Malaria and host erythrocyte channels

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ABBREVIATIONS

2-MeSADP: 2-methylthioadenosine 5´diphosphate

2-MeSATP: 2-methylthioadenosine 5´triphosphate

ADP: adenosine 5´ diphosphate

ADPβS: adenosine-5'-O-(2-thiodiphosphate)

ApnA: diadenosine polyphosphate

ATPγS: adenosine 5´-O-(3-thiotriphosphate)

ATP: adenosine 5´ triphosphate.

Bz-ATP: 2´ & 3´-O-(4-benzoylbenzoyl) adenosine 5´ triphosphate

BSA: bovine serum albumin

Ca^{2+}: calcium ion

$[Ca^{2+}]_i$: intracellular calcium concentration

$CaCl_2$: calcium chloride

CFTR: " Cystic fibrosis transmembrane conductance regulator "

Cl^-: chloride ion.

DIDS: 4,4'-diisothiocyanostibene-2,2' disulfonic acid

EGTA: ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid

$GdCl_3$: gadolinium chloride

HEPES: 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

K^+: potassium ion

KCl: potassium chloride

KGlutamate: potassium glutamate

NaCl: sodium chloride

NaGlutamate: sodium glutamate

NMDG-Cl: N-methyl-D-glucamine chloride

NPPs: New Permeability Pathways

NPPB: 5-nitro-2-(3-phenylpropylamino)-benzoic acid

nS: nanoSiemens

pA: picoAmpere

PKA: protein kinase A

PPADS: pyridoxalphosphate-6-azophenyl-2'-4'-disulfonate

pS: picoSiemens

RBCs: red blood cells

RVD: Regulatory Volume Decrease

***t*-BHP**: t-butylhydroxyperoxide

TEA: tetraethylammonium

TNP-ATP: 2',3'-*O*-(2,4,6-trinitrophenyl ATP)

UTP: uridine triphophate

SUMMARY

Malaria is caused by intracellular protozoan of the genus *Plasmodium*. The parasite is transmitted by the female *Anopheles* mosquito and within the human host it develops first in liver cells and then in erythrocytes. Parasitized human erythrocytes (RBCs) acquire new membrane permeabilities (*New Permeability Pathways; NPPs)* to meet the needs in nutrients and disposal of waste products of the intensively growing parasite. *In vitro* pharmacological inhibition of the *NPPs* results in parasite death. Therefore, the *NPPs* are potential targets for antimalarial drugs. In addition, the *NPPs* serve for entry routes of other drugs into the RBC thus delivering the drugs to the intraerythrocytic parasite. Moreover, a fraction of the *NPPs* has recently been shown to be activated in non-infected RBCs during aging and to trigger programmed cell death.

Functionally, the *NPPs* are organic osmolyte and anion channels with additional low but significant cation permeability. Therefore, suspending parasitized RBCs in isosmotic sorbitol solution leads to sorbitol entry, colloidosmotic swelling and eventually hemolysis. The rate of hemolysis in isosmotic sorbitol solution reflects the activity of the *NPPs*. Non-infected RBCs, in contrast, are not sorbitol permeable and stay intact when bathed in isosmotic sorbitol solution. *NPPs* and sorbitol hemolysis can also be induced in non-infected RBCs by oxidative stress. Upon oxidation and during infection the *NPP*s develop slowly suggesting complex signaling and reorganization of the oxidized/parasitized RBCs

This study investigates the role of ATP and purinoceptors in the induction of the *NPPs* upon oxidation or during *Plasmodium* infection by the use of hemolysis experiments, ATP luminescence assay, FACS analysis, patch-clamp whole-cell recordings, parasite *in vitro* growth assays and *in vivo* malaria infection of mice.

As a result, both, oxidation and *Plasmodium* infection, induce ATP release from the RBCs into the medium. Extracellular ATP and further purinoceptor agonists increase and antagonists (e.g. MRS2179, $P2Y_1$ receptors specific antagonist) as well as extracellular ATP degradation by apyrase decrease the induction of the sorbitol hemolysis in oxidized or parasitized RBCs. These data suggest the involvement of $P2Y_1$ and further metabotropic purinoceptors in the induction of the *NPP*s.

Accordingly, human RBCs express $P2Y_1$ protein and $P2Y_1$-deficient mouse erythrocytes exhibit a decreased *P. berghei* infection- or oxidation-induced sorbitol hemolysis as compared to their wildtype litter-mates.

Moreover, the non-specific purinoceptor antagonist suramin decreases *in vitro* the intraerythrocytic parasite amplification and DNA/RNA synthesis of *P. falciparum* as well as the induction of the *NPPs* in the membrane of the parasitized RBC. Furthermore, *P. berghei*-infected $P2Y_1$-deficient mice exhibit lower parasitemia and higher survival rates as compared to their wildtype litter mates.

Finally, this study focuses on the possible role of the *NPPs* for the programmed death of human RBCs. Aged RBCs express features characteristic for apoptosis in nucleated cells such as cell shrinkage, membrane blebbing and breakdown of the phosphatidylserine asymmetry of the plasma membrane. Programmed RBC death is triggered by the increased activity of a non-selective Ca^{2+}-permeable cation channel which – as an identified fraction of the *NPPs* - is also activated in *Plasmodium*-infected RBCs. Accordingly, the present study demonstrates that *Plasmodium* infection induces breakdown of the phosphatidylserine asymmetry of the parasitized RBCs, a process mimicked in non-infected cells by increased cytosolic free Ca^{2+} concentrations.

In conclusion, this study shows that the induction of the *NPPs* in *Plasmodium*-infected erythrocytes involves ATP release and purinoceptors signaling. It also demonstrates for the first time the protein expression of $P2Y_1$ receptors by human erythrocytes. Since chemical oxidation mimics the processes leading to the *NPPs* formation during *Plasmodium* infection, oxidative processes are probably involved in the signaling between the intraerythrocytic parasite and the RBC membrane. The inhibitory effect of purinoceptor antagonist and $P2Y_1$ deficiency on parasite development *in vitro* and *in vivo*, respectively, strongly suggests functional significance of the purinoceptor signaling for the malaria infection in human beings.

