon infection *by Plasmodium falciparum* is their sensitivity to trace of serum during recordings. Indeed, it has been shown that typical inwardly rectified phenotype of current/voltage relationship on infected cells may be modulated by remaining components of serum coming from cultivation medium. In infected cells serum presence has been shown to modify the membrane currents after malaria invasion of human RBCs, suggesting changes in channel activity. Using the *whole-cell* configuration, Staines and coworkers described that traces of serum (0.4%) in the bath induces a 4-times increase of the outward currents and a 2-times increase of inward currents in *P. falciparum* infected cells (Staines et al., 2003). Another work confirmed that serum stimulates an outwardly rectifying channels and sorbitol permeability (Staines et al., 2006). In year 2007, Bouyer et al. (Bouyer et al., 2007) demonstrated that alteration of the *whole-cell* currents in malaria-cultures by human serum might re-

sult from a change in the kinetics of already active, endogenous channels present in the erythrocyte membrane. Duranton and co-workers reported that interaction with serum albumin stimulates an anionic conductance in infected RBCs and suggested that high affinity and specificity of this relation might underlie the observed dependence of *P. falciparum* growth *in vitro* on serum albumin (Duranton et al., 2008). The phenomenon of serum effect may have a high physiological relevance, as the parasite can not growth without serum (Trager and Jensen 1976; to clarify with our above-mentioned facts, for long time the substitution of human serum as a component of culture medium has been seeking, till final use of serum albumin, so-called Albumax).

In this context, it was necessary to test if serum added during recordings can modulate membrane currents in the same extent in all RESA1 strains. Keeping in mind that serum activates maxi-anion channel (see chapter IV. 1) in healthy RBCs, and using pathophysiological conditions of malaria-infection for looking for triggering factors of endogenous PBR/VDAC up-regulated to so-called NPPs, we next used *whole-cell* configuration of patch-clamp to test the effect of serum on currents recorded from RESA1-WT, RESA1-KI and RESA1-KO. Again, both conditions of cultivation, namely using Albumax or serum, were used for comparison purpose. Trophozoite-stage cells were washed three times in physiological RnD solution to eliminate trace of culture medium. *Whole-cell* recordings were obtained with RnD in the bath and pipette 10 minutes after seal formation (control), 15 minutes after addition of 0.5% human serum to the bath (serum) and 5 minutes after inhibition with 100 μM NPPB added to the bath solution. Figure IV. 3. 12 shows results for the strains either from Albumax (Fig. IV. 3. 12A) or serum (Fig. IV. 3. 12B) medium. As demonstrated, for all RESA1 strains inwardly rectifying anion-selective currents in control and serum were observed (inhibition by NPPB was not shown for clarity of figures). Regardless of cultivation medium, addition of 0.5% serum increases current for all, wild type, revertant and mutant, compared to control when cells were in the physiological solution in the absence of serum. This confirmed that human serum component stimulates anionic conductance recorded in *whole-cell* currents. For the cells cultivated in Albumax (Fig. IV. 3. 12A) no differences between RESA1-WT, RESA1-KI and RESA1-KO were noticed. Dose-response inward

Figure IV. 3. 12: **Effect of serum induced *whole-cell* conductance of *P. falciparum* infected human red blood cells RESA1-WT, RESA1-KI, RESA1-KO.** (**A**) *I-V* curves corresponding to cells cultivated in Albumax media: control and after perfusion with 0.5% serum (WT: n=23, KI: n=12, KO: n=14 individual experiments). (**B**) *I-V* curves corresponding to cells cultivated in serum media: control and after perfusion with 0.5% serum (WT: n=8, KI: n=7, KO: n=7 individual experiments). Black and red solid lines corresponding to WT cells: control and after serum addition, respectively. Black and grey dash lines corresponding to KI cells: control and after serum addition, respectively. Black and green dotted lines corresponding to KO cells: control and after serum addition, respectively. *Whole-cell* recordings were obtained in RnD solution in bath and pipette (containing, in mM/l, 115 NaCl, 5 KCl, 10 $MgCl_2$, 5 $CaCl_2$, 10 glucose, 10 Hepes, adjusted to pH 7.40). Control current was measured 10 minutes after obtaining the *whole-cell* configuration. 0.5% serum was added to the bath solution and current was recorded after 15 minutes of stabilization. Inhibition with 100µM NPPB is not shown for clarity. No differences in serum effect were observed between strains cultivated in Albumax. Cells cultivated in serum media indicate differences in control as well as after serum perfusion. Serum induced conductance was much more efficient for strains from serum media. (**C**-**D**) The corresponding comparison of inward (G^-)/outward (G^+) conductances. Black bars, control conductance; light grey bars, after perfusion with 0.5% serum; dark grey bars, after perfusion with 0.5% serum and 100µM NPPB. (**C**) Strains cultivated in Albumax. No differences were observed. (**D**) Strains cultivated in serum media. Statistical significance was observed between RESA1-WT and RESA1-KO (* $P<0.05$).

Figure IV. 3. 12

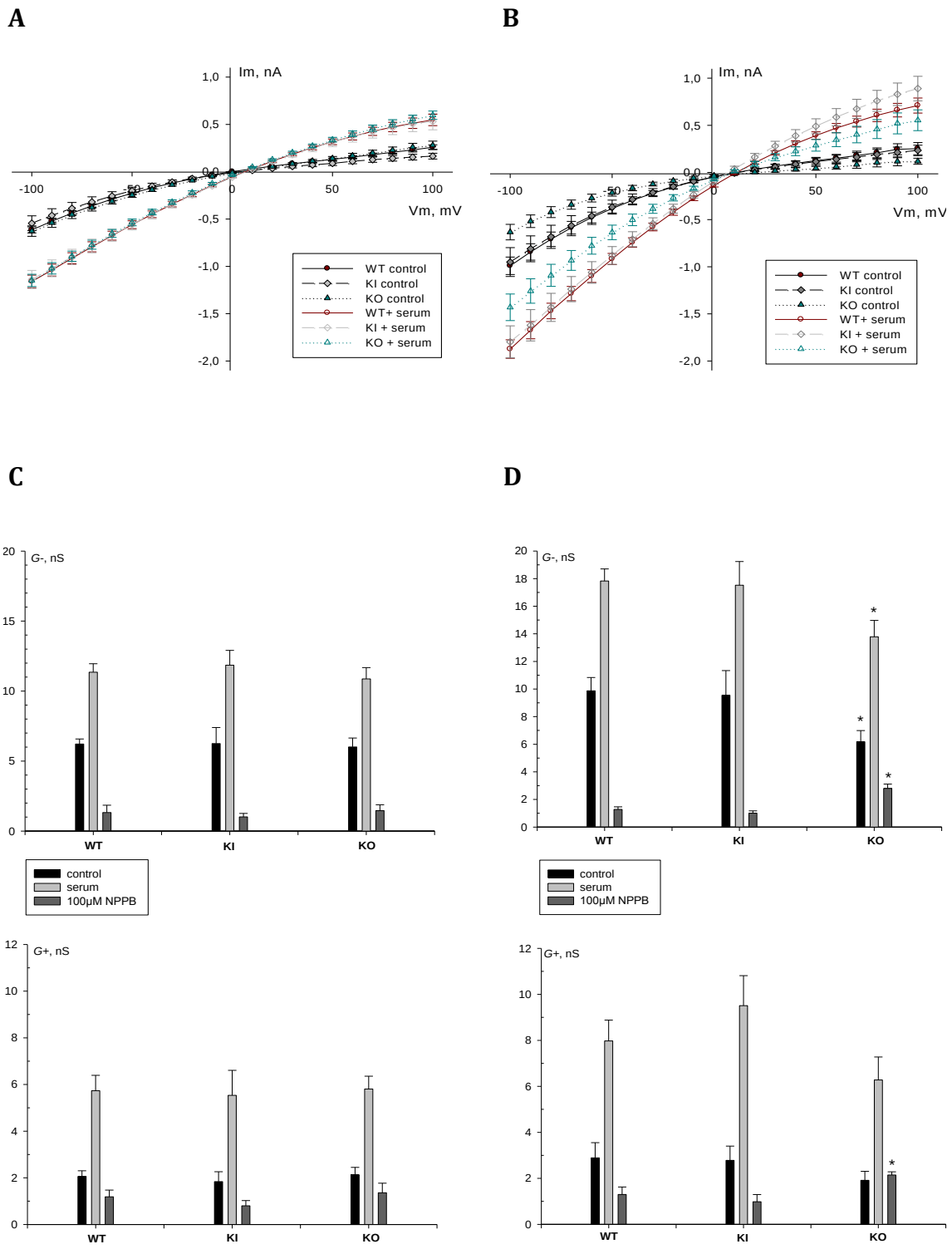


Figure IV. 3. 13

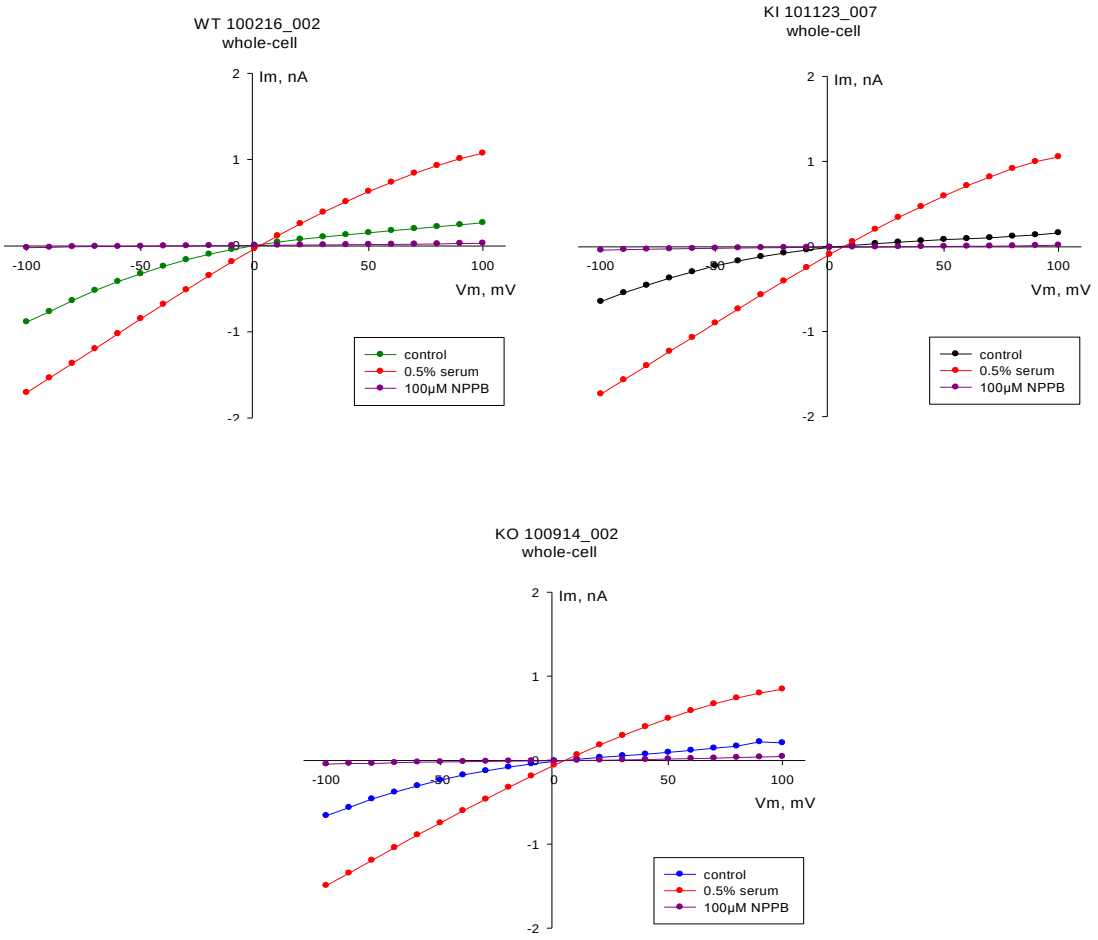


Figure IV. 3. 13: **Representative example of serum effect on *whole-cell* induced current on RESA1-WT, RESA1-KI and RESA1-KO.** I/V curves corresponding to one control cell of WT (up-left), KI (up-right) and KO (down), after 0.5% serum addition and inhibition with 100 µM NPPB.

(G^-) / outward (G^+) comparison (Fig. IV. 3. 12C) confirmed that serum addition to the bath induced 2-times increase of the inward and outward currents for all RESA1 strains. All currents were similarly inhibited by 100 μM NPPB. In contrast, other results were obtained for serum-cultivated RESA1 cells. Figure IV. 3. 12B shows that RESA1-WT and RESA1-KI exhibited larger control current compared with RESA1-KO. Serum increased 2-times the inward currents and 3-times outward currents of all strains, with the observed effect greater for wild type and revertant than for the mutant. The inhibition by NPPB was more efficient for WT and KI rather than for KO. Representative results are shown in Fig. IV. 3. 13. The corresponding comparison of inward/outward conductances (Fig. IV. 3. 12D) indicated significant differences in control, serum and inhibitory effect between RESA1-WT and RESA1-KO, more pronounced on inward currents. On the other hand, it should be noticed, that serum effect was more efficient (on outward currents) for strains from serum medium compared with Albumax-cultured. Since this phenomenon was especially manifested for RESA1-WT and RESA1-KI we could suggest that RESA1 protein modulates somehow NPPs activity.

