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\(^2\), Serge L.Y. Thomas\(^1\)

\(^1\) Centre National de la Recherche Scientifique, Université Pierre et Marie Curie Paris6, UMR 7150, Station Biologique, B. P. 74, 29682 Roscoff cedex, France,

\(^2\) Departments of Pediatrics and Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven CT 06520-8064, USA.

**Corresponding author:** Serge L.Y. Thomas, CNRS-UPMC, UMR 7150, Station biologique, B. P. 74, 29682 Roscoff cedex, France, Phone:+ 33 298 292348, Fax:+ 33 298 292310, Email: thomas@sb-roscoff.fr

Category: Red Cells, Iron and Erythropoiesis
Abstract

*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.

**Key Words:** Erythrocyte, maxi-anion channels, PBR, VDAC, *Plasmodium falciparum*, New permeability pathways

**Abbreviations:** NPP: new permeability pathways; PBR: peripheral benzodiazepine receptor; VDAC: voltage-dependant anion channel; ANT: adenine nucleotide transporter; TSPO: translocator protein
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. Initially, this transport was attributed to “new” permeability pathways (NPPs) exported by the parasite to the host membrane. However, later studies revealed that the current is due to up-regulation of endogenous channels 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. This channel displays complex gating and kinetics similar to those of ligand-gated channels.

Anions are transported through the human erythrocyte membrane by a two-component system: a large electroneutral exchanger mediated by band 3 and a four orders of magnitude smaller electrogenic component estimated at about 10 µS/cm² corresponding presumably to a small number of channels. 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. 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.

Our previous work employing 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. These channels are dormant under normal physiologic conditions, yet upon 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, can be expressed in plasma membranes alone, or as a component of the peripheral-type benzodiazepine receptor (PBR) complex. The PBR complex consists of at least three 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). The PBR is characterized by a primary distribution in tissues outside the central nervous system and by nanomolar affinity for the ligands PK 11195 > Ro5-4864 > diazepam. The TSPO component is considered to be primarily responsible for binding to PK 11195, while Ro5 4864 and other benzodiazepines may bind to all components of the PBR complex. 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.

This report demonstrates that the three principal components of the peripheral-type benzodiazepine receptor (PBR) are present and functional in the human erythrocyte membrane. Electrophysiologic studies demonstrate that PBR is responsible for the previously described erythrocyte membrane-associated maxi-anion current. PBR ligands reduce membrane transport and conductance in P. falciparum-infected erythrocytes and block the intraerythrocytic growth of P. falciparum in vitro. These data support the hypothesis that the dormant PBR mediates the band 3-independent anion conductance in normal erythrocytes, and, after up-regulation by P. falciparum, become the “new permeability pathways” in P. falciparum-infected erythrocytes. These channels are obvious targets for selective inhibition in
anti-malarial therapies, as well as potential routes for drug delivery in pharmacologic applications.
Materials and Methods

Chemicals. 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB), Isoquinoline 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) and the Benzodiazepine 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864), diazepam, chloroquine and human serum were purchased from Sigma (Saint Quentin Fallavier, France).

Cells and cell preparation. Venous blood from healthy volunteers upon written informed consent was drawn into heparinized vacutainers. Erythrocytes were washed three times by centrifugation and resuspension in large volumes of RPMI culture medium (Gibco BRL). The buffy coat was removed by aspiration after each wash. After the last wash, the cells were suspended at 50% hematocrit in RPMI and kept at 4°C.

Ramos cells were obtained from ATCC (CRL-1596) and cultured in RPMI with 10% fetal bovine serum. Human mobilized peripheral blood CD34-selected stem and progenitor cells were obtained from the Yale Center of Excellence in Molecular Hematology Cell Core and cultured in StemSpan SF expansion medium (StemSpan 09650) with estradiol (100 ng/ml), dexamethasone (10 ng/ml), human transferrin (200 ng/ml), insulin (10 ng/ml), Flt3 ligand (100 ng/ml), stem cell factor (100 ng/ml), interleukin-3 (50 ng/ml), interleukin-6 (20 ng/ml), insulin-like growth factor 1 (50 ng/ml), and erythropoietin (3 U/ml) for 9 to 14 days. Fluorescence-activated cell sorter analysis was used to analyze the cellular expression of CD71 (transferrin receptor) and CD235a (glycophorin A). Magnetic bead selection for CD71 (MACS 130-046-201; Miltenyi Biotech) and CD235a (MACS 130-050-501; Miltenyi Biotech) was used to purify an R3/R4 population of erythroid cells.