ZUSAMMENFASSUNG

Malaria wird durch intrazelluläre Protozoen der Gattung *Plasmodium* verursacht. Der Parasit wird durch weibliche Anopheles-Mücken übertragen. Im menschlichen Wirt entwickelt er sich zuerst in Leberzellen und dann in Erythrozyten. Infizierte humane Erythrozyten erwerben dabei neue Membran-Permeabilitäten (*New Permeability Pathways; NPPs),* über die sowohl der Parasit ernährt wird als auch dessen Abfallprodukte entsorgt werden. Pharmokologische Hemmung der *NPPs* tötet den Parasiten *in vitro* ab. Deshalb könnten die *NPPs* mögliche Zielstrukturen einer neuartigen Therapie gegen Malaria sein. Zusätzlich fungieren die *NPPs* als Eintrittspforten, über die weitere Antimalaria-Medikamente in den Erythrozyten zu den intrazellulären Parasiten gelangen können. Darüberhinaus werden Teile der *NPPs* auch in nicht-infizierten alternden Erythrozyten aktiviert, wodurch der programmierte Zelltod des Erythrozyten ausgelöst wird. Funktionell sind die *NPPs* organische Osmolyte- und Anionen-Kanäle, die auch sehr niedrige aber signifikante Kationen-Permeabiltäten aufweisen. Werden infizierte Erythrozyten in isosmotischer Sorbitollösung suspendiert, gelangt Sorbitol über die *NPPs* in die Zellen; die Erythrozyten schwellen kolloidosmotisch und hämolysieren letztendlich. Die Hämolysenraten in isosmotischer Sorbitollösung spiegeln dabei direkt die Aktivitäten der *NPPs* wider. Nicht-infizierte Erythrozyten sind dagegen nicht permeabel für Sorbitol und bleiben intakt, wenn sie in isosmotischer Sorbitollösung inkubiert werden. Die Sorbitol-Hämolyse und die *NPPs* können auch in nicht-infizierten Erythrozyten durch Oxidation induziert werden. Während Oxidation und Infektion entwickeln sich die *NPPs* langsam. Dies deutet auf komplexe Signalabläufe während des Umbaus des oxidierten/infizierten Erythrozyten hin. Diese Doktorarbeit untersuchte die Rolle von ATP und Purinozeptoren bei der Induktion der *NPPs* während Oxidation oder Plasmodium-Infektion unter Verwendung von Hämolyse-Experimenten, ATP-Lumineszenz-Bestimmungen, FACS-Analyse, Patch-Clamp Ganzzellableitungen, Parasiten *in vitro*-Wachstumstests und *in vivo* Malariainfektion von Mäusen. Als Ergebnis zeigt die Arbeit, dass sowohl Oxidation als auch *Plasmodium*-Infektion ATP-Freisetzung aus den Erythrozyten in das Medium induzierte. Ferner erhöhten extrazelluläres ATP und weitere Purinozeptor-Agonisten die Induktion der Sorbitol-Hämolyse in oxidierten und infizierten Erythrozyten

während Purinozeptor-Antagonisten sowie Degradation von extrazellulärem ATP durch Apyrase diese erniedrigten. Diese Daten deuten auf eine Beteiligung von $P2Y_1$ und weiteren metabotropen Purinozeptoren an der Induktion der *NPP*s hin. Damit übereinstimmend konnte die $P2Y_1$ Protein-Expression in humanen erythrozyten nachgewiesen werden. Ferner zeigten $P2Y_1$-defiziente Mauserythrozyten erniedrigte *P. berghei*-Infektion- bzw. Oxidation-induzierte Sorbitol Hämolyse im Vergleich zu Erythrozyten der Wildtyp-Geschwister. Auch hemmte der unspezifische Purinozeptor-Antagonist Suramin *in vitro* die intraerythrozytäre Parasitenvermehrung und -DNA/RNA-Synthese sowie die Induktion der *NPPs* in der Membran des infizierten Erythrozyten. Darüberhinaus zeigten *P. berghei*-infizierte $P2Y_1$-defiziente Mäuse eine niedrigere Parasitämie und längere Überlebenszeiten als ihre Wildtypgeschwister. Schlussendlich befasste sich diese Arbeit mit einer möglichen Rolle der *NPPs* für den programmierten Zelltod humaner Erythrozyten. Gealterte Erythrozyten zeigen Merkmale von apoptotischen kernhaltigen Zellen wie Zellschrumpfung, Abschnüren von Membranbläschen und Zusammenbruch der Phospholipid-Asymmetrie der Plasmamembran. Der programmierte Erythrozytentod wird durch die erhöhte Aktivität von nicht-selektiven Ca^{2+}-permeablen Kationenkanälen ausgelöst. Diese oder sehr ähnliche Kationenkanäle werden – als kleine Fraktion der *NPPs* - auch in *Plasmodium*-infizierten Erythrozyten aktiviert. Die vorliegende Arbeit konnte zeigen, dass die Plasmodium-Infektion den Zusammenbruch der Phospholipid-Asymmetrie des Wirtserythrozyten induzierte, ein Prozess, der in nicht-infizierten Zellen durch erhöhte zytosolische freie Ca^{2+} Konzentrationen imitiert werden konnte. Zusammenfassend zeigt die vorliegende Arbeit die Beiteiligung von ATP-Freisetzung und Purinozeptoren an der Induktion der *NPPs* in *Plasmodium*-infizierten Erythrozyten. Sie demonstriert auch zum ersten Mal die Expression von $P2Y_1$ Rezeptoren in humanen Erythrozyten. Oxidative Prozesse sind sehr wahrscheinlich an der Kommunikation zwischen intraerythrozytärem Parasiten und Erythrozytenembran beteiligt, da chemische Oxidation diejenigen Prozesse imitiert, die zur Bildung der *NPPs* während der *Plasmodium*–Infektion führen. Der hemmende Effekt von Purinozeptor-Antagonisten und $P2Y_1$-Defizienz auf die Parasitenentwicklung *in vitro* bzw. *in vivo* weist auf die funktionelle Signifikanz der Purinozeptor-Signaltransduktionskaskade für die Malariainfektion beim Menschen hin.

INTRODUCTION

1/ Malaria: general features and *Plasmodium spp.* life cycle.

A/ Introduction.

Malaria is the world's most prevalent vector-borne disease; approximately 300 to 500 million people worldwide are affected by malaria (over 90% of them in Africa according to the WHO, 2003) and it causes between 1 and 1.5 million death every year with a very high childhood mortality rate. It is estimated that 41% of the world's population is at risk. The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. The situation has become even more complex over the last few years with the increase in resistance to the drugs normally used to combat the parasite that causes the disease.