The first part of present results aimed at the electrophysiological study of currents activated in RBCs membrane upon *P. falciparum* infection. However, since 30 years, NPPs was defined as an increase of membrane permeability after invasion, notably for some substrates that are totally impermeant at the non-infected red cell membrane. Then, that is why hypothesis that RESA1 expression level may modulates NPPs had to be tested in this perspective using semiquantitative isosmotic sorbitol haemolysis. We focused mainly on cells cultivated in serum (MACS purified trophozoite-stage, *Materials and Methods*), but experiments from cells cultivated in Albumax were done for comparison in regard to electrophysiological data. Figures IV. 3. 14A, B show that the percentage of lysis in cells exposed simultaneously to isotonic sorbitol solution increases with time, rapidly in the first 20 minutes, indicating diffusional entry of sorbitol into infected RBCs via NPPs. For RESA1 strains cultivated in Albumax (Fig. IV. 3. 14A, mean of 3 experiments, ±SEM are not shown for clarity) no difference in this process between wild type and mutant was observed and the half-time of lysis ($t_{1/2}$ = 13') was the same for both

Figure IV. 3. 14

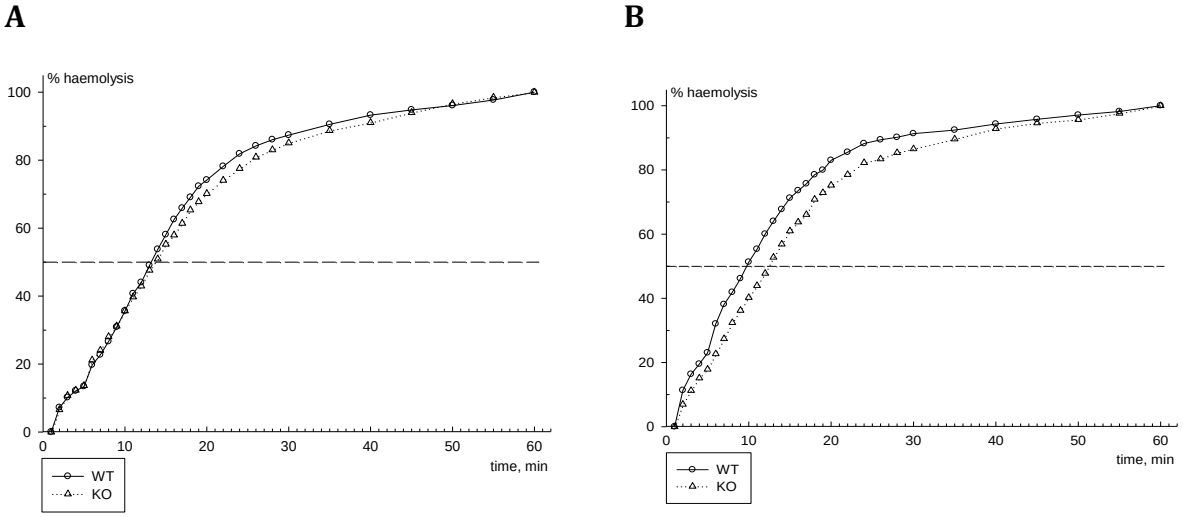


Figure IV. 3. 14: **RESA1-WT and RESA1-KO sorbitol haemolysis.** Sorbitol-induced haemolysis demonstrates that RESA1 could be involved in the modulation of new permeability pathways (NPPs) only if it is present in the erythrocyte membrane, which strongly depends on media composition. Time course of osmotic lysis were measured with a light scattering assay (transmittance). Suspensions of trophozoite-stage infected hRBCs were added to sorbitol lysis solution and haemoglobin release was monitored at 700 nm. Percentage of haemolysed cells is shown according to time. (**A**) Sorbitol haemolysis of RESA1-WT and RESA1-KO strains cultivated in Albumax. The figure is a mean of three independent experiments. ±SEM were removed for clarity. No difference between strains was observed. (**B**) Sorbitol haemolysis of RESA1-WT and RESA1-KO strains cultivated in serum. The figure is a mean of seven independent experiments. ±SEM were removed for clarity. Difference in osmotic lysis between RESA1 strains was observed. It should be notice that RESA1-WT haemolysed faster when cultivated in serum. For RESA1-KO the time of haemolysis was similar in both conditions.

strains. However, kinetic of lysis and $t_{1/2}$ for RESA1 strains were comparable with our previous data obtained for 3D7cultivated in Albumax (data not shown). Figure IV. 3. 14B presents haemolysis curves for RESA1-WT and RESA1-KO as a mean of seven independent experiments (±SEM are not shown for clarity), when cells were cultivated in serum medium. First, it indicates that for serum-cultured cells there is an obvious difference between wild type and mutant trophozoite-stage sorbitol haemolysis. Second, it shows that the lysis was delayed for mutant, with the half-time $t_{1/2}$ = 10' and $t_{1/2}$ = 13' for RESA1-WT and RESA1-KO, respectively.

Sorbitol induced haemolysis for RESA1-WT was more efficient for strain cultivated in serum compared to Albumax ($t_{1/2}$ = 10' and $t_{1/2}$ = 13' respectively). For RESA1-KO, independent on cultivation medium, we get the same kinetic curves ($t_{1/2}$ = 13' for both conditions). Interestingly, the lysis was similar for RESA1-WT (Albumax), RESA1-KO (Albumax) and RESA1-KO (serum). This could confirm our electro-physiological observations, that serum presence in the medium for cultivation is necessary for RESA1 protein effect on channel activity.

This set of experiments suggests that RESA1 protein could be involved in modulation of NPPs after *P. falciparum* malaria-infection.

IV. 3. 3. Discussion.

New Permeability Pathways (NPPs) are described so far as an increase of RBCs membrane permeability upon infection (Kirk, 2001). This membrane permeability is though to rely on channel/pore activation that takes place 12 hpi (Krugliak and Ginsburg, 2006). Nevertheless, molecular identity as well as triggering factors susceptible to explain sudden activation is still under debate even if endogenous PBR/VDAC complex has been recently proposed as a good candidate (chapter IV. 2, Bouyer et al., 2011). On the contrary, last results seeking at demonstrating that NPPs can be accounted by parasite exported proteins, pointed out the key role of CLAG3 proteins in NPPs formation (Nguitragool et al., 2011).

In this context, we made the hypothesis that other proteins exported by parasites during their intraerythrocytic life cycle may play crucial role in activation of membrane channels. Among around 400 of them, RESA1/Pf155 proteins are known to

be exported to the red cell membrane at the ring stage where by interacting with cytoskeleton, notably spectrin, they participate to the remodelling of host cells. Experiments performed on strains encoding one such protein and present in this part of the thesis suggest that RESA1 could at least partly contribute to the activity of NPPs. Patch-clamp data demonstrate statistically less membrane currents in RESA1-KO in contrast to parental (RESA1-WT) and revertant (RESA1-KI) strains. Moreover, haemolysis tests show that this process was kinetically delayed for mutant. However, knocking-down of *resa1* gene (in RESA1-KO) results in impairment of NPPs in efficiency.

This work demonstrates also that RESA1 protein needs special conditions to be involved in channels activity. We suggest here that components of normal human serum in cultivation medium play crucial role in this aspect. It should be noticed that this fact appeared after long time spent on working with RESA1 strains for this PhD thesis. Because huge number of electrophysiological results obtained from cells cultivated in Albumax medium did not indicate any differences between RESA1 strains we assumed missing something in protein expression. The probable cause, in our opinion, was cultivation conditions. As Albumax is routinely used in many malaria laboratories as medium for cultivation, and since it is known that most parasite strains are adapted to this condition (they grow and multiply properly), at the beginning we were using this medium for RESA1 strains. In addition, our laboratory 3D7 strain has been always cultivated in Albumax. Moreover, most of the studies conducted on Albumax-cultured parasites showed that properties and ability of merozoites to invade new erythrocytes are conserved, notably the receptors-ligands involved in pre-events (Cranmer et al., 1997). However, no direct comparison for rate of invasion between Albumax- and serum-cultivated parasites has been tested in parallel. Nevertheless, in year 2007, Frankland and coworkers (Frankland et al., 2007) strongly proved that although Albumax supports parasite growth it allows much less efficient presentation of PfEMP1 at the red blood cell surface compared to serum cultivation conditions. Accordingly, we can make the assumption that this apply also to RESA1 and account for the differences observed between WT and KO cultivated in serum, whereas membrane currents

measured in both strains cultivated in Albumax are similar. Since it is not confirmed yet by other tests (ex. western blotting and immunofluorescence for RESA1 strains from Albumax), it remains our suggestion that serum is probably necessary for correct export of RESA1 protein and/or its presence in the host erythrocyte membrane.

Although the conditions, as above-mentioned, 3D7 strain actually differs from RESA1-WT, but this could be due to the fact that 3D7 is an old strain and has been very long cultivated in our laboratory with huge number of asexual life cycles which could lead to loss of knobs (Langreth et al., 1979).

On the other hand, traces of serum have been reported to modulate membrane currents upon infection (Staines et al., 2003). Eventually, factors present into the serum, probably serum Albumin, have been shown to have high specificity and affinity for interactions with the infected cells (Duranton et al., 2008). Rather, their stimulatory properties resulted from binding to specific receptors in the erythrocyte membrane. Moreover, it has been suggested that serum interacts more directly with the channel protein and not via a complex signalling. We have demonstrated that serum stimulates dormant erythrocyte channels in healthy cells (see chapter IV. 1, Glogowska et al., 2010). But the mechanisms of this activation process either in non-infected or in malaria-infected RBCs remain totally unknown. However, since we have demonstrated the presence of PBR complex into the erythrocyte membrane (Bouyer et al., 2011), and that this complex contains TSPO protein, known to be associated with cholesterol import in mitochondria, it may suggest that serum stimulatory effect could corresponds to specific interactions with PBR complex. The functional and physiological significance played by serum for the intraerythrocytic *P. falciparum* development is obvious from the fact that this component is necessary to be present in the medium for malaria *in vitro* cultures (Asahi et al., 2005). Serum might act as a source of amino-acids for parasite development (El Tahir et al., 2003) and fatty acids used by the parasite for its membrane biosynthesis (Asahi et al., 2005). The results presented in this part of PhD thesis suggest also that the parasite is using serum factors for exporting proteins which remodel host erythrocyte membrane.

We have shown here that human serum modulates *whole-cell* current recordings in *P. falciparum* infected cells, which is in agreement with previous observations (Staines et al., 2003; Bouyer et al., 2011). Because this effect differentiates between RESA1 strains we could suspect that this protein modify somehow the properties of NPPs. The early results from our laboratory with 3D7 strain show that inwardly rectifying channel (IRC) support serum effect described before in *whole-cell* configuration. Single-channel recordings in *cell-attached* configuration made on RESA1-WT and RESA1-KO could bring in the future interesting informations on this subject.

Moreover, because human serum is the major component of blood plasma, it seems to be very important from the physiological point of view to understand its role played in channel activity in RBC membrane. Thus, pathophysiological conditions using *Plasmodium falciparum* infection could be a suitable model for solving such question.

Since we suggest that RESA1 plays a role in the activation of PBR/VDAC further experiments are necessary to confirm our hypothesis. For instance, haemolysis tests with the PBR ligands PK11195, Ro5-4864 and diazepam using RESA1 strains could bring new informations. For example, the values of IC_{50s} obtained with these ligands could be really interesting if differences are observed. Moreover, we should remember that stabilisation of red blood cell cytoskeleton by interaction of RESA1 with spectrin allow infected erythrocyte to overcome membrane fragilisation upon exposure at febrile temperatures (Silva et al., 2005; Pei et al. 2007; Mills et al. 2007). To investigate more accurately the fact that temperature increased rigidity and osmotic resistance of RESA1 cells osmotic lysis susceptibility assays under febrile mimicking conditions should be performed in the future studies. If we could observe the role on mechano- and osmotic- resistance provided by RESA1, concomitant activation of the anionic channels, we could confirm that RESA1 triggers activation of NPPs.