mRNA expression. RNA was prepared from Ramos, CD34+ stem and progenitor cells, and primary cultured erythroid cells (RNeasy mini kit; Qiagen) and treated with amplification-grade DNase I. cDNA was generated using an iScript cDNA synthesis kit.
according to manufacturer instructions (BioRad). Quantitative real-time PCR (Q-PCR) was performed on BioRad CFX96 System and analysed with qbase\textsuperscript{®}\textsuperscript{15,16} software. All samples were assessed for internal RNA integrity. Q-PCR primers were designed using Primer 3 Software. For each primer pair, amplification specificity was validated by gradient, standard curve, melting curve, and gel electrophoresis. Q-PCR was performed using iQ SYBR Green Supermix (BioRad). Relative expression was normalized to geometric mean of unvarying genes, OAZ1 (ornithine decarboxylase antizyme 1), HPRT1 (hypoxanthine phosphoribosyltransferase 1), UFM1 (ubiquitin-fold modifier 1), TBP (TATA box binding protein), PUM1 (pumilio homolog 1), and RPS13 (ribosomal protein S13). 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 ± standard errors of the means. Results were normalized to the gene with the highest expression level in each group.\textsuperscript{16} This technique provides the most stringent analyses of gene expression when comparing multiple genes over multiple cell types.\textsuperscript{17} Triplicate analyses were performed for each target gene.

*Preparation of erythrocyte ghosts.* Erythrocyte ghosts were prepared by successive cell washing in 5P8 lysis buffer prepared from 5P8x100 containing 0.5M NaH$_2$PO$_4$ (pH 8) and added with a protease inhibitor cocktail (Roche Complete®). After each wash the cells were centrifuged at 25 000g for 30 min at 4°C until clear supernatant and a white opalescent pellet was obtained.

*Immunoblotting.* Erythrocyte membranes were lysed in SDS buffer (SDS 4.5%, NaPi 150mM, EDTA 3mM, DTT 1mM, pH 7.6). Two volumes of membrane extract were mixed with 1 volume of SDS buffer, homogenized for 10 min, then sonicated. Proteins were resolved via SDS-PAGE on a 10% acrylamide gel and blotted onto nitrocellulose. Antibodies used were: polyclonal goat anti-TSPO raised against the C terminus of human TSPO (Everest Biotech), polyclonal rabbit anti VDAC 1,2,3 (Santa Cruz Biotechnology) and polyclonal
rabbit anti-ANT (Santa Cruz Biotechnology). Nitrocellulose membranes were incubated in a blocking solution consisting of non-fat milk in Tris buffer saline tween 20 (TBST) to avoid nonspecific binding. After labelling with rabbit and goat secondary antibodies conjugated with horseradish peroxidase (Santa Cruz), enhanced chemiluminescence detection (Typhoon, GE Health Care Life Sciences) was performed.

Immunoprecipitation. Erythrocyte ghosts were lysed in RadioImmunoPrecipitation Assay (RIPA) buffer, sonicated, then centrifuged at 10000g for 10 min. The supernatant was immunoprecipitated for 30 min with an anti-VDAC1, then incubated with μMACS protein G microbeads for another 30 min at 4°C. The magnetically labeled immune complex was loaded in a M column (Miltenyi Biotec), washed with RIPA buffer and eluted with Laemmli buffer. Samples were subjected to SDS-PAGE on 10% acrylamide gels, stained with colloidal Coomassie blue, and subjected to Western blot analysis for control and to NanoLC MS/MS for protein identification.

NanoLC MS/MS analysis. Analysis was performed by Innova Proteomics (Rennes, France). Mass spectra of VDAC, ANT, TSPO and PBR were obtained using a nanoLC-LTQ-Orbitrap-XL: nanoLC Ultimate 3000 (Dionex) and LTQ-Orbitrap-XL (Thermo Electron). All spectra were processed by the software Proteome Discoverer 1.0 (Thermo Scientific) with combined analysis via Sequest (Thermo Scientific) et Mascot (Matrix Sciences) and Peaks algorithms for protein identification.