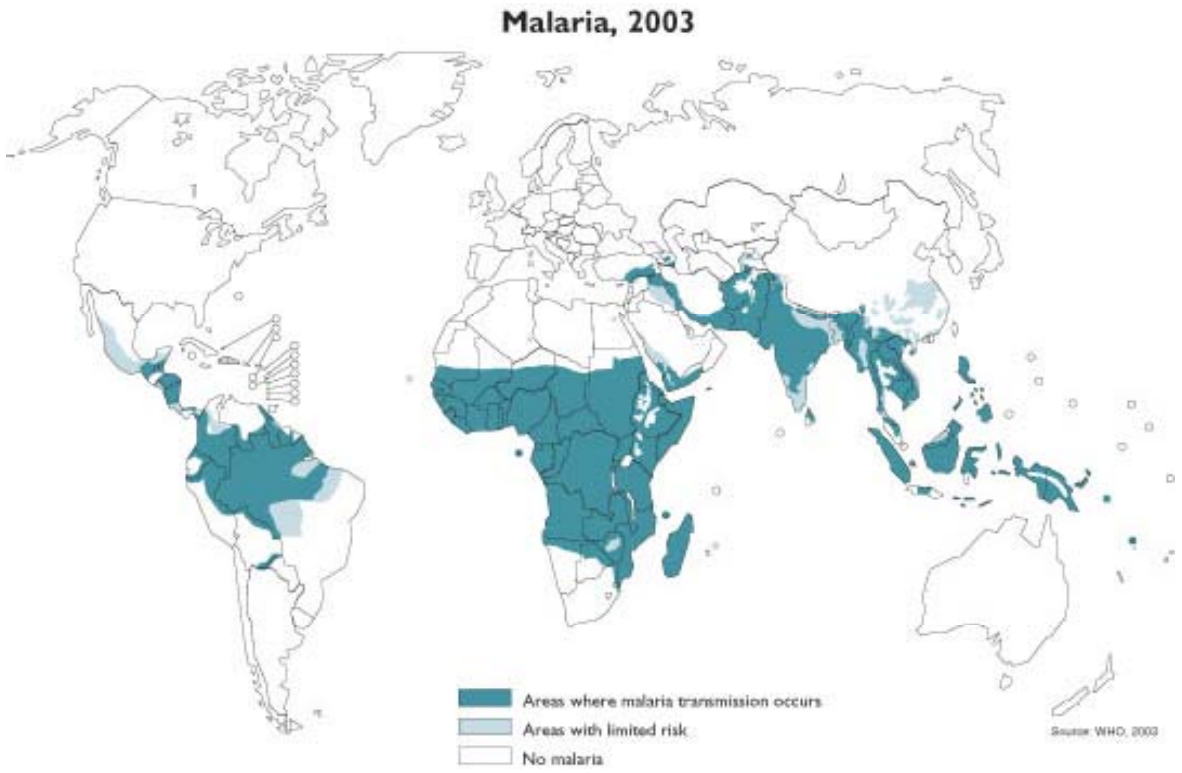


Fig. 1 Malaria distribution in 2003 according to the World Health Organisation (www.who.int)

Malaria is an endemic disease caused by intracellular protozoan parasites of the genus *Plasmodium*. Protozoa are unicellular eukaryotic organisms, which, during the course of a complex life cycle, invade the red blood cells of their vertebrate hosts. Four species of *Plasmodium* can produce the disease in its various forms:

- *Plasmodium falciparum.*
- *Plasmodium vivax* (Indian sub-continent and Central America).
- *Plasmodium ovale* (relatively unusual outside Africa).
- *Plasmodium malariae* (found in most endemic areas especially sub-Saharan Africa).

P. falciparum is the most widespread parasite species in Africa, New Guinea and Haiti and the most dangerous of the four. Untreated it can lead to fatal cerebral malaria. The prevalence of *Plasmodium falciparum* and *Plasmodium vivax* infection is similar in Asia, Oceania and South America.

Malaria parasites are transmitted from one person to another by the female *Anopheles* mosquito (Fig. 2). The males do not transmit the disease as they feed only on plant juices. There are about 380 species of Anopheles mosquito, but about 60 are able to transmit the parasite. Like all other mosquitoes, the *Anopheles* breed in water, each species having its preferred breeding grounds, feeding patterns and resting place. Their sensitivity to insecticides is also highly variable.

Fig. 2 *Anopheles* mosquito

Plasmodium develops in the gut of the mosquito and is passed in the saliva of the female infected insect to the host´s blood each time it takes a new blood meal.

The malarial form is called sporozoite (uninucleated, lancet-shaped, approximately 1X7µm). The sporozoites circulate in the blood for a short time (about 30 minutes) and then settle in the victim's liver where they invade the parenchymal cells and multiply, this stage is known as pre-erythrocytic or exoerythrocytic schizogony (Fig. 3).

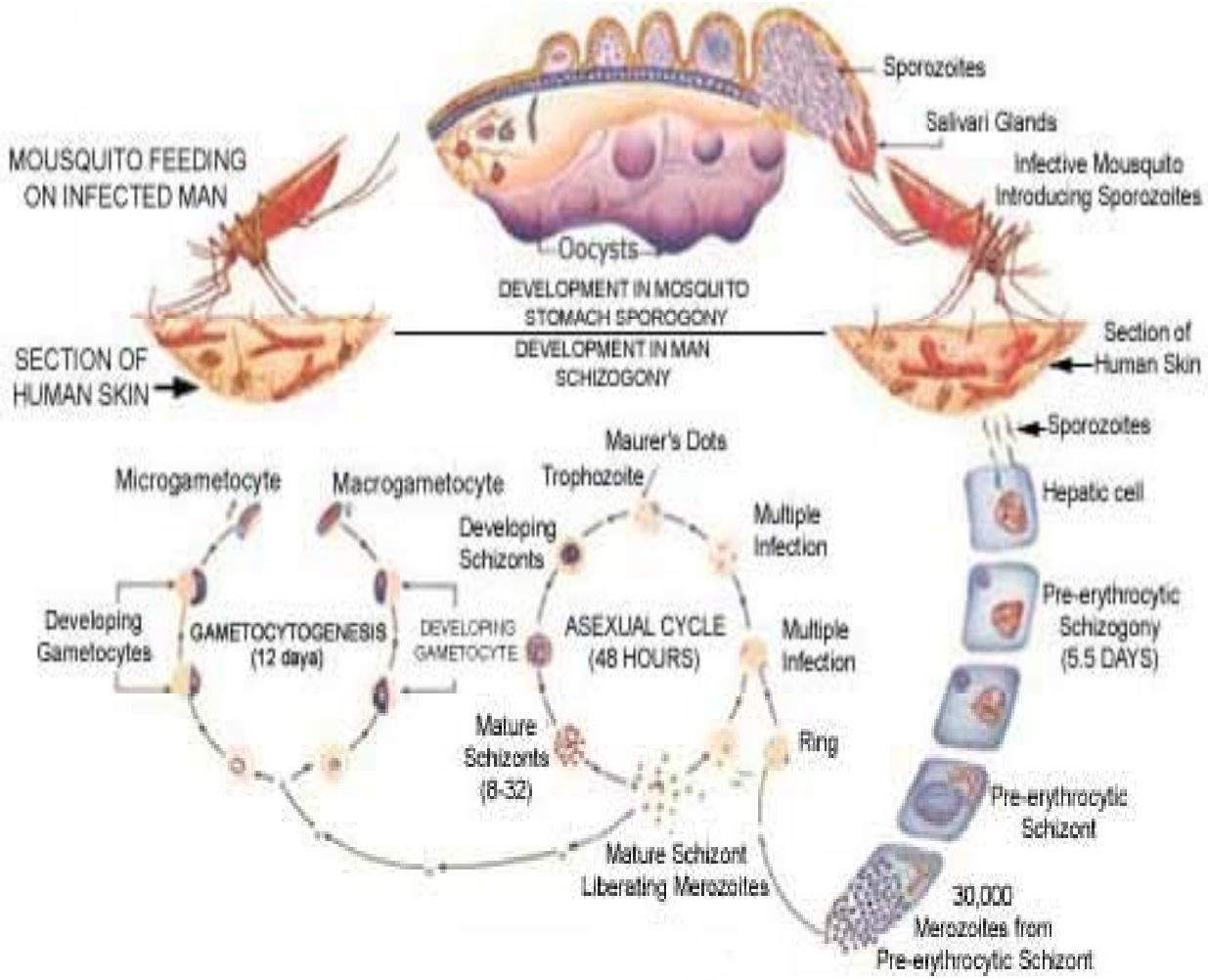


Fig. 3 Life cycle of *Plasmodium falciparum* (World Health Organisation).

B/ Asexual and sexual cycle (exo and intraerythrocytic schizogony).

- The liver stage (exoerythrocytic schizogony).

The sporozoites enter a replication phase and multiply into spherical, multinucleate liver-stage schizont which contain 2,000 to 40,000 uninucleate merozoites. This phase is asymptomatic and lasts between 5 and 21 days,

depending on the species of *Plasmodium (in P vivax* and *P ovale* infections, maturation of liver-stage schizonts may be delayed for as long as 1 to 2 years). The liver cells then lyse and free merozoites pass into the blood. Each merozoite can then infect one erythrocyte. (Sherman, 1998)

-The blood stage (intraerythrocytic schizogony).