For the purpose of this PhD thesis we speculate that RESA1 protein plays a key role in modulation/activation of NPPs. However, during intraerythrocytic development numerous proteins are exported from the parasite to the RBCs. Since

Figure IV. 3. 15

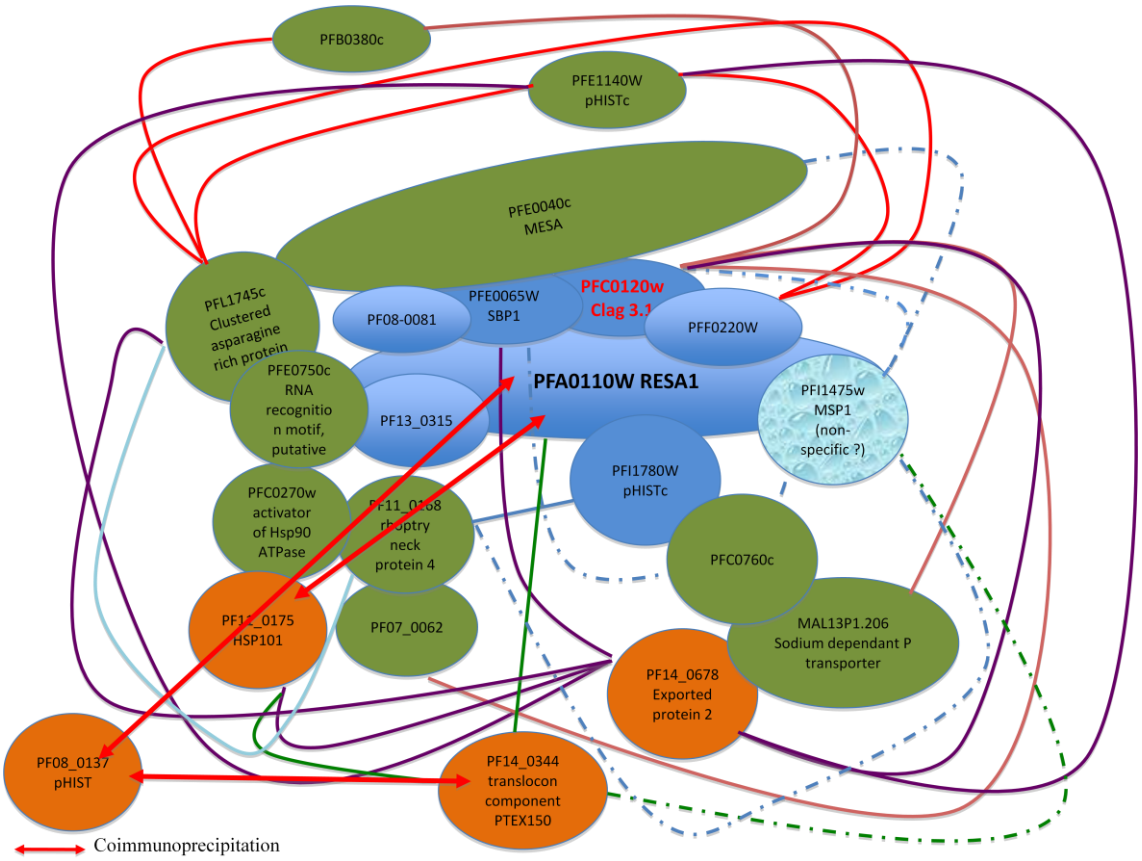


Figure IV. 3. 15: **Interaction of RESA1 with other proteins in the red blood cell membrane infected by *Plasmodium falciparum*.** The figure represents schematically connection of RESA1 with other *P. falciparum* protein family members. The coimmunoprecipitated with RESA1 are indicated with a red arrow (from S. Bonnefoy).

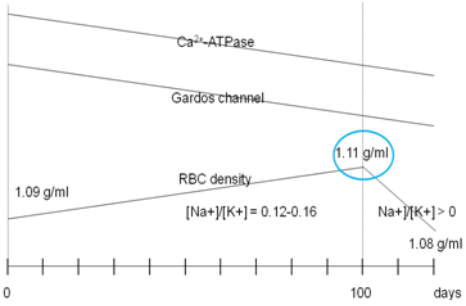
RESA1 is connected with many proteins (see Fig. IV. 3. 15) we can not exclude implication of some other components in this process. For instance, evidence for parasite-encoded CLAG3 proteins as contributors to solute uptake (Nguitragool et al., 2011) brought new insight into anionic channels in malaria-infected cells. It is unclear now and remains to be determined of whether CLAG3 form a novel anionic channel or whether it acts as subunit in a channel complex involving another parasite-encoded proteins or endogenous RBC channels. However, because CLAG proteins have been previously observed to form part of a high-molecular-weight complex that assembles in the rhoptry organelles of mature merozoite-stage parasites, and because these proteins are strictly connected and interacted with RESA1, we could next hypothesise their common role in modulation/activation of NPPs.

Despite diversity, most of exported proteins have at least one common link: they share a role in remodelling of host red blood cell membrane. This process occurs at different intraerythrocytic stages. For instance, most cytoadherence-responsible parasite-derived proteins are produced in early ring-stage, e.g. spectrin-binding protein 1 (SBP1), membrane-associated histidine-rich protein (MAHRP), ring-expressed antigen-1 (REX1) (see chapter *Theoretical background*, Table II. 2 and II. 3). This is continued when the parasite transforms into a trophozoite stage (knob-associated histidine-rich protein (KAHRP), *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), *Theoretical background*, Table II. 2 and II. 3). Although, some proteins responsible for cell deformability and rigidity are expressed at schizont stage (RESA) or even in the gametocyte (STEVOR). Thus it is likely that a repertoire of parasite proteins in infected red blood cells may play a collective role. However, it is noteworthy that proteins for which export have been reported are mainly proteins involved in virulence. Thus, this could account for the fact that these proteins are dispensable *in vitro* for full completion of the intraerythocytic life cycle. Nevertheless, this may imply that such proteins are not directly involved in the generation of the NPPs *per se*, but indirectly may modulate the level of NPPs activity instead. Then it would be really interesting to test this hypothesis in rodent model when homologues exist or to test in a systematic way all the repertoire of exported proteins known to be addressed to the membrane or involved in interaction with cytoskeleton components. More broadly, it is logical that understand-

ing of function and role(s) of proteins exported by the parasite to the host membrane is an important aspect for the biology and pharmacology of the malaria parasite.

The up-to-now available informations, although still divergent, clearly show that the endogenous anionic channels, called after new permeability pathways in *P. falciparum* infected cells, depend on parasite proteins, either as their components (Alkhalil et al., 2004; Nguitragool et al., 2011) or their modulators (e.g. RESA1). Accordingly, it has been reported that resuspension of the cells in a chymotrypsin-free medium (chymotrypsin treatment of intact RBCs resulted in NPPs inactivation) demonstrated the progressive reappearance of the NPPs, which was dependent on the parasite viability and ability for protein secretion (Baumeister et al., 2006). On the other hand, several parasite protein-kinases belonging to the FIKK protein-kinases multigene family are exported to the RBC cytosol that might modulate the activity and specificity of inactive membrane anionic channels (Nunes et al., 2007). Another illustration of the importance of parasite proteins in the up-regulated induction of the host erythrocyte membrane endogenous dormant anionic transporters is the specific and high affinity interaction of human serum factors with the surface of infected RBCs that stimulates anionic conductance (Duranton et al., 2008), observed already in non-infected human red blood cells (Glogowska et al., 2010).

IV. 4.



Physiological role of human erythrocyte channels: A unifying hypothesis for senescence, sickle cells and malaria.

Forth objective:

IV. 4. Physiological role of human erythrocyte channels: A unifying hypothesis for senescence, sickle cells and malaria.

IV. 4. 1. Introduction.

The three above-presented parts of this PhD thesis described electrophysiological characteristics, molecular identity and partly regulation of anionic channels in the resting non stimulated red cell membrane in health and disease. In order to address the physiological role played by these channels in human erythrocytes, specific experiments mimicking physiological stress and transit in capillaries should be carried out. These experiments are technically difficult to perform and were not an object of this work. However, the literature contains lots of data concerning senescence, sickle cell anaemia and malaria which, in view of what we described above, could be interpreted in a model for a dynamic role of channels in red cell physiology and pathophysiology. This model is based on a unique cascade of events and specific experiments on cell aging and malaria infection are presented in this chapter to test this hypothesis.

As already postulated, in the 'resting cell', channels are inactive but they can be potentially activated under experimental conditions. For instance, membrane deformation upon seal formation (Dyrda et al., 2010) triggers transient increase in calcium permeability, leading to Gardos channel activation resulting in transient potassium efflux accompanied by transient generation of anion efflux. This leads to water loss and cell volume decrease. As suggested, the possible physiological role of ionic channels depends a lot from the molecular nature and regulation of anionic conductance. Therefore, in our proposed model Ca^{2+} plays crucial role as a first key initiating this cascade process. Logically, Gardos channels are the second link of this chain.

Figure IV. 4. 1

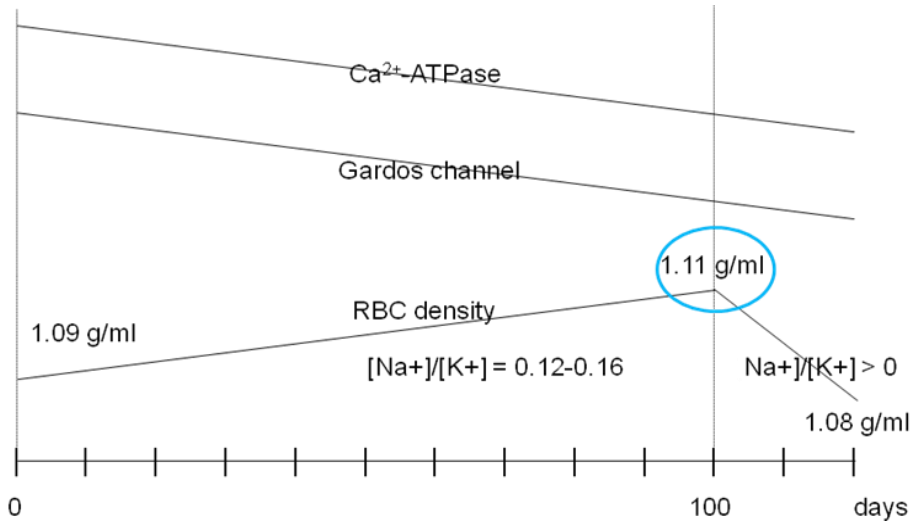


Figure IV. 4. 1: **Schematic changes occurring in RBCs during senescence.**

Figure IV. 4. 2

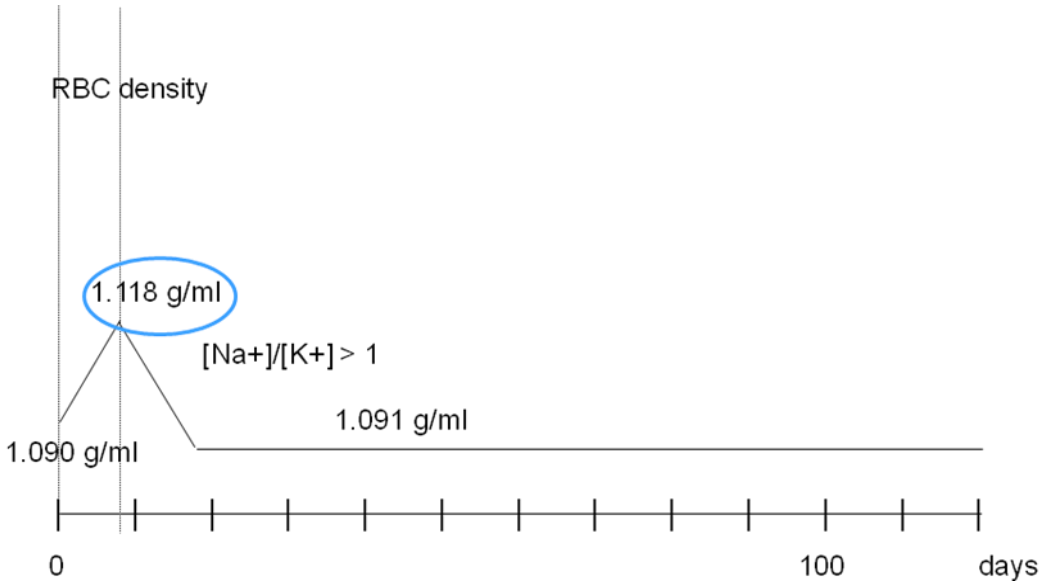


Figure IV. 4. 2: **Schematic changes occurring in RBCs during sickle cell anemia.**

The first interesting situation is the process of erythrocytes aging. Senescence is characterized by a progressive decline of the calcium ATPase and of the Gardos channel activities. In addition, when getting older red cell loose potassium and chloride and become progressively denser (from the value 1.09 g/mL, to 1.11 g/mL for young and old erythrocytes, respectively), but the ratio of Na^+/K^+ remains about 0.12 - 0.16. The interesting point is that a few days before the end of their life span, perhaps very shortly before removal, the red cells undergo a sudden transformation. Cationic conductance (P_{cat}) becomes activated resulting in a complete loss of gradients ($Na^+/K^+ > 1$) and RBCs return to initial density (1.08 g/mL) (Fig. IV. 4. 1). What does this phenomenon mean? It seems very likely, that the only way to explain the process of densification without real change in the gradients is a very slow activation of Gardos channels and the only way to explain the reverse process of return to initial density is a sudden activation of a non selective cationic conductance. A mathematical model (developed by Lew and Bookchin, 1986) confirmed that calcium Ca^{2+} triggers this phenomenon, while the values of membrane potential E_m varies very little but these variations increase rapidly with time according to changes in membrane polarization.
We carried out here some experiments for checking Gardos channel activity in the human RBCs depending on their age.