Immunostaining. Analyses were carried out on intact RBC according to Campanella and co-workers.18 Cells were washed twice in PBS containing 5mM glucose, and then fixed for 5 min in 0.5% acrolein in PBS. They were rinsed three times then permeabilised in PBS containing 0.1M glycine (rinsing buffer) plus 0.1% Triton X-100 for 5 min and again rinsed 4X 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 blocking buffer (PBS
containing 0.05mM glycine, 0.2% fish skin gelatin (GE-Healthcare). Staining of fixed, permeabilized RBCs was performed by using antibodies diluted in blocking buffer. Primary antibodies used were: mouse anti-porin 31HL (Calbiochem), goat anti-TSPO Nter and goat anti-ANT Nter (Santa Cruz). The secondary antibodies were goat anti-mouse and donkey anti-goat both coupled to AlexaFluor 488nm (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 de Roscoff, BP 74, Place Georges Teissier, 29680 Roscoff.

Controls were performed using a primary antibody generated against the Gardos channel protein known to have similar expression levels (approx. 100-200 copies per cell). A goat IK1-Cter antibody (Santa Cruz) was used as primary antibody and goat anti-Alexafluor 488 (Molecular probes) as secondary antibody. Three sets of additional negative controls were carried out. A first series of experiments, where primary antibody was omitted, validated the specificity of the secondary antibody raised against the primary antibody. A second set, where the primary antibody was replaced by the corresponding non immune IgG (goat and mouse) at the same final concentration, showed negative staining, giving evidence for the specificity of the primary antibody. A third set, where the primary antibody was blocked by the corresponding peptide (when commercially available), showed the specificity of the antibody.

Plasmodium growth assays. Plasmodium falciparum 3D7 cultures were synchronized at ring stage by two successive exposures to a 5% (W/V) sorbitol solution at 48h intervals. Parasitemia was adjusted to 1.5%, and cultures were grown for 72h in 96-wells plate in triplicate. Hematocrit was 2% with increasing drug concentrations in the culture media. Half the drug-containing culture media was replaced at 24 and 48h. At 72h, parasitemia was evaluated using flow cytometry according to the protocol previously described,19 using a Cell
Lab Quanta™ SC (Beckman Coulter) cytometer equipped with a plate reader. Briefly, a TRIS-saline solution with 138mM NaCl and 20mM TRIS (pH 8.8) was prepared, filtered on 0.22µm membrane. SYBR Green I (Sigma) was added to a final concentration of 1.5X, and solution was distributed to a 96-wells plate (300µl/well). 2 minutes before reading, cultures were resuspended, and 15µl of culture was added to each wells of the former plate. Analysis of cell populations (infected /non infected cells) was performed using Cell Lab Quanta® SC MPL Analysis software (Beckman Coulter). At least 20 000 cells (infected or non infected) were counted for each well.

Sorbitol hemolysis. For standard semi-quantitative hemolysis assays, hemoglobin release was used to estimate lysis time. Culture suspensions (2-5% parasitemia) were washed three times in culture medium without serum and resuspended at 50% hematocrit. Time courses started with the addition of a 0.4 ml aliquot of cell suspension to 3.6 ml of the sorbitol iso-osmotic solutions (300 mM sorbitol, 10 mM hepes, 5 mM glucose, pH 7.4) to give a cell concentration of approximately 0.5.10⁸ cells/ml. Experiments were performed in triplicate. At predetermined intervals (0, 2.5, 5, 10, 15, 30, 60 min), 0.5 ml aliquots of the suspension were transferred to microcentrifuge tubes containing 0.5 ml of ice-cold “stopping solution” (400 mM sucrose in H₂O). Tubes were centrifuged for 30 seconds. Next, 0.2 µl of the supernatant solution was transferred into 96-well plates for spectrophotometric estimation of hemoglobin concentration by absorption at a wavelength of 540 nm (A₅₄₀). In all experiments, the A₅₄₀ value corresponding to full hemolysis of trophozoite-infected erythrocytes was estimated from the final A₅₄₀ value achieved in the supernatant solution from infected cells suspended in an iso-osmotic sorbitol. When drugs were tested, the percentage of inhibition was determined relative to non-treated cells when hemolysis was at maximum. Data analyses were carried out according to Krugliak and Ginsburg.²⁰ The % 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 % lysis, $a$ is the maximal lysis, $x$ is the sampling time, $x_0$ is the t1/2 of lysis, and $b$ is the variability of cells in the population.