Inside the red blood cells, the parasites develop into two forms: an asexual and a sexual form. This strategy of living inside the red blood cells of its host helps the parasite to protect from the harmful or lethal effects of antibodies or cellular defence mechanisms. Once the merozoite has entered the erythrocyte, it differentiates within the parasitophorous vacuole from ring forms (the parasite indeed appears as a thin ring) to trophozoite (~ 15 h after invasion). At this stage, the erythrocyte has lost its smooth biconcave discoid aspect and becomes more spherical and covered of small electro-dense protusions on its surface called “knobs” (Langreth *et al.*, 1978; Sherman *et al.*, 2004). These morphological changes are accompanied by a progressive increase of metabolic and biosynthetic activity of the parasite (Sherman, 1983; Kirk *et al.*, 1991). 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 rupture and release merozoites ready to invade new red blood cells. This cycle lasts approximately 48 h for *P. falciparum*.

The sexual cycle results in the production of male and female gametocytes, which are taken up by the female *Anopheles* with the blood meal (Sinden & Smalley, 1979). Within the mosquito’s gut, the male and female gametocytes fuse to form a zygote, which multiplies and differentiates into sporozoites within the salivary gland. The *Anopheles* mosquito can now initiate the cycle again.

C/ Clinical features of malaria infection.

The symptoms of malaria are recurrent chills, fever and sweating. In typical cases, the symptoms peak roughly every 48h, when successive generation of merozoites are released from infected erythrocytes. Malaria also leads to anaemia, weakness and splenomegaly (Sherman, 1998). The large number of merozoites released can eventually block the capillaries, causing intense headaches, renal

failure or obstruct the blood vessels in the brain causing cerebral malaria which is mostly fatal (Sharma *et al.*, 1995). Malaria is diagnosed by the clinical symptoms and microscopic examination of the blood. The symptoms (fever, shivering, pain in the joints and headache) can normally be cured by antimalarial drugs and quickly disappear once the parasite is killed. In certain regions, however, the parasites have developed resistance to certain antimalarial drugs, particularly chloroquine. Patients in these areas require treatment with other more expensive drugs. Cases of severe disease including cerebral malaria require hospital care.

2/ Membrane transport in the non-infected erythrocytes.

The control of red blood cell (RBC) volume, pH, membrane potential and ion content results from the interaction of many passive and active transport systems, cytoplasmic buffers, and from the charge and osmotic properties of hemoglobin and other impermeant solutes (Fig. 4).

The human erythrocytes possess a range of transporters and channels, which contribute to maintenance of the cell integrity, its stability and deformability in response to shear forces of blood circulation. The transporters also play a role in the major function of the erythrocytes i.e. the O_2 delivery from the lung to the tissues and the CO_2 removal from the tissues to the lungs. Table 1 and 2 provide the normal erythrocyte biophysical parameters and the electrolyte composition of plasma.

Band-3 (or AE1) is the major erythrocyte membrane protein (approximately 1 million of copies per erythrocyte); it is a Cl^-/bicarbonate anion exchanger. Band-3 activity enhances the blood CO_2-carrying capacity and assists in acid–base homeostasis (LaCelle & Rothsteto, 1966; Gunn *et al.*, 1973; Cabantchik, 1999). By interacting with lipids and proteins, AE1 tethers the membrane skeletal multiprotein complex to the membrane and confers mechanical integrity and viscoelasticity upon RBCs, allowing them to withstand the shear forces of circulation and squeeze through capillaries (Jay, 1996).

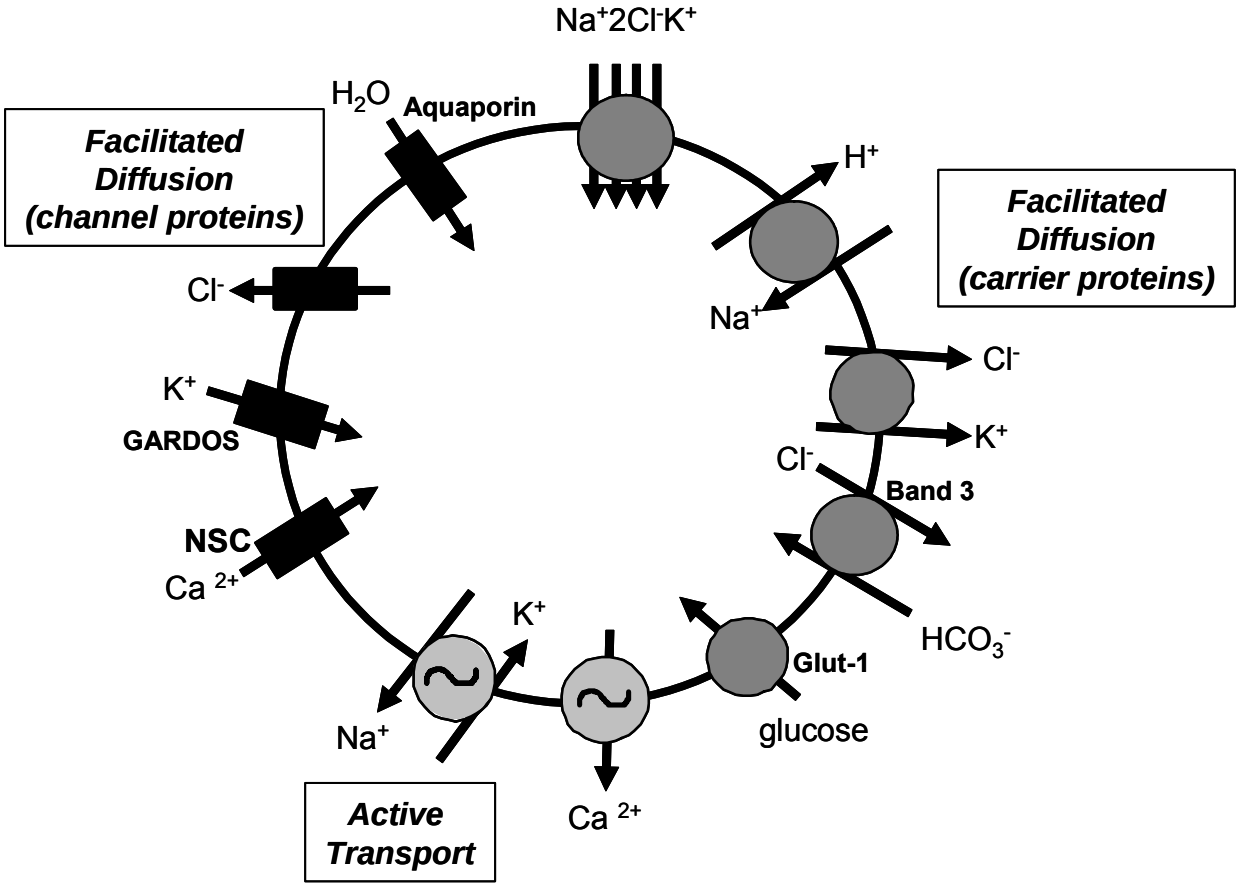


Fig. 4 Principal channels and transporters of human erythrocytes.