The second interesting situation is sickle cell disease where upon deoxygenation haemoglobin polymerizes and thus induces membrane deformation and calcium entry. A reversible, poorly selective cationic pathway for small, inorganic monovalent and divalent cations (P_{sickle}) is activated (Tosteson et al., 1952; Tosteson, 1955; Bookchin and Lew, 1981; Ortiz et al., 1990; Tiffert et al., 1993; Lew et al., 1997). Depending on the calcium influx resulting from stochastic opening of calcium permeation pathways, the Gardos channel can be activated with reversible (or not) consequences on loss of KCl and water (dehydration) and subsequent anion efflux through available anion permeability pathways. This phenomenon leads to the existence of two populations of typical cells: red cells with very high density (1.118) obtained only 7 - 8 days after release in the circulation, with the typical falciformed shape and responsible of vaso-occlusive events in the microcirculation, and dehy-

Figure IV. 4. 3

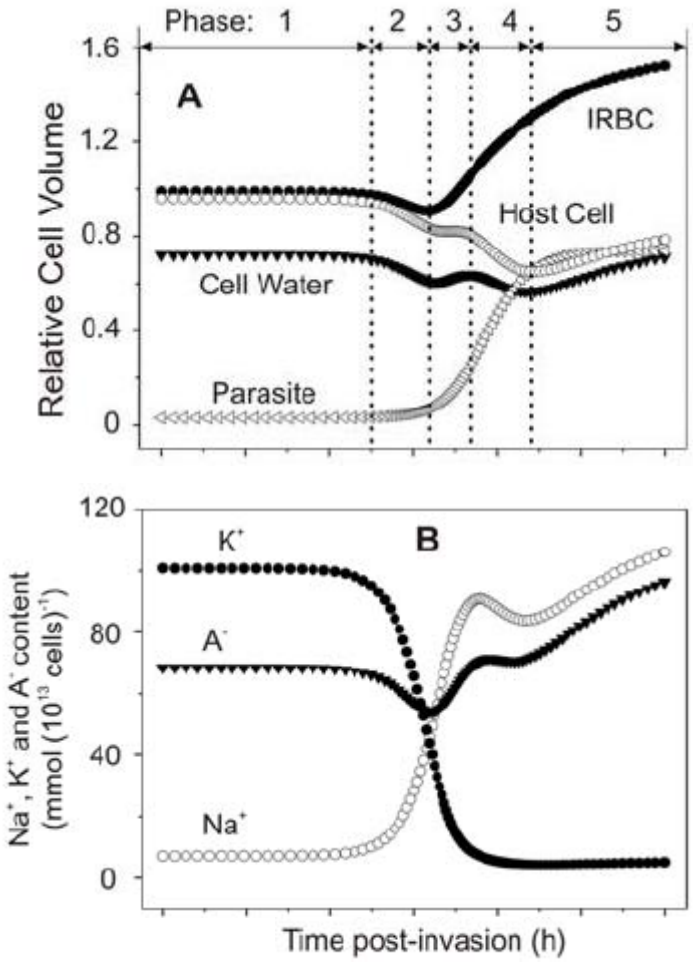


Figure IV. 4. 3: **Predicted changes of human red blood cell after malaria-parasite infection** (Mauritz et al., 2007)**.** (**A**) Volumes of parasite, host cell water, host RBC and IRBC, relative to IRBC volume at time t=0. (**B**) Na^+, K^+ and diffusible anion content of IRBCs in mmol $(10^{13}$ cells$)^{-1}$ (10^{13} cells is taken to represent the approximate volume of a litre of normal, uninfected packed red blood cells with a mean cell volume of 100 fl). IRBC, infected red blood cell.

Hypothesis in this work: Phase 1: initial quiescence; both types of channels, the Gardos and the VDAC, are silent. Phase 2: K^+-driven net fluid loss; Gardos channel becomes active probably due to a suddenly increased calcium permeability followed by an activation of the VDAC channel in its 'closed states' selective for cations what may explain the Na^+ movement. Phase 3: Na^+-driven fluid gain; initial gradients for Na^+ and K^+ are progressively abolished but the net fluxes of Na^+ and anions into the cell persist long after the net K^+ flux has returned to the baseline level. Phase 4 (fluid loss; maximal rate of Hb consumption) and phase 5 (sustained swelling) are not taken into account here.

dration-resistant red cells, very light (below 1.091) and displaying a reversed Na^+/K^+ ratio but with the same shape as the hyperdense cells, indicating that they could have the same origin. Figure IV. 4. 2 presents changes in RBCs occurring during sickle cell anemia. There are obviously striking similarities between this phenomenon and the cascade of events described in senescence, except the timing. It seems once more that cationic conductance (P_{cat}) allows progressive Na^+ gain to exceed progressive K^+ loss at some stages of sickle cells.
Although the pathophysiological role of Gardos channel is already known in sickle cell anemia (Lew et al., 2005), the precise mechanism of its contribution remains unclear. However, this pathophysiological situation was not studied in this work.

Third aspect is malaria disease. We have shown that after infection *P. falciparum* up-regulates endogenous PBR/VDAC channel to so-called new permeability pathways (NPPs), which allows the parasite to transport of nutrients and release waste products. An important point to underline is that NPP, like P_{cat} and P_{sickle}, may also be a cationic selective channel, with the selectivity masked by the huge background anionic permeability when tested only for cation selectivity. According to mathematical model proposed by Mauritz and co-workers (Mauritz et al., 2009) we can speculate (see Fig. IV. 4. 3) that after malaria invasion, during phase 1 of host cell life cycle, all transporters in the red cell membrane remain essentially unchanged and we can hypothesize that both types of channels, Gardos and VDAC, are silent. During phase 2, the initial phase of NPP activation, Gardos channel becomes active probably due to a suddenly increased calcium permeability, followed by an activation of the PBR /VDAC channel in its 'closed states' (which are known to be selective for cations and then may explain the Na^+ movement) and transient dehydration. We should remember that VDAC is voltage-dependent and thus we may also hypothesize that this activation is due to the hyperpolarization resulting from Gardos activity and/or modifications in membrane rigidity. During phase 3, the K^+ efflux, which initially exceeds Na^+ influx, rapidly returns to near-zero baseline levels as the K^+ gradient is dissipated and the direction of net fluid movement is reversed following the reversal of the gradients. It seems that the net fluxes of Na^+ and anions into the cell persist long after the net K^+ flux has returned to baseline levels but

Figure IV. 4. 4

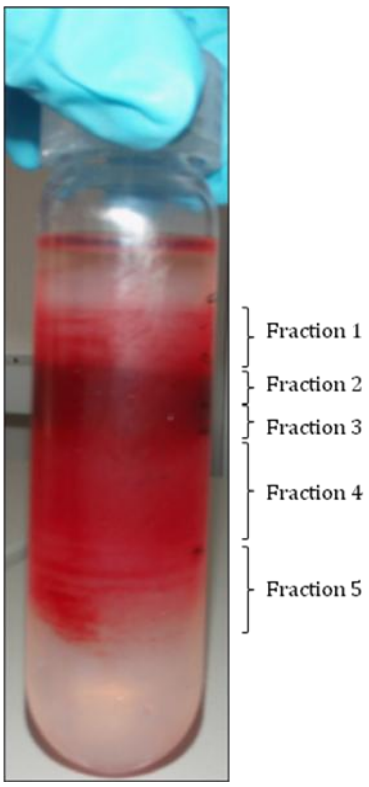


Figure IV. 4. 4: **Human red blood cells separated by Percoll gradient centrifugation.** Fraction 1: young erythrocytes; 2, 3, 4: mature erythrocytes; 5: old erythrocytes.

the Na^+ influx is progressively balanced by a large efflux of anions produced by maximal Hb consumption. We have described previously up-regulation of endogenous anionic channel leading to activation of NPPs. However, it was demonstrated that Gardos channels do not participate to the generation of NPPs phenomenon (Kirk et al., 1992), but the susceptibility of being activated in infected cells was never tested. For this reason, this study was also aimed at providing an answer to the question of Gardos channel ability to function in *P. falciparum*-infected cells?

IV. 4. 2. Results.

In the first step, performed experiments were designed to test the activity of Gardos channel in healthy human RBCs (the same protocol as used in Dyrda et al., 2010) according their age. For that purpose, erythrocytes were separated using centrifugation in Percoll buffer solution (III. 8, *Materials and Methods*). Self-performed gradient allowed to obtaining five fractions of different red cell densities (Fig. IV. 4. 4). Fraction 1 with the lightest cells contains young erythrocytes; 2, 3 and 4 correspond to the mature erythrocytes; 5 contains the oldest cells. Using *cell-attached* configuration of the patch-clamp the activity of the Gardos channel was monitored with 115 mM NaCl in the bath and 150 mM KCl in the pipette (Table III. 6, *Materials and Methods*). Recordings were obtained from each fraction during 3 days following the separation process, in order to check the possible changes in channel activity corresponding to *in vitro* storage. Figure IV. 4. 5 presents representative data obtained from each fraction. Surprisingly, the results from these series of experiments indicate no differences in the Gardos channel activity, neither between fractions nor with the time of storage.

Secondly, we monitored Gardos channel activity (*cell-attached* configuration of patch-clamp, 115 mM NaCl in the bath and 150 mM KCl in the pipette) in malaria infected human erythrocytes. For that purpose we used the common *Plasmodium falciparum* laboratory strain 3D7. As shown in Fig. IV. 4. 6E, F Gardos channel was found to be still active in infected erythrocytes (as a positive control, Gardos channel activity recorded in healthy human RBCs, Fig. IV. 4. 6A).

Figure IV. 4. 5

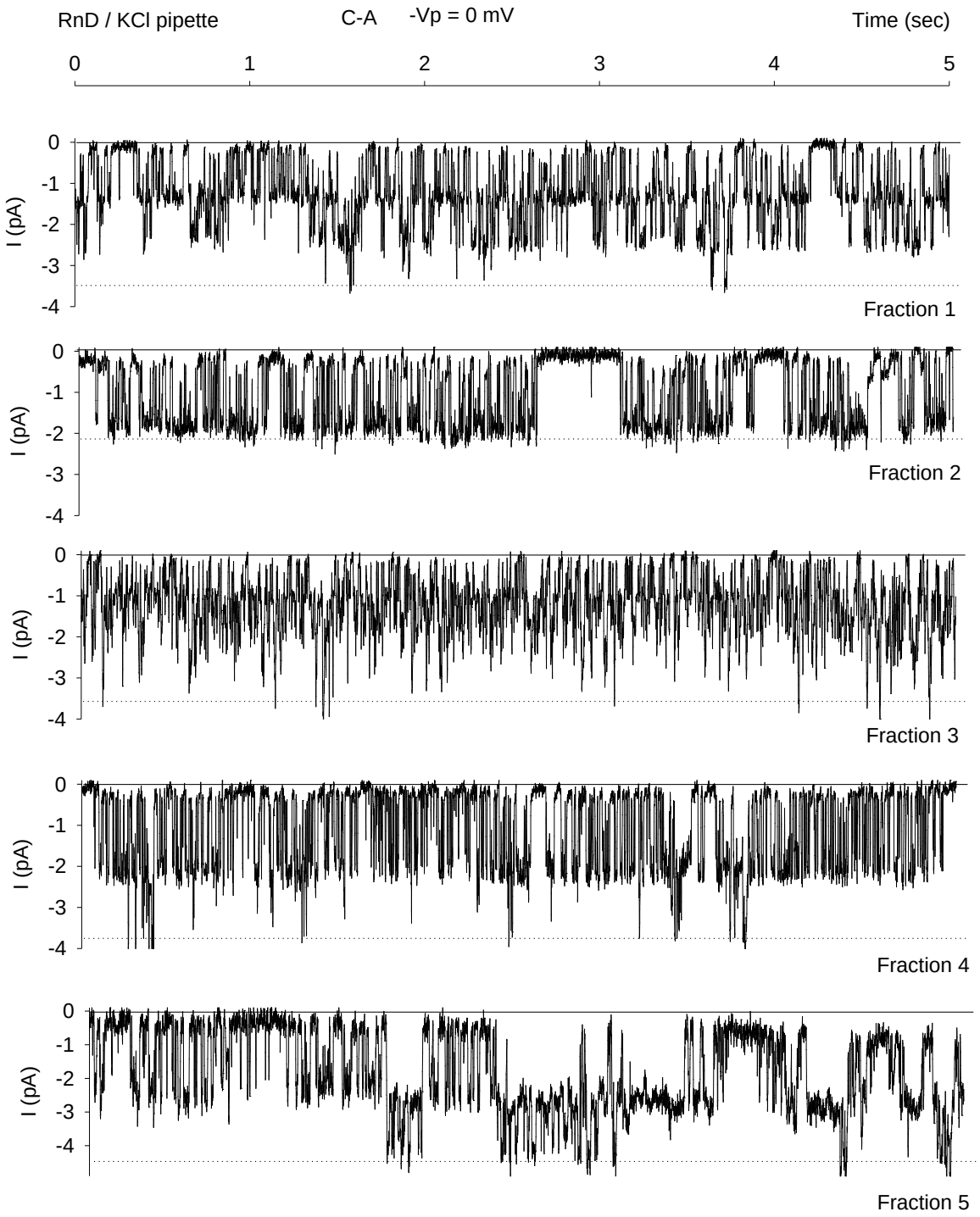


Figure IV. 4. 5: **Gardos channel activity recorded in Percoll-separated human red blood cells.**