**Electrophysiology.** The whole-cell configuration of the patch-clamp technique was assessed by the development of small capacitance transient and reduction of access resistance. Cation movements across the membrane from the exterior (bath) to the cytoplasmic side is defined as inward current and shown as downward deflection in whole cell recordings. Seal resistances were 4–20 GΩ. Patch pipettes (tip resistance 10–20 MΩ) were prepared from borosilicate glass capillaries (GC150 TF-10, Clark Medical Instruments, Phymep, France) pulled and polished on a Werner Zeitz DMZ programmable puller (Augsburg, Germany). The ruptured patch whole-cell configuration was used to record whole-cell currents. Whole-cell currents were recorded using a RK400 (Biologic, France) amplifier, with voltage command protocols generated and the currents analyzed using the WCP Software (WCP V3.3.3. Software, Strathclyde, UK) 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. Data for the construction of $I–V$ curves were the mean current measured between 200 and 400 ms. Since temperature has no influence on whole cell currents in infected erythrocytes (data not shown), all experiments were performed at room temperature.

**Statistical analyses.** Data are given as mean values ± S.E.M. Significance was assessed using the Fisher $F$ test and Student’s $t$ test.
Results

Our previous work demonstrated that a unique type of maxi-anion channel with multiple conductance levels mediates band 3-independent anion conductance across the erythrocyte membrane.\textsuperscript{5} To address our hypothesis that this anion conductance is mediated by a voltage dependent anion channel (VDAC), we sought molecular and physiologic evidence for a VDAC in the erythrocyte. Because VDACs may exist alone or as part of a PBR complex consisting of VDAC, TSPO, and ANT proteins, we performed quantitative RT-PCR of mRNA isolated from cultured human primary erythroid cells, CD34+ hematopoietic stem and progenitor cells, and Ramos cells, a Burkitt lymphoma cell line, using primers specific for the 3 known VDAC genes (VDAC1, VDAC2, VDAC3), the 2 known TSPO genes (TSPO, TSPO2), and the 3 of the 4 known ANT genes (ANT1, ANT2, ANT3). ANT4 has been shown to be testis-specific. PBR expression has previously been described in hematopoietic cells, particularly lymphoid-derived cells,\textsuperscript{21} thus CD34+ hematopoietic stem/progenitor cells and Ramos cells (lymphoma cells) were chosen as parallel hematopoietic cell types for study. In the test RNA samples, the OAZ1 gene amplicon demonstrated the least variability, thus all data was normalized to OAZ1. All three VDAC genes were expressed in erythroid cells, with expression of VDAC 2 and 3 predominating (Fig.1). Both TSPO genes were highly expressed in erythroid cells, even though TSPO2 was not expressed in stem or lymphoid cells (Fig.1). There were low levels of ANT1 expression and higher levels of ANT2 and ANT3 expression in erythroid cells (Fig.1). There was significant expression of all 3 ANT genes in lymphoid cells.

We next sought evidence of a VDAC protein in the human erythrocyte membrane either alone or interacting with ANT and TSPO proteins to form a PBR complex. The evidence for VDAC being present in the membrane, among the proteins present in the RBC membrane (Fig.2A), is given by 4 bands appearing between 29-30 kDa and 36 kDa on Western blots.
(Fig.2B). The different isoforms described in the VDAC literature indicate that VDAC isoforms correspond to molecular weights ranging between 30 and 36 kDa which is in keeping with the 4 lower bands visible in Fig.2B. In addition, experiments using the VDAC1 antibody (data not shown) show a faint band at 29-30 kDa and a marked band at 58 kDa indicating that this isoform is predominantly present as dimers. Therefore the 58 kDa band of Fig.2B may reasonably be interpreted as VDAC1 dimers. With regard to trimers, if any, they should be located in a range varying between 90 and 110 kDa. This range is also the range of weight corresponding to the Band 3 protein. In this context, interpretation of the large band appearing between 90 and 120-130 kDa on Fig.2B remains difficult but we cannot exclude that this band corresponds to oligomerization of VDAC proteins or to monomers associated with the band 3 protein. Figure 2B also demonstrates the presence of the two 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 at the expected size.