Principal membrane transporters are aquaporin 1 (a water channel), Glut1 (a glucose transporter), a Ca^{2+} ATPase (a calcium pump), some amino acids transporters such as the glutamate transporter, a Na^+/H^+ exchanger (NHE1), an oxidized gluthatione (GSSG) transporter (GSTP), a nucleoside transporter (NT1), a urea transporter and K^+, Na^+ and Ca^{2+} channels. The erythrocyte possesses at least four well-characterised K^+ transport mechanisms: the KCl cotransporter, a NaK2Cl cotransporter, a Na^+/K^+ ATPase (a sodium pump) and a Ca^{2+}-activated K^+ channel. The activation of the Ca^{2+} -sensitive K^+ channel (also called Gardos channel) has been demonstrated to play a key role in the regulation of cell volume (Gardos, 1958; Lew *et al.*, 1982; Brugnara *et al.*, 1983). This K^+ selective channel plays an important role in sickle-cell dehydration (Bookchin *et al.*, 1991; Lew & Bookchin, 1991) and may confer protection against immune hemolysis (Halperin *et al.*, 1989). When fully activated, these channels cause membrane hyperpolarization near the K^+ equilibrium

potential, providing a driving gradient for rapid KCl and water loss, rate-limited by Cl^- permeability.

The activity of a non selective cation channel (NSC) has also been described in human erythrocyte by using patch-clamp techniques (Christophersen & Bennekou, 1991; Bennekou, 1993; Kaestner *et al.*, 1999; Kaestner *et al.*, 2000). Recently, it has been shown that oxidation of the RBC membrane (Duranton *et al.*, 2002) or energy depletion (Duranton *et al.*, 2002) stimulate also a non selective cation conductance which promotes apoptosis and finally hemolysis of human erythrocytes. Energy depletion presumably impairs the replenishment of GSH and thus weakens the antioxidative defence of the erythrocytes (Mavelli *et al.*, 1984; Bilmen *et al.*, 2001). The non selective cation channels are further activated by removal of intracellular and extracellular Cl^- (Huber *et al.*, 2001; Duranton *et al.*, 2002). They are probably identical to the Na^+ and K^+ permeability activated by incubation of human erythrocytes in low ionic strength (LIS) medium (LaCelle & Rothsteto, 1966; Jones & Knauf, 1985; Bernhardt *et al.*, 1991). The nonselective cation channels are permeable to Ca^{2+} (Kaestner *et al.*, 2000; Duranton *et al.*, 2002). Cellular influx of Ca^{2+} through the cation channels stimulates the Ca^{2+} sensitive K^+ channels in erythrocytes as previously described (Brugnara *et al.*, 1993; Pellegrino & Pellegrini, 1998; Shindo *et al.*, 2000). The activation of the channels leads to hyperpolarization of the cell membrane driving Cl^- in parallel to K^+ into the extracellular space. The cellular loss of KCl favours further cell shrinkage and presumably participates in the triggering of erythrocyte apoptosis (Lang *et al.*, 2004).

ATP is especially needed for the activity of the calcium and the sodium pumps, which play an important role in the maintenance of the cell volume. The human erythrocyte indeed maintains a high intracellular K^+ concentration (around 140 mM) and low intracellular Na^+ concentration (around 5 mM) through a well-characterized pump-leak mechanism (Tosteson & Hoffman, 1960). Na^+ is pumped out of the cell, and K^+ is pumped into the cell via the ouabain-sensitive Na^+-K^+-ATPase (Post *et al.*, 1967), which thereby generates electrochemical gradients for both ions (see Table 1 for the normal electrolytes blood concentrations). The pumping counterbalances the "leak" of the two ions down their respective concentration gradients via various cotransporters, exchangers, and channels. The net result, in normal human erythrocytes, is a steady-state cytoplasmic [Na^+]-to-[K^+] ratio of 0.12-0.16 (Bernstein,

1954). Furthermore, erythrocytes make use of the concentration and/or electrical components of this gradient to energize the movement of different solutes across the membrane via secondary active transporters (i.e., symporters and antiporters). The Na^+/H^+ exchanger is an example of one such transporter which plays a key role in the regulation of intracellular pH, using the energy in the Na^+ gradient to extrude H^+ (Kaloyianni *et al.*, 2001).

Cations :		
- **Sodium Na^+ ……**	142	
- **Potassium K^+ …… ……**	4.5	150 mmol/l
- **Calcium Ca^{2+}……..**	2.5	
- **Magnesium Mg^{2+} ……..**	1	
Anions :		
- **Chloride Cl^- ……**	104	
- **Bicarbonate HCO^{3-} ……..**	24	
- **Phosphate HPO_4^{2-} ……**	2	150 mmol/l
- **Proteins ……**	14	
- **Others ……**	6	

Table 1 Normal values of the major electrolytes of human plasma (from the manual (Blacque Belair, 1991)).

Hematocrit	**Men: 40-54%** **Women: 37-47%**
pH	**7.38 to 7.42**
Hemoglobin concentration	**Men: 160 g/l** **Women: 140 g/l**
Erythrocyte count	**Men: $5*10^6$/µl** **Women: $4.5*10^6$/µl**
MCV (mean corpuscular volume)	**Men: 80-98 fl** **Women: 96-108 fl**
MCH (mean corpuscular hemoglobin)	**17-31 pg**
MCHC (mean corpuscular hemoglobin concentration)	**32-36%**

Table 2 Normal human blood erythrocyte parameters (from the manual (Blacque Belair, 1991)).

3/ *Plasmodium* infection and erythrocyte membrane changes.

To accomplish nutrient uptake and disposal of waste products the malaria pathogen *Plasmodium falciparum* permeabilizes host-erythrocytes for a large variety of solutes (Kutner *et al.*, 1982; Ginsburg *et al.*, 1983), including organic and inorganic anions (Kirk *et al.*, 1994; Ginsburg & Kirk, 1998; Staines *et al.*, 2001b), cations (Kirk & Horner, 1995b; Staines *et al.*, 2000; Staines *et al.*, 2001a; Duranton *et al.*, 2003), carbohydrates, amino acids, nucleosides (Sherman, 1983; Ginsburg *et al.*, 1985), and small peptides (Ginsburg *et al.*, 1985; El Tahir *et al.*, 2003; Lew *et al.*, 2004). A parallel increase of the host erythrocyte membrane permeability and of the intraerythrocytic parasite metabolic and biosynthetic activity (from 12-16 h post-invasion onwards) has been observed. The *in vitro* growth of *P. falciparum* in culture indeed requires the presence in the culture medium of a hexose sugar, a purine source (Sherman, 1979; Sherman & Greenan, 1984), seven amino acids (cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine) (Polet & Conrad,

1968) and panthotenate (Divo *et al.*, 1985). A major question arises then to know whether the increased membrane permeability is due to an increase in the activity of endogenous host cell systems or if new permeability pathways are created from parasite-encoded proteins exported to the RBC membrane.

A/ Induction of the New Permeation Pathways (NPPs).

Many studies have reported an increased transport in *P. falciparum* infected erythrocyte with characteristics similar to the endogenous transport systems found in non-infected human erythrocytes. For example, glucose, nucleosides and some amino acids have been shown to enter the RBC via endogenous transporters at a rate which is probably sufficient to meet parasite's metabolic requirements (Kirk *et al.*, 1996; Krishna *et al.*, 2000).