Figure IV. 4. 6

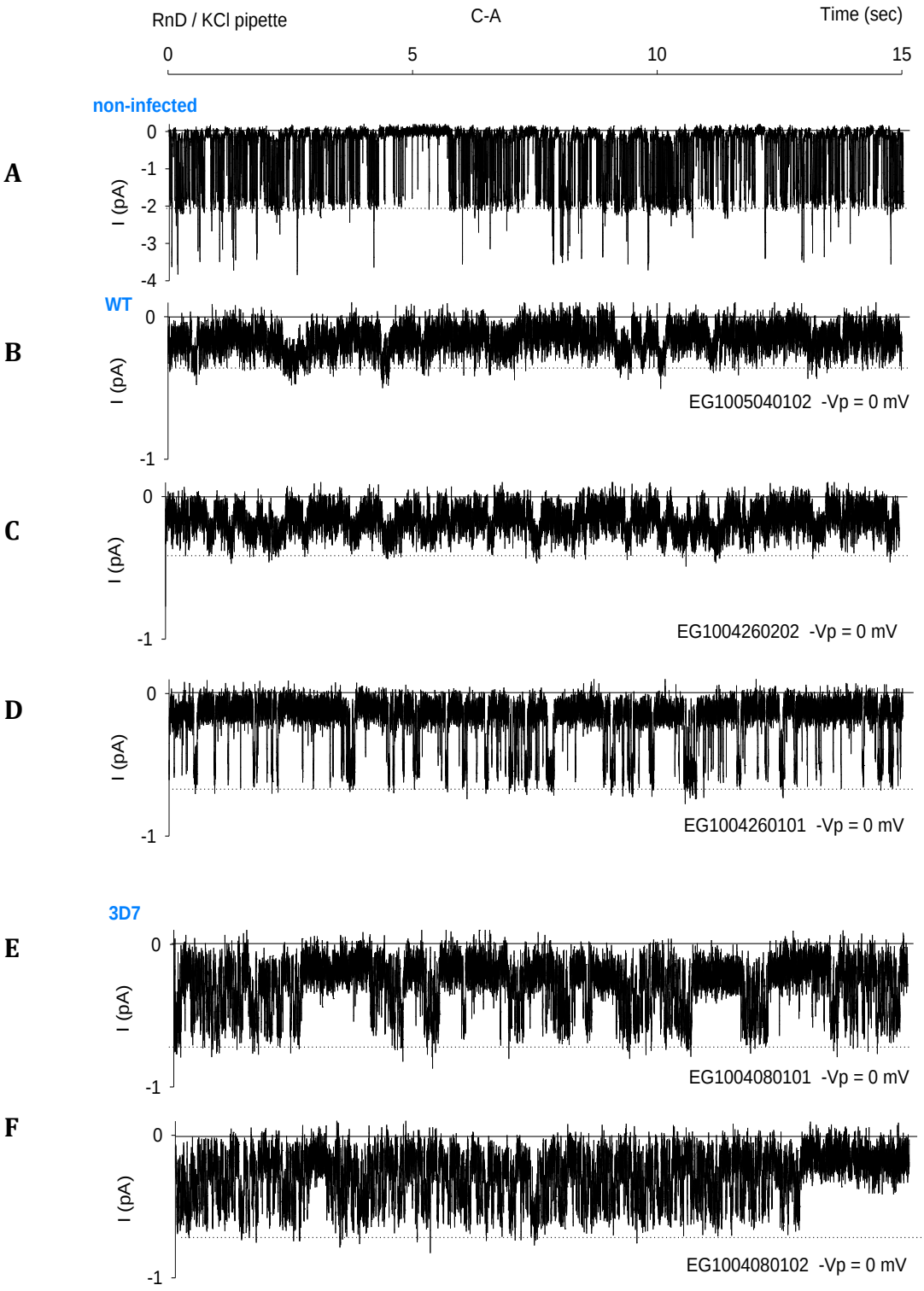


Figure IV. 4. 6: **Gardos channel activity recorded in healthy and *P. falciparum* infected human RBCs: RESA1-WT and 3D7 strains** (**A**) typical Gardos channel activity obtained in non-infected RBCs. (**B-D**) Gardos channel recorded in malaria infected RBCs, RESA1-WT strain. (**E-F**) Gardos channel recorded in malaria infected RBCs, 3D7 strain.

Figure IV. 4. 7

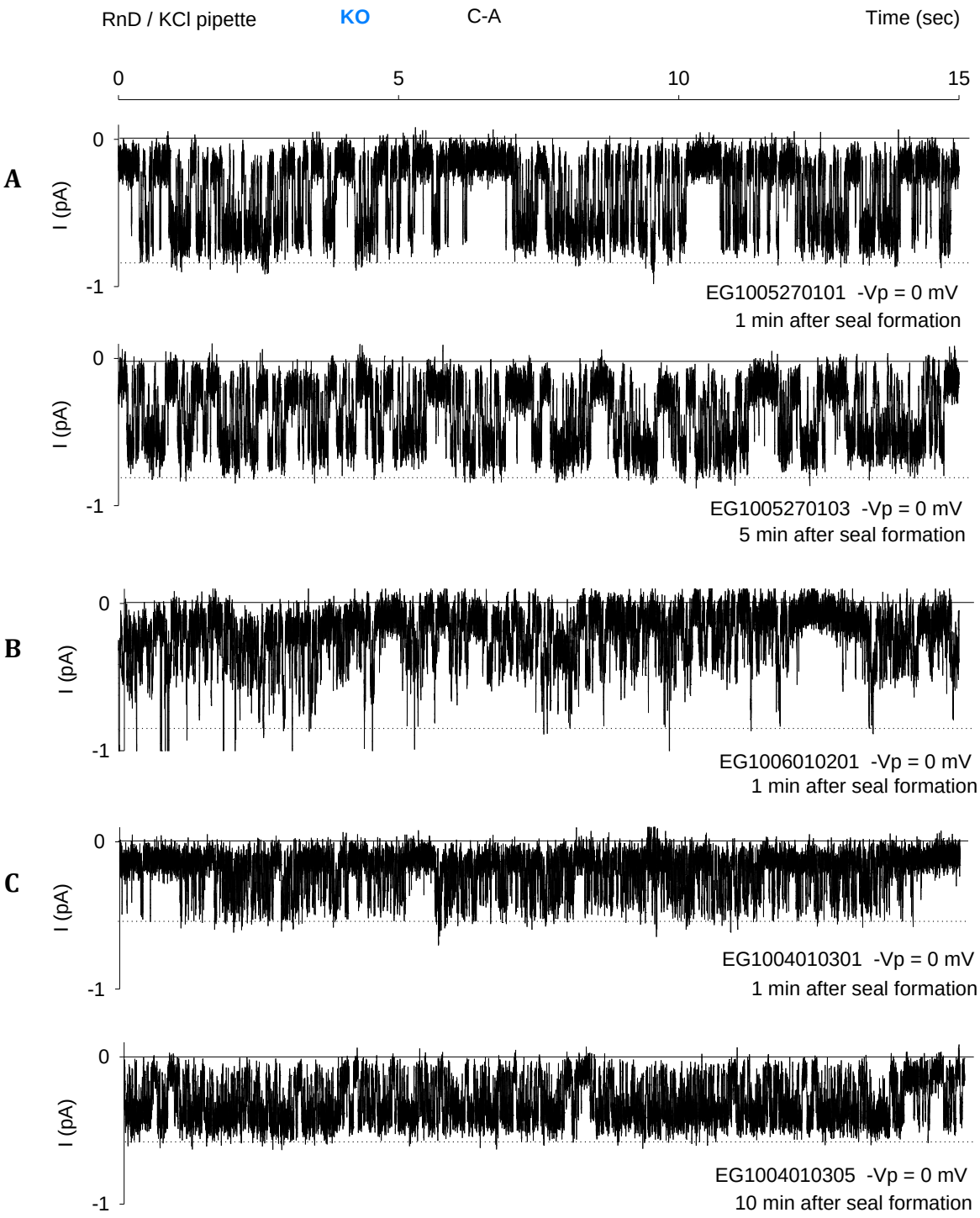


Figure IV. 4. 7: **Gardos channel activity recorded in *P. falciparum* infected human RBCs RESA1-KO strain.** Example of data obtained from 3 independent cells (**A-C**). Channel activity recorded 1', 5' and 10' after seal formation.

Furthermore, we used also RESA1-WT and RESA1-KO strains. At resting membrane ($-V_p$ = 0 mV), when cultivated in serum medium RESA1-WT shows no spontaneous Gardos channel activity upon deformation during seal formation (Fig. IV. 4. 6B, C). In contrast, once cultivated in Albumax, this strain shows typical Gardos channel activity (Fig. IV. 4. 6D). Moreover, when the same protocol was applied on RESA1-KO similar Gardos channel activity was recorded whatever the cultivation medium, and high channel activity was maintained up to 5 or 10 min after seal formation (Fig. IV. 4. 7A, C).

These results indicate that Gardos channel is still able to be active in infected human RBCs. Furthermore, they may confirm assumption made in former chapter regarding the role of RESA1 protein in stabilizing the erythrocyte membrane, and by doing so its role in modulation of channel activities upon infection.

To summarize, the data obtained in this part of the thesis confirm that Ca^{2+}-sensitive K^+ channel is active in healthy erythrocytes and demonstrate that it is still able to function in pathophysiological conditions after malaria infection.

IV. 4. 3. Discussion.

The circulating RBC represents the product of a biochemical and physiological maturation that occurs during the differentiation and proliferation processes of haematopoiesis. In the bone marrow, an undifferentiated stem cell undergoes a series of cell divisions stimulated by the hormone erythropoietin to produce the sequential cell types: the pro-erythroblast, the basophilic, polychromatic and orthochromatic erythroblasts and finally the polychromatic erythrocytes, called also reticulocytes. Almost 40 years ago, morphological changes during erythroid cell maturation have been already described by Bessis (Bessis, 1973), from which loss of nucleus, cease of RNA production and densification are the most obvious changes. Alterations in membrane structure and function involving changes in phospholipid composition, transport of amino acids, sugars, Ca^{2+}, Na^+ and K^+ have been also reported during the RBCs differentiation process (Kemp et al., 1975).

During their life span erythrocytes undergo changes leading to dissimilarities between young and old cells. Senescence has been characterized by a progressive decline of the calcium ATPase and of the Gardos channel activities (Tiffert et al., 2007), change in cells geometry (Canham et al., 1969), alteration in some enzymes e.g. hexokinase, acetylcholinesterase, glutamate oxaloacetate transaminase (McLellan and Thornalley, 1989; Shimizu and Suzuki, 1991), and vitamins (Burton et al., 1986) according to age of RBCs. Two other reports (Hyun et al., 2006; Rizvi et al., 2006) showed reduction of membrane redox activity related to aging. Differences observed among red blood cells population enabled the development of separation methods such as filtration or more recently gradient centrifugation (Lutz et al., 1992). Using Percoll gradient centrifugation Romero and co-workers (Romero et al., 1997) separated light and dense RBCs from freshly withdrawn human blood. Measurements of intracellular Ca^{2+} in different fractions indicated higher level in dense, old cells (31.2 ± 13.0 nM) compared to light, young cells (8.4 ± 2.82 nM). However, these experiments were performed using fura-2, which regarding its possible interference with haemoglobin could not be relevant (Kaestner et al., 2006). Other authors, using atomic absorption spectrometry, demonstrated a two-fold increase in Ca^{2+} content in the old fraction (Percoll- fractionated) in comparison to the light fraction of human RBCs (Lutz et al., 1992). Their observations suggest rising of intracellular Ca^{2+} content in the red cells during their aging *in vivo*. On the other hand, the intracellular Ca^{2+} level is directly controlled by the activity of the ATP-dependent Ca^{2+} pump. Since it has been shown that ATP content decreases upon aging of human RBCs (Lutz et al., 1992) it is expected that this may lead to an increase of the intracellular Ca^{2+} level (Romero et al., 1997).

As previously reported, ionic channels are usually dormant, but their activity has been shown in some experimental or pathological situations such as senescence (Lew et al., 2007), sickle cell anemia (Lew and Bookchin, 2005) and malaria (Ginsburg, 1983; Ginsburg and Kirk, 1998). The role played by these channels still remains poorly understood. By using patch-clamp this part of present PhD thesis was aimed to study Gardos channel activity in healthy human RBCs depending on their age, and in *Plasmodium falciparum* infected. The first set of experiments did not in-

Figure IV. 4. 8

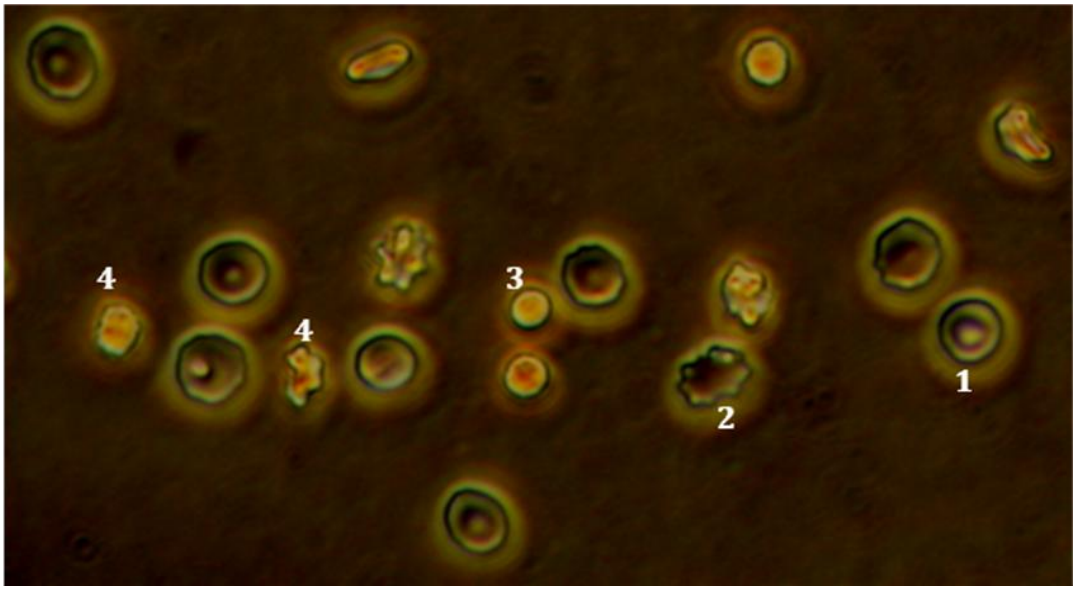


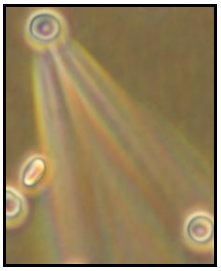
Figure IV. 4. 8: **Light microscopy observation of the shape of human erythrocytes.** Typical shapes of erythrocytes: 1 - discocyte, 2 - echinocyte, 3 - spherocyte, 4 - sphere-echinocyte.

dicate any alterations in channel activity, as it was expected, between erythrocytes at different age. Possible explanation could be the limitation due to the patch-clamp method. For instance, getting good seal takes some time and may vary from one cell to another. In recordings where seal formation takes more than 2 minutes we could loose relevant informations on initial channel activity upon deformation immediately after membrane deformation. Once the seal is established membrane deformation induced by the glass pipette is not under experimental control and appears directly after the first contact between cell and pipette.