In another set of experiments, because a few hundred copies of proteins were not detected by mass spectrometry with total extracts, membrane extracts were immunoprecipitated with an anti-VDAC1 (Nter) and complexed with protein G-coated microbeads, then subjected to SDS-PAGE stained with Coomassie blue. The 29-36 kDa bands showed only a very faint signal, consistent with the presence of very low levels of proteins in the RBC membrane. Nevertheless, NanoLC MS/MS analysis of this band clearly identified ANT1,2,3 proteins as well as VDAC3 which co-precipitated with VDAC1. TSPO was only detected with a low level of certainty.

Finally, we used immunostaining for localization of the three PBR proteins (Fig.2C). The use of anti-porin 31HL antibody demonstrated the presence of VDAC in the RBC membrane. The same conclusion was obtained for TSPO using anti-TSPO (NCter) antibody
and for ANT using anti-ANT (Nter) antibody. These results were confirmed by experiments using anti-VDAC1-Nter (Santa Cruz) and anti-TSPO-Cter (Everest Biotech) (data not shown) and indicate that the above results are not due to contamination with other blood cells.

These data are consistent with previously published reports. Analysis of mRNA expression data from GeneAtlas U133A indicate that 3 isoforms of VDAC, 2 isoforms of ANT and 2 isoforms of TSPO have been found in progenitor cells (http://biogps.gnf.org). VDAC3, TSPO and ANT expression is ubiquitous and essentially nonchanging at the median level from CD34+ cells to CD71+ progenitor cells. A population of receptors with nanomolar affinity for PK 11195 was found in all blood cells in rank order of density: lymphocytes > platelets > erythrocytes and the population of binding sites in erythrocytes was evaluated to 110 ± 22 per cell. TSPO protein and VDAC protein have been described in the erythrocyte membrane.

Because the NPP, allowing both organic and inorganic anions, electroneutral molecules and organic and inorganic monovalent cations to pass, displays many common characteristic features with the PBR/VDAC and because the ligands of PBR and are known as potent inhibitors of *Plasmodium falciparum* growth in infected RBCs *in vitro* 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. Cultures of the malaria parasite *Plasmodium falciparum* were exposed for 72 hrs to increasing concentrations of PBR ligands PK11195, Ro5-4864 and diazepam. Growth inhibition curves are shown in Fig.3A. The effects of ligands were compared to those of the reference antimalarial drug chloroquine (CQ) and inhibitor of anionic channels NPPB. The dose dependence curves show that although the inhibitory effects of the ligands remained far below the CQ effect, the IC$_{50}$ for PK11195 was ~ ten times lower than for NPPB whilst Ro5-4864 and diazepam displayed similar IC$_{50}$ as NPPB, in the range 100 µM. We cannot discard the
possibility that at the concentrations used PBR ligands act on non-PBR systems, but if this interaction was via any inhibitory effect of channel activity, we could expect a reduction of cell swelling and hemolysis occurring when infected cells containing the new permeability pathways are exposed to isotonic solutions containing sorbitol. This appearance of increased permeability to otherwise impermeable solutes, was recorded more than two decades ago.\textsuperscript{2,27} Figure 3(B-D) shows that the percentage of lysis in cells exposed simultaneously to isotonic solutions containing sorbitol and to various concentrations of the three ligands is considerably decreased/delayed. Data analysis carried out according to Krugliak and Ginsburg method\textsuperscript{20} allowed accurate quantification of the t1/2 of lysis that is inversely proportional to the permeability and of maximal extent of lysis. The derived half-times (t1/2) plotted against the ligand concentration showed that the membrane permeability was significantly decreased (P<0.001) when concentration reached 50 µM, 1 µM and 10µM for PK11195, Ro5-4864 and Diazepam, respectively. In addition, the maximal extent of lysis declined significantly (P<0.001) at 100 µM, 50 µM and 100 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.3 (E,F), an infected RBC displays a reduction in whole-cell membrane conductance at both positive and negative potentials over the first 15 min following addition of PK11195 at the concentration of 100 µM. The dose-dependence of inhibition evoked after only 15 min by the three ligands is presented in Fig.3 for inward (G) and outward (H) currents and confirms that the immediate reduction in the membrane permeability of \textit{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. With regard to the quickness of the observed
response, hemolysis and electrophysiology experiments suggest a direct effect on channel activity rather than an effect on parasite fitness.
Discussion