Nevertheless, for some specific nutrients, transport via endogenous transport systems of the host RBC is too slow to meet the parasite's developing needs. For a few nutrients (e.g. glutamate or panthotenic acid) the RBCs lack the appropriate transporters, the increased permeability induced by *P. falciparum* infection shows functional (pharmacological, kinetics) characteristics quite different from that of the endogenous erythrocyte membrane transport systems: these permeabilities have been called New Permeation Pathways (*NPPs*) (Ginsburg *et al.*, 1983; Kutner *et al.*, 1987; Cabantchik, 1990; Kirk *et al.*, 1991; Thomas & Lew, 2004). Two research groups, Hagai Ginsburg in Jerusalem and Kiaran Kirk in Oxford have characterised the *NPPs* by mainly using two transport measurement techniques, radiotracer flux and hemolysis measurements.

The main characteristics of the *NPPs* are defined below:

- They are induced between 10 and 20 h post erythrocyte-infection (Elford *et al.*, 1985) and remain stable until the end of the development of the parasite.
- They are permeable to a wide variety of low-molecular weight solutes (Ginsburg *et al.*, 1983; Sherman, 1983; Kirk *et al.*, 1991). As we can see in Fig. 5 the *NPPs* present a major permeability for Cl^- and for the negatively charged compounds but they are also permeable to electroneutral solutes (such as sugars) and present a weak but measurable permeability for cations.

The rate of permeation seems influenced by the size and the hydrophobicity of the solute.

- They are inhibited by a broad variety of anion transport blockers such as NPPB, furosemide and glibenclamide (Breuer *et al.*, 1987; Kutner *et al.*, 1987; Kirk & Horner, 1995b, a). *In vitro*, pharmacological inhibition of the *NPPs* results in parasite death (Kirk, 2001). An established method used to study the specificity and the selectivity of the *NPPs* (cf. Material and methods part) is to suspend the *P. falciparum* infected erythrocytes in an isosmotic solution of compounds such as sorbitol which pass through the *NPPs* freely but have little or no permeability in the non infected erythrocytes together with some potential inhibitors of the *NPPs* (Lambros & Vanderberg, 1979; Ginsburg *et al.*, 1983).
- The *NPPs* have been found to be non-saturable at physiological concentrations (up to 10 mM).

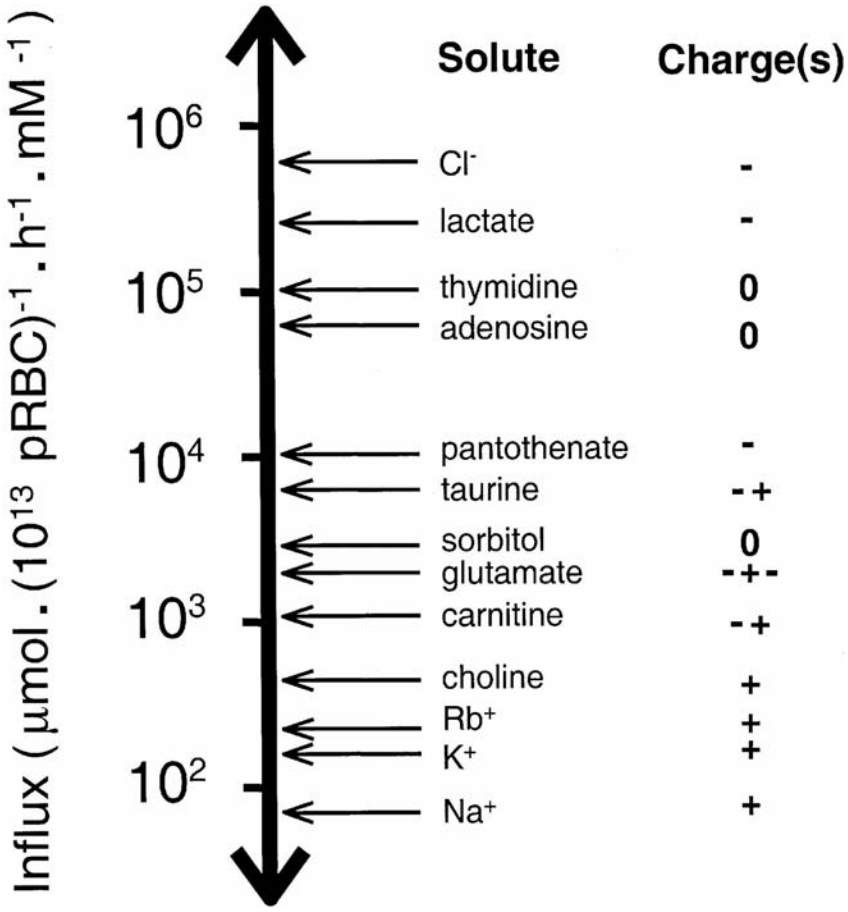


Fig. 5. Relative rates of transport of solutes through the *NPPs* from (Kirk, 2001).

In recent years, electrophysiological techniques, such as patch-clamp, have been used to characterise changes in the conductance properties of the RBC membrane following malaria infection.

The first detailed characterisation of the electrophysiological properties of the infected human RBC came from the group of Desai (Desai *et al.*, 2000b) who reported that in RBCs infected by mature parasites, the whole-cell current was 150-fold larger than that of the non-infected RBCs. The increased current was attributed to the activity of an inwardly rectifying anion channel (small conductance <10 pS) that was present at an estimated 1000 copies per RBC. The ion selectivity and the pharmacological properties of this channel were very close to that found by radiotracer flux measurements and hemolysis. However, recent studies performed in our laboratory (Huber *et al.*, 2002) have shown the presence of at least two distinct anion conductances, differing by their inhibitor sensitivity and their voltage-dependence and serum sensitivity. One was outwardly rectifying and presented the closest pharmacological profile to that of the parasite-induced permeability described previously (Huber *et al.*, 2002). The joint effort of three laboratories could resolve the apparent discrepancies concerning the exact number of anion conductance, as it was shown that the activation of the outwardly rectifying conductance requires the presence of serum (Staines *et al.*, 2003). In addition, the outwardly rectifying conductance was shown to be permeable to various organic osmolytes (Duranton *et al.*, 2004). In sharp contrast, the inwardly rectifying conductance did not show significant permeability for organic osmolytes but was found to be activated by cell swelling. Recently, the molecule generating the volume sensitive inwardly rectifying current was identified as a member of the ClC super-family, ClC-2, (Huber *et al.*, 2004).

The treatment of parasitized RBCs by reducing agents diminished these conductances as well as the rate of hemolysis in isosmotic sorbitol. Interestingly, it was shown that conductances with similar properties were activated in non-infected RBC upon oxidation by peroxides. Therefore it has been postulated that **the *NPPs* are endogenous host human RBC channels** activated upon infection by *Plasmodium* by **oxidative stress**.

A third conductance has been described, i.e. a non-selective cation conductance found both in parasitized and in oxidized non-infected RBCs (Duranton *et al.*, 2002; Duranton *et al.*, 2003).

In summary, it is clear from the recent electrophysiological studies of malaria-infected RBCs that the membrane of the parasitized erythrocytes presents a much higher electrical conductance than that of the uninfected erythrocytes. Therefore, the *NPPs* represent a real challenge from a clinical point of view as they represent potential targets for drugs both directly or as routes capable of mediating the entry of cytotoxic drugs into the parasite. However, the identity and the number of channels are still under study.