On the other hand, it should be noticed that we observed a link between shape of the patched erythrocyte and its channel activity. As shown in Fig. IV. 4. 8, in our experimental conditions, different cell shapes in the human red cell population were identified: discocytes, echinocytes, spherocytes, sphere-echinocytes. Gardos channel activity differed for different shapes (Fig IV. 4. 9, Fig. IV. 4. 10, Fig. IV. 4. 11 and Fig. IV. 4. 12). Full Gardos channel activity was observed in discocytes (Fig. IV. 4. 9), reduced Gardos channel activity was found in sphere-echinoctes (Fig. IV. 4. 10), and residual or almost no activity was recorded in spherocytes (Fig. IV. 4. 11). Echinocytes displayed no channel activity and observed recordings looked like leakage currents (Fig. IV. 4. 12). The fact that in most cases the experimentator choose in preference to patch-clamp discocyte, as it is the physiological shape of normal RBC, rather than other forms, could explain why for all fractions studied above we got the same pattern of activity. Therefore, further investigations are necessary to determine the percentage of different erythrocyte shapes in all fractions (is there difference or not?). Preliminary observations suggest that when getting older the cells become more spherical. The same situation was seen for *in vitro* storage. At day 3, echinocytes and spherocytes exceeded the number of discocytes especially in the fraction 5 (data not shown). This could also depend on the sex, age and life style of blood donor. However, all these indications should be kept in mind for the future studies.

Additionally, we observed in some cases that membrane deformation upon seal formation induces probably reorganization in cytoskeleton and results in changes of Gardos channel activity. In the patched dyscocyte, Gardos channel was recorded directly after getting good seal (in less than 10s after touching the cell with the tip

Figure IV. 4. 9



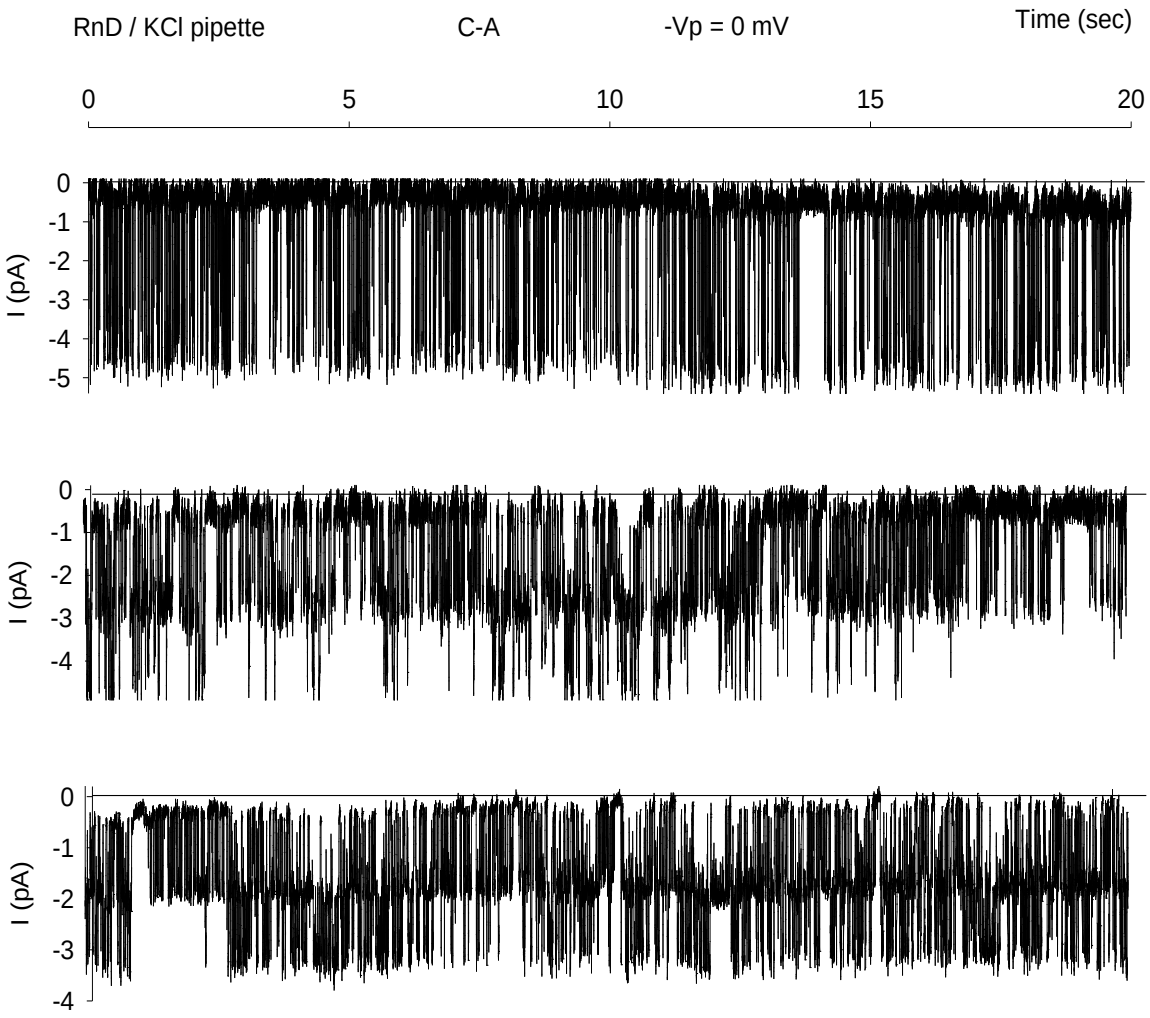


Figure IV. 4. 9: **Typical Gardos channel activity recorded in dyscocytes.**

Figure IV. 4. 10

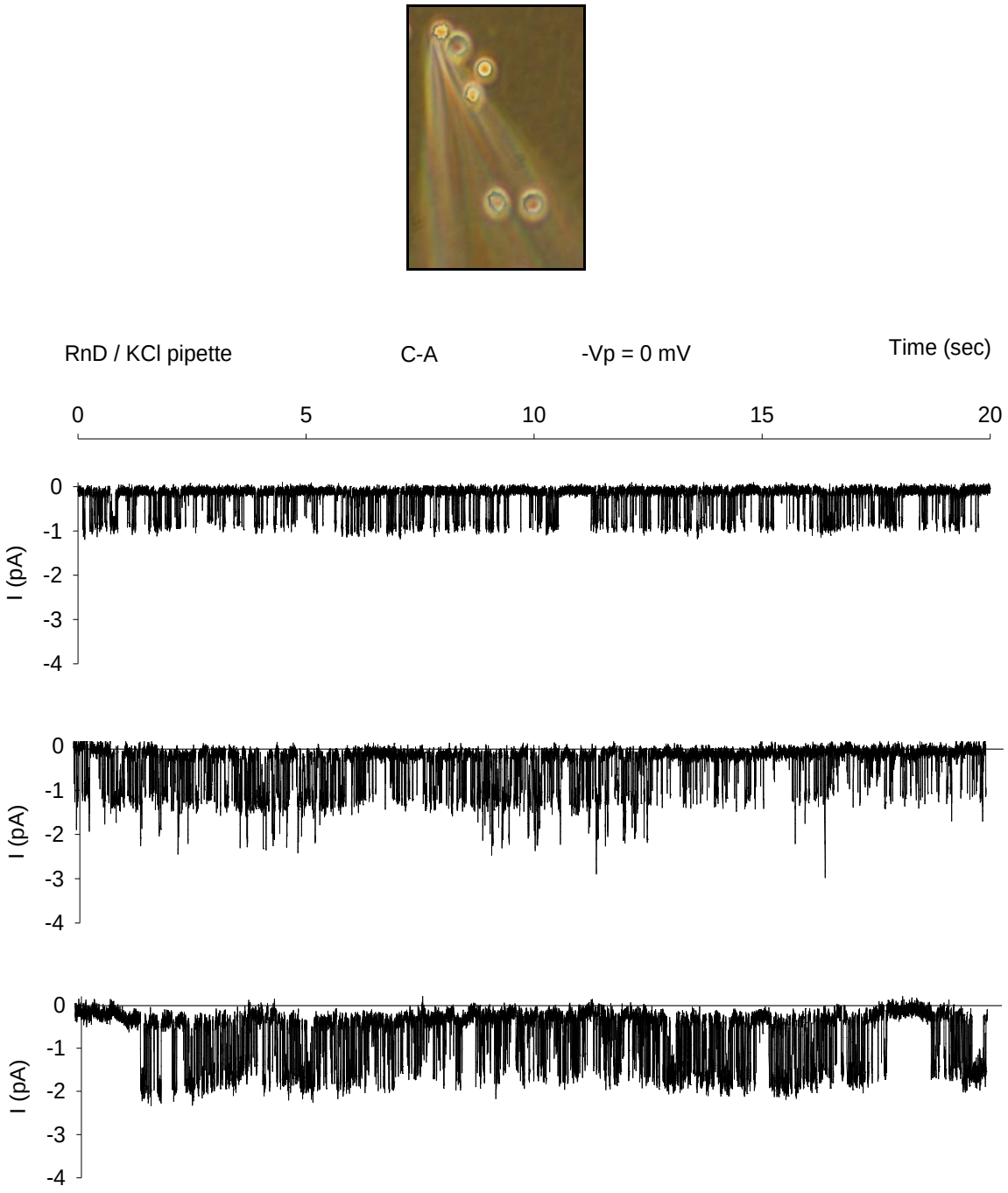


Figure IV. 4. 10: **Typical Gardos channel activity recorded in sphere-echinocytes.**