Seeking clues for the molecular identification of the RBC native maxi-anion channel, this study links together for the first time the presence of PBR proteins in the RBC membrane and the NPP up-regulated in infected RBC. The experimental data substantiate the hypothesis that dormant PBR/VDAC become the so-called “new permeability pathways” in infected erythrocytes after up-regulation by *P.falciparum*.

A basic RBC membrane anionic conductance of less than 100 pS,\(^{28}\) is in fair agreement with the value calculated from experiments on cell suspensions\(^ {29}\) and indicates that most of the time, the channel carrying the anionic conductance is deactivated, but that it could be transiently activated. The molecular nature of the gating mechanism is still unclear, but activation could take place by voltage changes as is the case for VDAC, caused for example by transient activation of the Gardos channel upon membrane deformation as recently shown.\(^ {30}\) 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).\(^ {31}\) Highest VDAC conductance states are observed at low potentials with a marked preference for anions (e.g. thiocyanate, phosphate, and chloride), whereas the selectivity is favourable to small cations at higher positive or negative potentials.\(^ {31}\) Mitochondrial VDAC is located at the interface between the mitochondria and the cell cytosol and appear to control the fluxes of ions and metabolites in and out of the mitochondria.\(^ {32}\) The same role could be played by VDAC in the PBR complex between the RBC interior and the extracellular milieu in health and in disease.

In health, depending on the conformational state, this channel could be involved in a large range of dynamic changes in red cell homeostasis and membrane permeability.\(^ {32}\) We may conclude from our experiments that VDAC forms a PBR complex with TSPO and ANT molecules clearly identified in the RBC membrane despite their low quantity. A population of
approx. 100-150 PBR complexes is more likely since it fits with our previous
electrophysiological evaluation of anion channels and with the calculated number of
receptors with nanomolar affinity for PK 11195. It is likely that a hundred copies of a
pathway for small ions (Na+, K+, Cl-,...) as well as large anions (glutamate, ATP,...)
large cations (Tris,...) and divalent cations, such as Ca2+, plays an important role in the
physiological processes of metabolite transport and volume regulation in the erythrocyte. It
is tempting to explain the observation that the oldest RBCs are light, high in Na and low in
K by final activation of VDAC in its low-conductance (cationic) mode, thus reversing the
densification trend prevailing in younger cells.

We previously demonstrated that the channels up-regulated by P. falciparum are of
endogenous origin and, since the present work demonstrate that PK11195, Ro5-4864 and
diazepam block parasite growth and induce rapid reductions in permeation and conductance,
we suggest that the new permeability pathways are largely carried by PBR/VDAC even
though we cannot discard the possibility that ANT and TSPO contribute in the global
conductance. Early pharmacological studies using isotopic fluxes and sorbitol lysis
experiments came to the conclusion that NPP is a large poorly selective anion channel with a
selectivity SCN>I>Br>Cl>acetate>lactate>glutamate corresponding to the Eisenman
sequence number1 for anions and also allows sugars, purines, amino-acids and organic and
inorganic cations to pass, as is the case for VDAC. NPP displays a selectivity over 0.6 kDa
for organic solutes similar to VDAC (almost 1.0 kDa) they both allow cation
movements, carry ATP and are modulated by serum components, oxydo-reduction
states and PKA-dependent phosphorylation. The pore radius of NPP was estimated at
0.70 nm and the VDAC pore radius calculated to be 0.85 nm. The fact that NPP properties
resemble those of VDAC provides further support to the conclusion that the two channels are
one and the same protein. Thus, a single species of multipotent channel able to transport
different structurally unrelated solutes has the potential to meet the parasite requirements for
selective nutrient uptake, removal of waste products and volume regulation by playing
sequentially with a wide range of conductances, selectivities and permeabilities controled by
several modulators such as small molecules (calcium\textsuperscript{37,38}, glutamate,\textsuperscript{34} reactive oxygen
species\textsuperscript{51}), kinases or associated proteins.\textsuperscript{48,49} Although it appears that NPPs are not parasite-
engendered channels as previously suggested,\textsuperscript{3} it is conceivable, in the light of recent
findings,\textsuperscript{52} that some parasite-encoded proteins such as those encoded by the two clag\textsuperscript{3}
genes, contributes to up-regulation of this native pathway.\textsuperscript{53} In our electrophysiological
single-channel recordings, it is evident that the NPP are permanently active at the low
conductance levels but may display occasionally (in \textit{approx.} 5\% of recordings) brief episodes
of high conductance initially attributed to superimposition of another type of outwardly
rectifying anion channel.