B/ Effect of the NPPs on ion equilibrium and cell volume.

- Na^+ and K^+

The strategy of living inside the cells of its host helps the parasite to escape the host's immune system. However, it does pose significant challenges to the parasite. The interior of the host erythrocyte represents a highly unusual extracellular environment as it initially has high K^+ concentrations, low levels of Na^+ and only submicromolar levels of Ca^{2+}. It has long been recognized that in mammalian erythrocytes infected with malaria parasites (*Plasmodium* spp.) there is a marked perturbation of the normal Na^+/K^+ ratios (Dunn, 1969; Sherman & Tanigoshi, 1971). The low $[Na^+]/[K^+]$ is maintained within the erythrocyte cytosol in the hours after invasion by the parasite (the ring stage; (Ginsburg *et al.*, 1986; Lee *et al.*, 1988)). As the parasite matures, however, the $[Na^+]/[K^+]$ in the host cell cytosol increases. Using *Sendai* virus to permeabilize the plasma membrane of human erythrocytes infected with mature (trophozoite) stage forms of *P. falciparum* and thereby release the ions in the host cell compartment for analysis, Ginsburg *et al.* (Ginsburg *et al.*, 1986) estimated the $[Na^+]/[K^+]$ in the erythrocyte cytosol to increase 10-fold, to ~1.25. Using X-ray microanalysis, Lee *et al.* (Lee *et al.*, 1988) obtained evidence for an even greater perturbation, estimating the $[Na^+]/[K^+]$ in the cytosol of *P. falciparum* trophozoite-infected human erythrocytes to be ~11.6. This value implies an almost complete loss of the normal transmembrane Na^+ and K^+ gradients across the RBC

membrane. In contrast to its host erythrocyte, the intracellular parasite maintains a low cytosolic $[Na^+]/[K^+]$ (estimated using different techniques to be between 0.06 and 0.17) throughout the intraerythrocytic cycle (Ginsburg *et al.*, 1985; Lee *et al.*, 1988). The mechanisms by which this is achieved are not clear yet.

The mathematical model proposed by Staines *et al.* (Staines *et al.*, 2001b) according to Lew and Bookchin (Lew *et al.*, 2003) shows the time-dependent increase of the permeabilities to Na^+ and K^+ throughout the intraerythrocytic parasite development (Fig. 6A). The permeabilities were calculated from the furosemide-sensitive influx of $^{86}Rb^+$ in parasitized RBCs reflecting the rate of transport of K^+ through the Na^+/K^+ ATPase. Direct measurements of $K^+(^{86}Rb^+)$ transport via the Na^+/K^+ pump in trophozoite-stage-infected erythrocytes showed that in the period 24-36h postinvasion, the pump activity is increased (up to twice its normal value) in response to the increased cytosolic $[Na^+]$ and leakage of ions via the *NPPs* (Kirk *et al.*, 1991; Staines *et al.*, 2001b) (Fig. 6B). However, the increase is not sufficient to maintain the normal transmembrane Na^+ and K^+ gradients. From 36 to 48 h postinvasion, the flux of ions via the *NPPs* increases, whereas the activity of the Na^+/K^+ pump undergoes a progressive decrease (Staines *et al.*, 2001b). Fig. 6C shows the predicted cytosolic Na^+ and K^+ concentrations throughout the parasite intraerythrocytic cycle. From 12 hours post-invasion there is a decrease of intracellular $[K^+]$ and a symmetrical increase of $[Na^+]$ until both ions reach their extracellular concentrations. In parallel, the predicted relative cell volume (Fig. 6D) slightly decreases from 12 to 24h post-invasion (due to the fact that the efflux of K^+ exceeds the influx of Na^+), then increases from 24 h post invasion onwards, reaching a predicted value of 1.8 at 46h post-invasion.

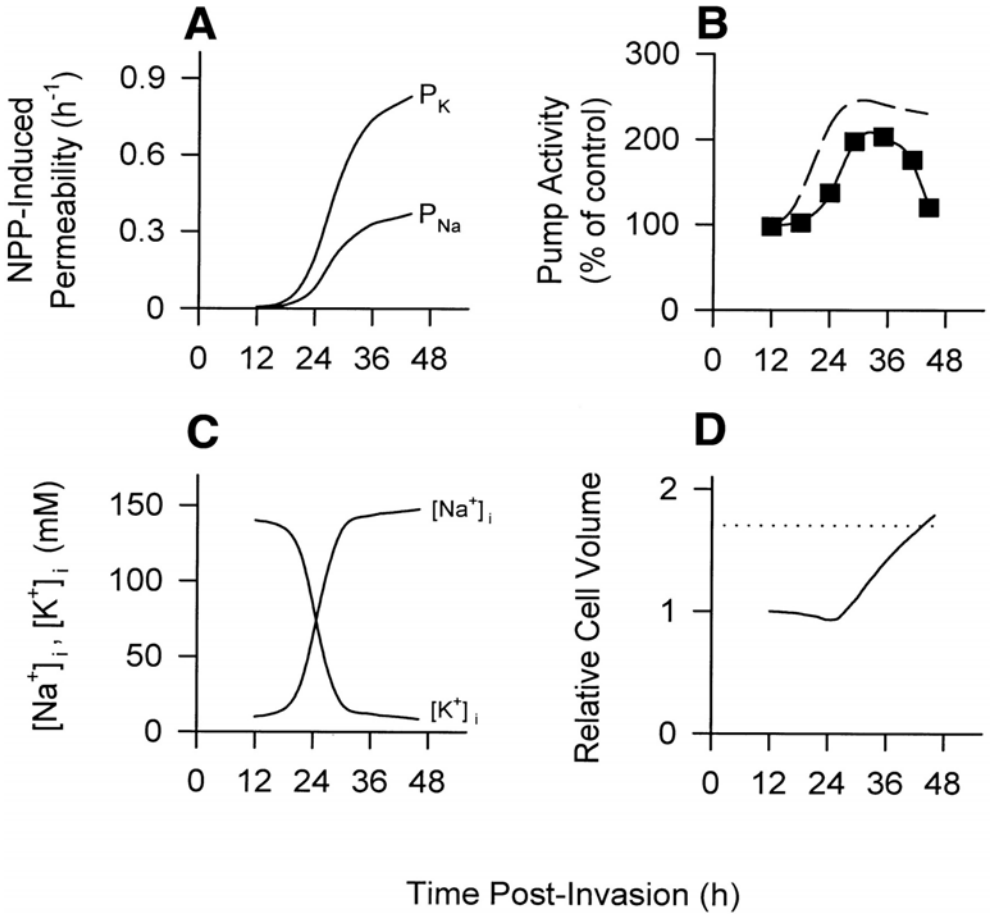


Fig. 6 from (Staines *et al.*, 2001b) Mathematical modelling of the *plasmodium*-induced changes in Na^+ and K^+ transport and physiological consequences over the 48h intra-erythrocytic development cycle of the parasite. This modelling was carried out using the integrated Lew-Bookchin erythrocyte model. **A.** Time-dependent increase in the basal permeabilities of the RBC membrane to Na^+ (P_{Na}) and K^+ (P_K) arising from the furosemide-sensitive *NPPs*. **B.** Time-dependent variation in the activity of the Na+/K+ pump. The symbol (■) shows the measured Na+/K+ pump activity. Predicted changes in cytosolic Na^+ ($[Na^+]_i$) and K^+ ($[K^+]_i$) concentrations (**C**) and in relative cell volume (**D**) following parasite invasion (at t=0).