Figure IV. 4. 11

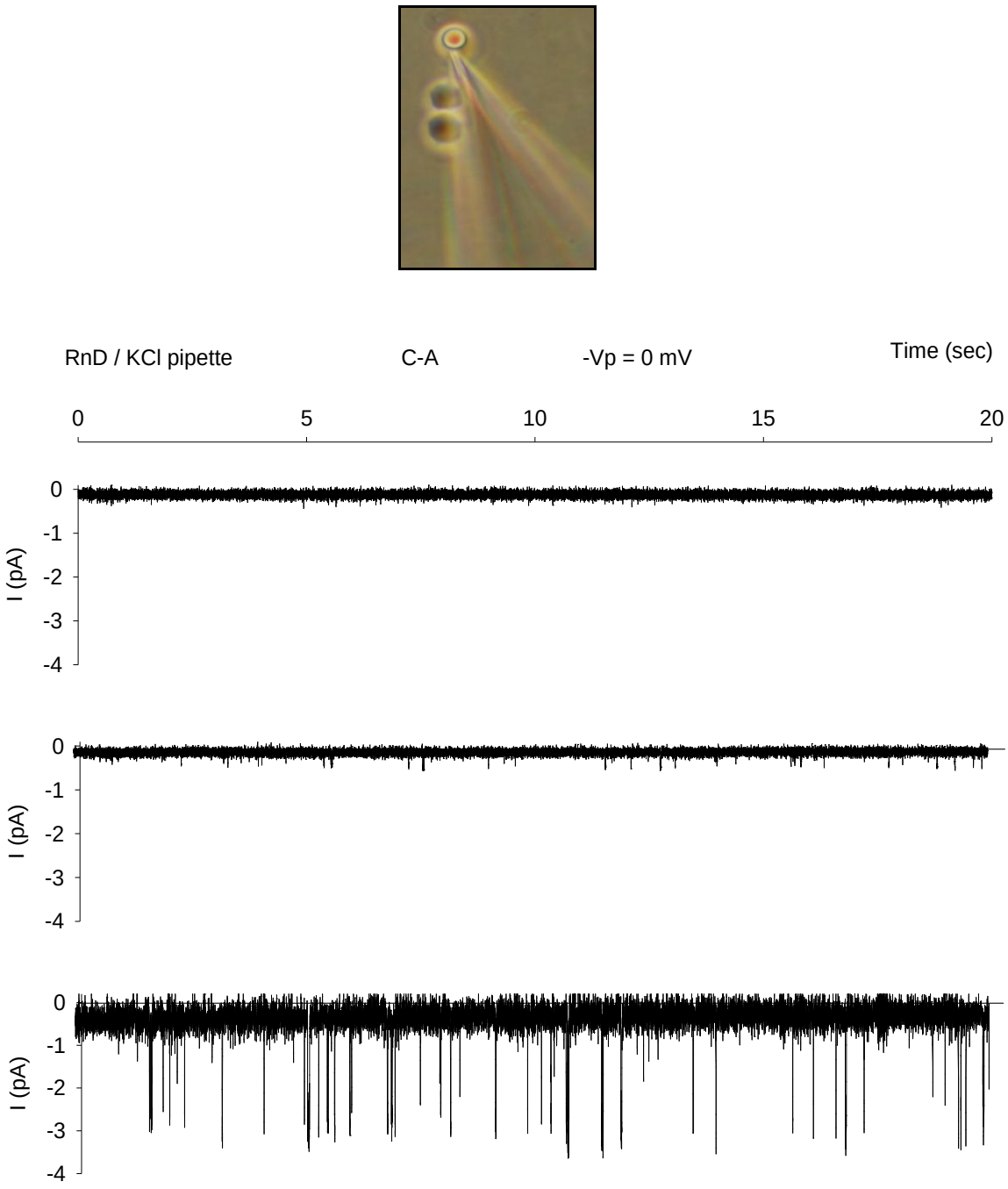


Figure IV. 4. 11: **Typical Gardos channel activity recorded in spherocytes.**

Figure IV. 4. 12

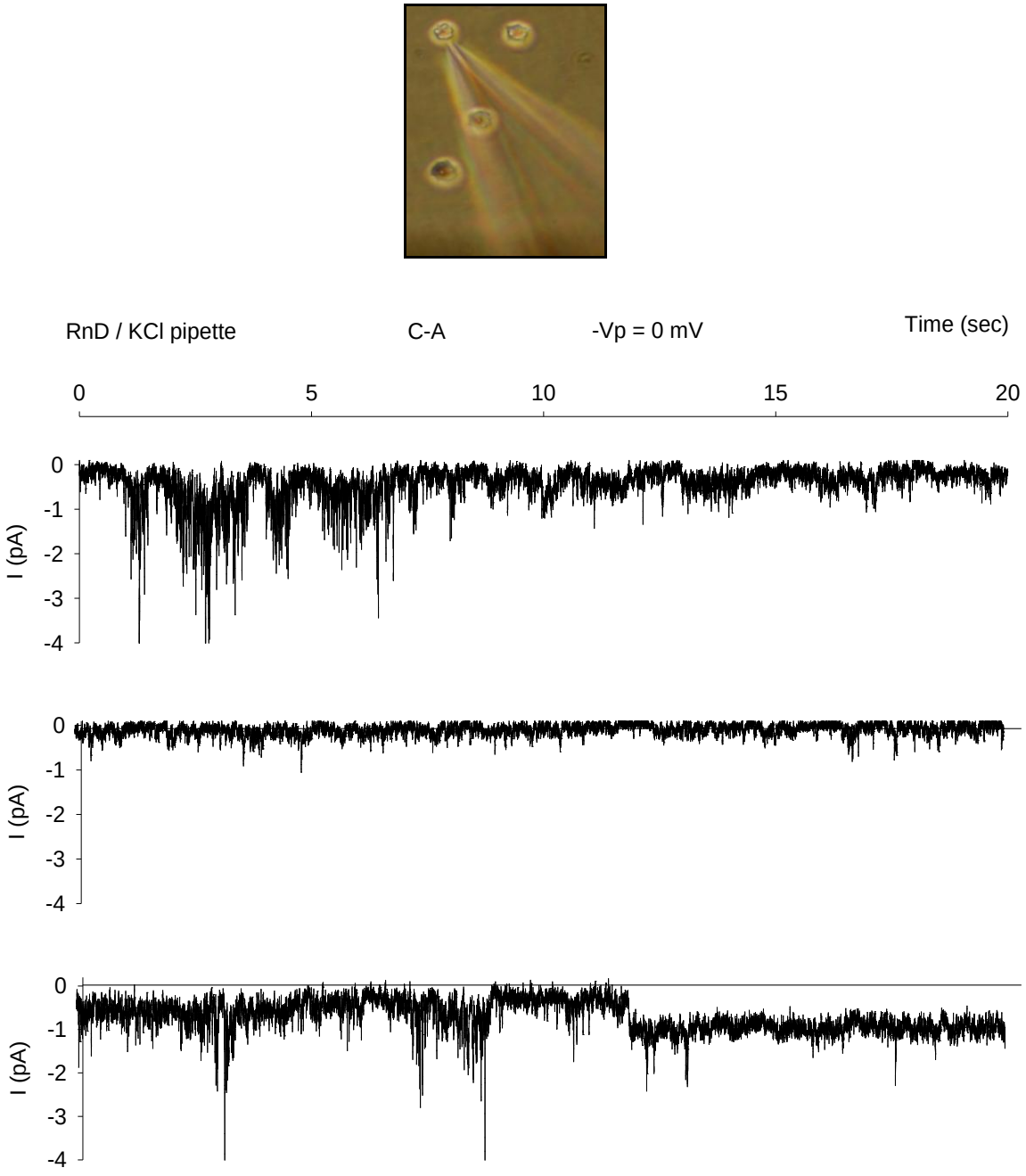


Figure IV. 4. 12: **Typical recordings observed in echinocytes.**

Figure IV. 4. 13

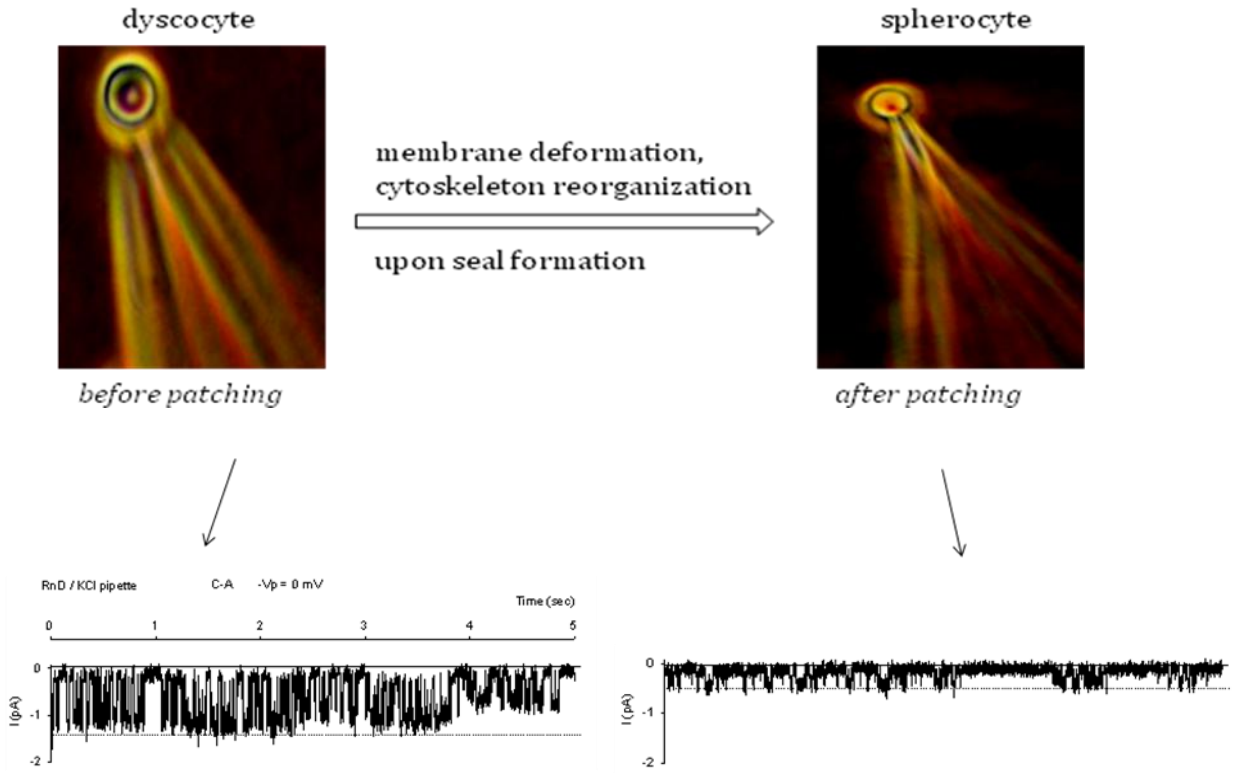


Figure IV. 4. 13: **Change in erythrocyte shape induced during the patch-clamp seal formation. Corresponding Gardos channel activity observed directly after seal formation (in patched dyscocyte) and 10s after (in spherocyte).**

of the pipette). During the first 10s change in the erythrocyte shape from dyscocyte to spherocyte was frequently observed, and subsequent decrease of Gardos channel activity was obtained. The described observations are presented in Fig. IV. 4. 13. This suggests strongly that changes in the red cell membrane properties could play an important role in the susceptibility of channels to be active.

The well documented P_{sickle} indicates that a poorly selective Ca^{2+} permeability pathway is present in human RBC and may become active in deoxygenated RBCs from sickle cell anemia patients (Ney et al., 1990; McGoron et al., 2000; Lew et al., 2002; Lew and Bookchin 2005; Lew et al., 2005; Oyewole et al., 2008). What about channel activity in malaria pathogenesis? Preliminary data suggest that Gardos channel is still active in all tested malaria strains: 3D7, RESA1-WT and RESA1-KO.

The existence of Ca^{2+}-sensitive K^{+}-selective channel in the human red blood cell membrane could lead to the first risk of disorders caused by cell dehydration. In physiological solutions, the Gardos channel activity drives the membrane potential toward the equilibrium potential for potassium and hyperpolarizes the cell, generating a favourable gradient for negative-ions (Cl^- or HCO_3^-) to leave the cell via parallel anionic conductive pathways. The resulting net loss of potassium and anions is tightly coupled to osmotic-driven water loss, leading to cell dehydration. The capacity of Gardos channels to mediate rapid RBC dehydration when fully activated far exceeds those of the other transporters. Full dehydration by maximally activated Gardos channels may take only 2 or 3 min. Fortunately, at physiological Cl^- and HCO_3^- concentrations the anionic permeability is one order of magnitude lower than potassium permeability which gives the anionic permeability a powerful restrictive effect on the speed and extent of RBC dehydration attainable during brief periods of Gardos channel activation.

With global regard to ionic channels in the human red cell membrane and their physiological role it was necessary to study in details anionic permeability together with Gardos channel. It is our preliminary hypothesis that processes discussed here may be mediated by a single cascade of channel activation. The calcium permeability pathway PCa is the first step induced by membrane deformation

as shown in shear stress or sickling. This pathway could be a specific calcium channel or a particular functional state of a VDAC channel, or permeation through any other protein of the PBR complex, or even passive diffusion through stretched RBC membrane. PCa's role has been clearly evidenced in sickle cells and is considered as a good candidate to account for periodic Ca^{2+} influx resulting from shear-stress-induced membrane deformations in the microcirculation. Concerning malaria, PCa's participation in the activation of NPP is still unclear. The second link of the chain is the Gardos channel, which has the potential to induce either fast dehydration as evidenced in sickle cell anemia or slow dehydration resulting from periodic and brief Ca^{2+} influx as suspected in cell aging. Whatever its physiological or pathological origin, increased density results in a fall in cell water content, hyperpolarization, cell acidification, increase in $[Ca^{2+}]i$, variations in redox status and modified interactions with elements of the cytoskeleton. The third phase is VDAC channel activation from an inactive mode (or perhaps PCa mode) to the cationic non-selective functional mode. A further switch from NSC mode to the large conductance anionic mode of the VDAC channel is expected in *P. falciparum*-infected cells where it could be the pathways for organic compounds, the so-called NPPs, a phenomenon never observed in any other situations where VDAC is suspected to participate.

Taken together, it appears that activation of the single Gardos channel is a threat to cellular homeostasis, but at the opposite the combined effects of the Gardos channel and anionic channel (VDAC) make possible rapid and reversible changes of ionic composition and cell volume. We do not believe that a physiological role of a process of Gardos channel activation could be to permit the cell to loose volume rapidly because volume recovery is too slow as we have shown in another work (Cueff et al., 2010). Rather, a physiological role could be to allow the cell to modify its deformability properties by permitting an increase in calcium concentration remaining in a range below the activation threshold for the Gardos channel. Or, better, the stochastic activation of large calcium permeability in a very small population of cell could permit to trigger transient anion release, notably ATP, with immediate vasodilatory effect in the narrowest of capillaries. This thereafter is more

a benefit than a threat for cellular homeostasis and may effectively influence the respiratory function of RBCs (Thomas et al., 2011) by allowing ATP release, which eventually will act as a purinergic signalling triggering factor for vasodilatation, permitting then a better oxygen capacity of the blood.

However, still many questions remain open. For instance, the pathway of Ca^{2+} entry to the cell is still unidentified. It is obvious that the activity of Gardos channel is tightly controlled by $[Ca^{2+}]i$. We have demonstrated that local deformation of the human RBC activates a calcium conductive pathway resulting in increased intracellular calcium level and subsequent Gardos channel activity. This has been supported by observations of increased Ca^{2+} permeability induced by physiological shear stress (Johnson and Gannon 1990; Johnson and Tang 1992; Johnson, 1994) and by reports of mechanosensitive calcium influx into RBCs (Cordero and Romero 2002; Bao et al., 2004; Brain et al., 2004).

Maybe, mechanosensitive channels are the pathways for calcium entry? Or maybe red cell vacuoles (vesicles), generated during red blood cell aging play a crucial role in this process? Investigations are in progress in our laboratory on this subject.

CHAPTER V

GENERAL CONCLUSIONS AND PROSPECTS

V. GENERAL CONCLUSIONS AND PROSPECTS.

V. 1. Concluding remarks.

The human red blood cell membrane is endowed with various transporters. Among them, from the point of this PhD thesis, channels are the most important: i) Ca^{2+}-dependent K^+ channels (Gardos), ii) non selective cationic channels (NSC), iii) voltage-dependent anionic channels (VDAC). These conductive pathways are activatable *in vitro* and susceptible for activation in physiological and pathophysiological conditions such as senescence, malaria and sickle cell anemia. This ability for activation results in a very high conductances (Glogowska et al., 2010) which subsequently and presumably could threat red cell homeostasis.

My studies were carried out with the intention addressed to:
1 / the electrophysiological characterization of anionic channels in red cell membrane,
2 / the molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells,
3 / the activation of anionic channels by *Plasmodium falciparum* and possible involvement of RESA1 protein in this process,
4 / physiological role of human erythrocyte channels in health and disease.