The present work demonstrates that peripheral-type benzodiazepin receptors are an
important new contributing factor for consideration in understanding red cell physiology and
pathophysiology of malaria and other diseases.\textsuperscript{54} It validates the PBR complex as an
antimalarial target and suggests that the pharmacopoeia of benzodiazepine as well as
benzodiazepine scaffolds for the production of new inhibitors\textsuperscript{55} could become a novel strategic
approach for future antimalarial chemotherapies.
Acknowledgments

We thank Drs J.F. Hoffman, P. Bennekou and V.L. Lew for invaluable advice and help during the course of this work. In addition we thank D. Vaulot and D. Marie (Station Biologique de Roscoff) for expert assistance in flux cytometry experiments. This study was supported by research funding from ANR (-08-MIEN-031-02) and the Doris Duke Charitable Foundation (PGG). EG is supported by ANR (-08-MIEN-031-02).

Authorship

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.

The authors declare no conflict of interest.
References


Figure legends

**Figure 1: 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 $\Delta \Delta CT$ method (where $CT$ is threshold cycle), with results presented as means ± standard errors of the means. Results were normalized to the gene with the highest expression level in each group. Triplicate analyses were performed for each target gene.

**Figure 2: VDAC, ANT and TSPO detection in human red blood cell ghosts**

Samples (15 µg 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 (1:1,000 dilution), rabbit polyclonal anti VDAC 1-2-3 (1:100 dilution) or polyclonal goat anti-TSPO raised against the C terminus of human TSPO (1:1,000). 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. It is to be noted that, according to the supplier assessment (Everest Biotech), the TSPO protein could not be expected at 18 kDa but rather at 36 kDa. These blots are representative of 12 replicates. Immunofluorescence experiments (C) were performed on
smears prepared as described in Material and methods. Dilution were 1/5 for primary and 1/20 for secondary antibodies. Scale bars represent 10µm.

**Figure 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 h at 37°C. Their effects were compared to effects of antimalarial drug chloroquine (CQ) and anionic channels 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 three separate experiments.

(B,C,D) In sorbitol lysis experiments, culture suspensions were prepared as described in Materials and Methods. The effects of diazepam (B), PK11195 (C) and Ro5-4864 (D) added at t = 0 min of lysis experiments at concentrations below and above their IC₅₀ were tested by comparison to non-treated cells when haemolysis was at maximum at t = 60 min. Each count was made in triplicate and each point on the curves correspond to the mean (± SEM) of three 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 (VT) from -100 to +100 mV in 10 mV steps for 500 ms from a holding potential (VH) of 0 mV in the whole-cell configuration of the patch-clamp technique before and 15 min after addition of a ligand. The examples of panels E and F were obtained before and 15 min after addition of 100 µM PK11195 to the bathing solution containing (in mmol/l) 115 NaCl, 5 KCl, 10 MgCl₂,
5 CaCl₂, 10 Hepes, 10 glucose, 1% human serum, pH 7.4. The pipette solution contained 155 NMDG-Cl, 1 MgCl₂, 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 three different ligands were assessed by calculating the percent 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 1
Figure 2
Figure 3
Erythrocyte peripheral type benzodiazepine receptor/voltage-dependent anion channels are upregulated by *Plasmodium falciparum*

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