Each corresponding chapter gives details description and discusses the objectives. Summarizing:

First objective:

Further clues on electrophysiological characterization of anionic channels in human red cell membrane.

Experiments performed in this part gave description of the electrophysiological activity of anionic membrane conductance in human erythrocyte. We demonstrated existence of another pathway for anions, *maxi-anion channel* able to giving much higher conductance than ground leak mediated by electroneutral exchanger (band 3). This study showed that the diversity of anionic channel activities recorded in normal human erythrocytes corresponds to different kinetic modalities of a unique type of maxi-anion channel with multiply conductance levels, gating properties and pharmacology, depending on conditions (Glogowska et al., 2010).

Second objective:

The molecular identity and regulation of anionic channels in the physiology and pathophysiology of the human red blood cells.

To this statement the present work provides very important elements. We identified a *voltage-dependent anion channel (VDAC)*, existing in the membrane as a component of PBR (Bouyer et al., 2011). This part of the study linked together physiological and pathophysiological situations of human red cell, showing that PBR/VDAC in healthy cells is dormant and could be activated in experimental conditions leading to very high anionic conductances. After *P. falciparum* infection this channel is up-regulated to NPPs.

Third objective:

The activation of anionic channels by *P. falciparum* and possible involvement of RESA1 protein in this process.

This part of the thesis, using pathophysiological conditions of malaria infection focussed on regulation of anionic channels. We showed that proteins exported by the parasite to the host erythrocyte membrane play crucial role in activation of these channels. One such protein, RESA1 is an obvious and promising candidate which influences new permeability pathways. Additional investigations are necessary to

confirm this theory and to give more details of appropriate mechanism. For instance, experiment using immunoprecipitations with antibodies raised against the different proteins thought to be involved in NPPs (RESA1, VDAC, TSPO, ANT) could help to understand the physical relationship between these proteins.

Forth objective:

Physiological role of human erythrocyte channels: A unifying hypothesis for senescence, sickle cells and malaria.

Although many interesting things concerning channels in red cell membrane were discovered in this work still many questions remain open and the roles played by these channels are lacking. Using literature knowledge concerning senescence, sickle cell anemia and malaria we made a unifying hypothesis based on a cascade model for a dynamic role of channels in red cell physiology and pathophysiology. For that purpose additional experiments on cell aging and malaria infection were performed. The results obtained for healthy cells separated according to their age did not indicate differences in Gardos channel activity, as it could be expected but we noticed that activity depends rather on the erythrocyte shape, since we observed different channel activity in discocytes, echinocytes or spherocytes. This fact should be clarified in further studies. Another possibility is that calcium contained vesicles could be involved in channels activation. Investigations are in progress in our group to clarify this point.

In malaria, the results obtained for 3D7, RESA1-WT and RESA1-KO strains indicated Gardos channel activity at 0 mV imposed potential. This demonstrated that Gardos channels are still active in infected erythrocytes. Additionally, the role played by RESA1 protein in channel activity in RBCs, resulting from membrane stabilization, was also indicated.

V. 2. Discussion and perspectives.

The present work confirms that the human red blood cell is equipped with powerful tools to induce very rapid variations in cell volume, acid-base and electrolytes balances to effectively influence their respiratory function. My PhD thesis is help-

ing to advance the knowledge of the specific role played by ionic channels, both in the membrane of health and disease human erythrocytes. This work shows that it would be simplistic to consider that these channels are relics or residues. Even if they appear, in the current state of knowledge, rarely spontaneously active, this apparent inactivity is probably the result of experimental *in vivo* conditions which reproduce only very little mechanical and physico-chemical influences on erythrocytes in circulating blood. With a mean cardiac output of 5 L/min and a volume of blood of 5 L, the red cell of healthy adult human must perform a round trip every single minute. This means that a RBC has to squeeze approximately 160,000 times (in absence of muscular exercise) through pulmonary and tissue capillaries with a diameter lower than its own. There is increasing evidence that, in case of excessive tissue demand, a signalling pathway is activated resulting in the release of ATP acting in a paracrine fashion to increase vascular calibre. The permeability pathways or ionic channels involved in this process are not well understood yet (recent publication Sridharan et al., 2012 gives an evidence that VDAC is an ATP conduit in human erythrocytes which opens new horizonts for the further studies), but it is reasonable to consider that sequential activation process of calcium entry through PCa plus Gardos channel activation plus PBR/VDAC activation in either of its two modes (cationic or anionic) provide the cell with a necessary but rather great machinery. In addition, to matching oxygen delivery with local need, these pathways give the RBC the ability to adjust *in situ* its own membrane deformability and cell volume. Single activation of Gardos channel represents a threat for cell homeostasis. Instead, the coexistence of Gardos channel and VDAC, enabling induction of inverse volume variations, combined with efficient Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase constitute much more advantage than a threat for RBC homeostasis.

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ANNEX

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Ion channels in human red blood cell membrane: Actors or relics?

Serge L.Y. Thomas *, Guillaume Bouyer, Anne Cueff, Stéphane Egée, Edyta Glogowska, Céline Ollivaux

Centre National de la Recherche Scientifique, Université Pierre et Marie Curie Paris6, UMR 7150, Station Biologique, B. P. 74, 29682 Roscoff cedex, France

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ABSTRACT

During the past three decades, electrophysiological studies revealed that human red blood cell membrane is endowed with a large variety of ion channels. The physiological role of these channels, if any, remains unclear; they do not participate in red cell homeostasis which is rather based on the almost total absence of cationic permeability and minute anionic conductance. They seem to be inactive in the "resting cell." However, when activated experimentally, ion channels can lead to a very high single cell conductance and potentially induce disorders, with the major risks of fast dehydration and dissipation of gradients. Could there be physiological conditions under which the red cell needs to activate these high conductances, or are ion channels relics of a function lost in anucleated cells? It has been demonstrated that they play a key role in diseases such as sickle cell anemia or malaria. This short overview of ion channels identified to-date in the human red cell membrane is an attempt to propose a dynamic role for these channels in circulating cells in health and disease.

Introduction

The transport of oxygen from lungs to tissues and the reverse transport of CO_2 are mediated by two specialized molecular structures: hemoglobin and an anion exchange carrier in the red blood cell (RBC) membrane. Encapsulation of ~7.2 mmol of impermeable hemoglobin per liter of intracellular water in a cell moving within a plasma environment that has a much lower protein concentration creates a huge osmotic pressure. However, as explicitly formulated in the "pump-leak" concept [1], the risk of colloid-osmotic swelling and bursting is prevented by a very low membrane permeability to cations, allowing the pumps Na^+/K^+-ATPase and Ca^{2+}-ATPase to extrude the residual Na^+ and Ca^{2+} leaks at minimal metabolic cost. Anion movements through the RBC membrane are essentially linked to the respiratory function: a million copies of electroneutral Cl^-/HCO_3^- exchanger per cell allow 85% of the CO_2 produced in the tissues to be transported in the blood as HCO_3^- ions; one hundred copies of anion channels, allowing conductive permeation, clamp the RBC membrane potential (E_m) at the equilibrium potential for Cl^- ions (E_{Cl}), thereby permitting the exchangers to work optimally in absence of electrochemical gradients for anions movements. The molecular nature of these anion channels is still unknown and, under steady-state, their activity remains negligible which is in accord with the estimates of the whole-cell conductance in normal un-stimulated cells, i.e. about 70 picoSiemens (pS) [2] and the 40–50 pS calculated from the suspension experiments [3]. It appears from this picture that there is very little room for ionic channel activity in the maintenance of RBC homeostasis. However, it is known that the red cell membrane is endowed with a large variety of cation channels [4–8] and anion channels [2,9–14] activable under experimental conditions where they can potentially give very high single cell conductances capable to threaten homeostasis. These channels are usually dormant but have been shown to be active in some pathological situations. What does this mean? Could there be physiological conditions under which the red cell needs to activate these high conductances, or are the channels relics of a past function susceptible to be re-activated by pathogens?

Gardos channels

Among these conductive pathways, 100–200 copies of Ca^{2+} sensitive K^+ channel, also called "Gardos" channel [15] or IK1[16], are expressed in human RBC membrane [17–20]. These channels are activated by Ca^{2+} ions interacting with calmodulin when intracellular Ca^{2+} level is increased from a physiological level of 20–50 nmol l^{-1} to above a threshold of 150 nmol l^{-1} [21,22], maximal activation being obtained around 2 μmol l^{-1}. Under physiological conditions, a full experimental activation of Gardos channels induces massive outward K^+ flux and membrane hyperpolarization [23]. The sudden shift of E_m from E_{Cl} toward the equilibrium potential for K^+ ions (E_K) creates a driving force for anion extrusion through conductive permeation pathways [8,24–27]. The water loss accompanying the parallel exit of K^+ ions and anions results in dramatic dehydration. When induced experimentally, the extent of this process is totally dependent on the membrane conductive permeability for anions (P^G_A) and the K^+ efflux from the red cells shows a marked dependence on the nature of the anion present in the suspending medium. This explains why SCN^- or NO_3^- ions, more permeant than Cl^-, were frequently used to

* Corresponding author at: CNRS-UPMC, UMR 7150, Station Biologique, B. P. 74, 29682 Roscoff cedex, France. Fax: +33 298 292310.
E-mail address: thomas@sb-roscoff.fr (S.L.Y. Thomas).

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ABSTRACT

“ Electrophysiological characteristics, molecular identity, regulation and physiological role of membrane anionic channels of the human erythrocyte in health and disease. “

Red blood cells are a classic model system for studying how ions, nutrients and other solutes cross the plasma membrane. Whereas erythrocyte’s membrane transporters, i.e. exchangers, pumps and cotransporters have been well characterized, ionic channels, particular anionic conductances still remain undefined. Molecular identity, regulation and especially physiological role of these conductive pathways are unclear, in spite of the evidences of their involvement in the physiological process of senescence of RBCs, as well as in pathological conditions such as sickle cell disease or malaria.

The present work, using patch-clamp electrophysiological technique and biochemical assays show that:

1/ The diversity of anionic channel activities recorded in normal, as well as in *Plasmodium falciparum*-infected human erythrocytes, corresponds to different kinetic modalities of a unique type of maxi-anion channel with multiply conductance levels, gating properties and pharmacology, depending on conditions.

2/ The molecular identity of these anionic channels corresponds to the voltage dependent anion channel (VDAC), one of the component of the peripheral-type benzodiazepine receptor (PBR) present in the RBCs membrane.

3/ The dormant, endogenous VDAC becomes the “new permeability pathways” in infected erythrocytes after up-regulation by *P. falciparum* and is activated by the Ring-infected Erythrocyte Surface Antigen (RESA), protein exported by the parasite to the host RBC membrane.

Finally, the present work contributes to the understanding of the specific role played by the ionic channels in the RBCs membrane, both in health and disease. It shows that it would be simplistic to consider that these channels are relics or residues of a lost previous function during evolution or maturation process of erythrocytes.

Keywords: red blood cells, maxi-anion channel, voltage dependent anionic channel (VDAC), ring-infected erythrocyte surface antigen (RESA), *Plasmodium falciparum*, new permeability pathways (NPPs), patch-clamp

Résumé

“ Détermination des caractéristiques électrophysiologiques, de l’identité moléculaire, de la régulation et du rôle physiologique/patho-physiologique des canaux anioniques de la membrane des érythrocytes humains “

Les érythrocytes sont un modèle classique pour l’étude du transport des ions, des nutriments et de divers solutés au travers de la membrane cellulaire. Les agents du transport électro-neutre tels que les échangeurs et les co-transporteurs ont été particulièrement bien décrits, par contre, les voies de conductance (canaux ioniques) sont longtemps restées peu étudiés, voire ignorées. Leur identité moléculaire, leur régulation et leur rôle physiologique ne sont donc pas clairement établis malgré leur implication évidente dans des processus physiologiques comme la sénescence ou pathologiques comme la drépanocytose ou le paludisme. Le présent travail de thèse a fait appel à la technique électrophysiologique du ‘patch-clamp’ et à diverses méthodes biochimiques pour démontrer que :

1/ La diversité des courants anioniques enregistrés au travers de la membrane de l’érythrocyte humain sain, ou infecté par le parasite du paludisme *Plasmodium falciparum,* correspond à différents états d’activité d’un type unique de canal ‘maxi-anionique’ comportant des niveaux de conductance, des modes d’activation et des propriétés pharmacologiques variables selon les conditions physico-chimiques ambiantes.

2/ L’identité moléculaire de ce canal anionique est de type ‘voltage dependent anion channel (VDAC)’. Il est l’une des trois composantes d’un récepteur ‘peripheral-type benzodiazepine receptor (PBR)’ présent dans la membrane érythrocytaire.

3/ Le canal VDAC, généralement peu actif en condition expérimentale de référence, correspond, lorsqu’il est activé, à la nouvelle voie de perméation ‘new permeability pathway’ décrite dans la membrane de l’érythrocyte infecté par *P. falciparum.* L’activation résulte alors en partie de l’insertion dans la membrane érythrocytaire de protéines plasmodiales de type ‘Ring-infected Erythrocyte Surface Antigen (RESA)’.

Ce travail contribue à l’élucidation de la nature exacte des canaux ioniques présents dans la membrane érythrocytaire et avance une hypothèse unificatrice quant au rôle joué par ces canaux ioniques dans diverses situations physiologiques ou pathologiques.

Mots-clés: Erythrocytes, maxi-canaux anioniques, voltage dependent anionic channel (VDAC), ring-infected erythrocyte surface antigen (RESA), paludisme, *Plasmodium falciparum*, new permeability pathways (NPPs), patch-clamp.