Lew *et al.* (Lew *et al.*, 2003) provided a model predicting the distinct contributions of the parasite and host cell to the overall volume change of infected RBCs (Fig. 7). The parasite volume increases monotonically whereas the host cell volume shows 4 phases: (1) initial shrinkage due to KCl loss exceeding NaCl gain through developing *NPPs* , (2) swelling due to sustained net NaCl and water gain after K^+ gradient dissipation, (3) volume decrease due to transfers of cytoplasm from host to parasite exceeding swelling tendency from net NaCl gain, this period is corresponding to digestion of the hemoglobin (see Fig. 8), (4) swelling due to the net NaCl gain and the colloidosmotic swelling. By 48 hours the host volume is decreased to about 80 % of its original volume.

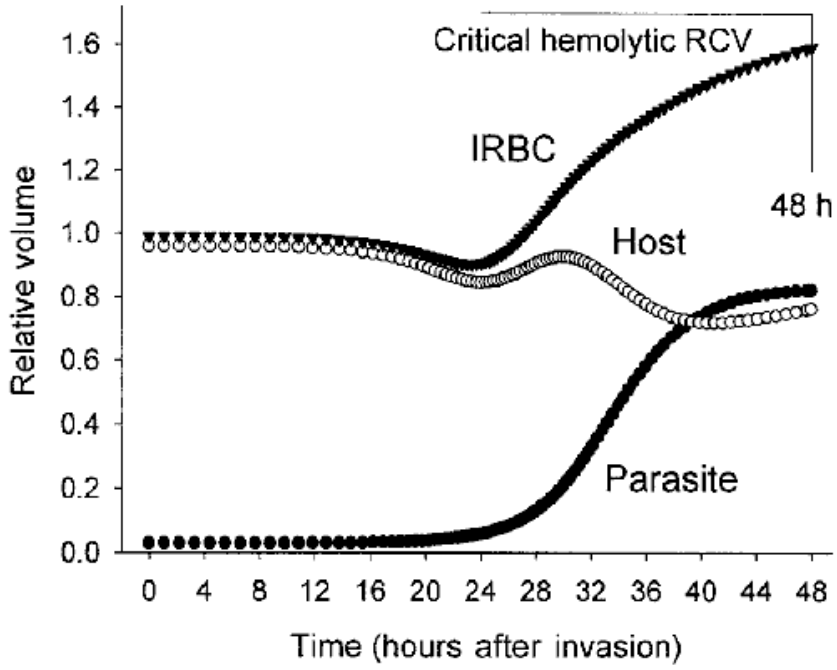


Fig. 7 from (Lew *et al.*, 2003) Predicted changes in relative volume of infected RBCs (▼), host cell (○) and parasite (●) following invasion.

- H^+

The malaria parasite fully depends on glycolysis as its energy source for its intense metabolic and biosynthetic activity. It consumes glucose and produces lactic acid at a rate 100 times higher than that of the non-infected RBC. This high metabolic activity therefore generates intracellular acidification. In addition *in vivo*, malaria infection often leads to acidosis of the patient. In *P. falciparum*, the intracellular pH within the parasite has been estimated to be within the range 7.2-7.4 (Bosia *et al.*, 1993; Saliba & Kirk, 1999), compared with a pH in the host cell cytosol of ~7.1 (Bosia *et al.*, 1993; Wunsch *et al.*, 1997).

- Ca^{2+}

The Ca^{2+} content of *Plasmodium*-infected erythrocytes increases as the parasite matures (Krogstad *et al.*, 1991). An increase in the influx of extracellular Ca^{2+} into infected erythrocytes is evident at later stages of parasite development. In infected erythrocytes, Ca^{2+} is almost exclusively localized in the parasite compartment and but changes little in the cytosol of the host cell (Tanabe *et al.*, 1982; Tanabe, 1990; Marchesini *et al.*, 2000). The importance of Ca^{2+} in supporting the growth of

intraerythrocytic parasites and the invasion of erythrocytes by the merozoite has been assessed by depletion of extracellular Ca^{2+} with chelators (Wasserman *et al.*, 1982; Brand *et al.*, 2003) or by disturbance of the metabolism and transport of Ca^{2+} with a variety of Ca^{2+} modulators (Staines *et al.*, 1999). Recently, patch-clamp experiments have demonstrated that infection with *P. falciparum* induces in the trophozoite stage a 7-8-fold increase in the cation conductance of the host RBC. The infection-induced cation conductance is nonselective for monovalent cations (Na^+, K^+, Cs^+, etc.), Ca^{2+}-permeable, dependent on extracellular Cl^- and sensitive to various inhibitors (Duranton 2003). Experimental evidence (Saliba & Kirk, 1999) suggests that *Plasmodium* possesses in its plasma membrane a proton pump. By operating this proton pump, parasites extrude H^+ and thus generate an electrochemical gradient of protons (an internal negative membrane potential and a concentration gradient of protons) across the parasite plasma membrane. The electrochemical gradient apparently drives inward movement of Ca^{2+} by mean of a Ca^{2+}/H^+ antiporter (Kramer & Ginsburg, 1991) and, possibly, glucose from the cytosol of infected erythrocytes.

4/ Oxidative stress and activation of the *NPPs* in *Plasmodium*-infected erythrocytes.

It has been shown that the high metabolic and biosynthetic rate of the blood stage *P. falciparum* parasite leads to the generation of large quantities of reactive oxygen species (ROS) (Breuer *et al.*, 1983; Atamna & Ginsburg, 1993; Ginsburg & Atamna, 1994). These ROS induce an oxidative stress to the malaria-infected erythrocyte and mainly come from the degradation of host hemoglobin by the parasite (see Fig. 8). Indeed hemoglobin provides the major source of amino acids required for the parasite protein synthesis as well as a source of iron required for the synthesis of iron-containing proteins such as ribonucleotide reductase or superoxide dismutase. The degradation of hemoglobin by *P. falciparum* leads to the production of toxic free haem (ferri/ferroprotporphyrin IX) redox active by-products and H_2O_2. The free haem is neutralized by sequestration into a golden-brown crystalline form called hemozoin or malaria pigment (hemozoin consists of dimers of ferriprotoporphyrin IX, methemoglobin and plasmodial proteins), by degradation, by reaction with gluthatione or binding to some proteins. However, even 0.5% of the free haem non-neutralized causes redox damage to the host erythrocyte membrane and proteins (Tilley *et al.*, 2001). The degradation of hemoglobin plays an important role in the host erythrocyte volume regulation (Lew *et al.*, 2003; Lew *et al.*, 2004).

As discussed before, the high cytosolic Na^+ concentration leads to cell swelling and eventually colloidosmotic hemolysis of the host erythrocyte. However, *P. falciparum* parasite digests more hemoglobin than needed for protein synthesis and discharges hemoglobin-derived amino acids via the *NPPs* leading to a decrease of the oncotic (colloidosmotic) pressure. Therefore, the *NPPs* mediate the regulatory volume decrease (RVD) of the volume-stressed infected RBC similar to organic osmolyte and anion channels in other mammalian cell types (Strange *et al.*, 1996).

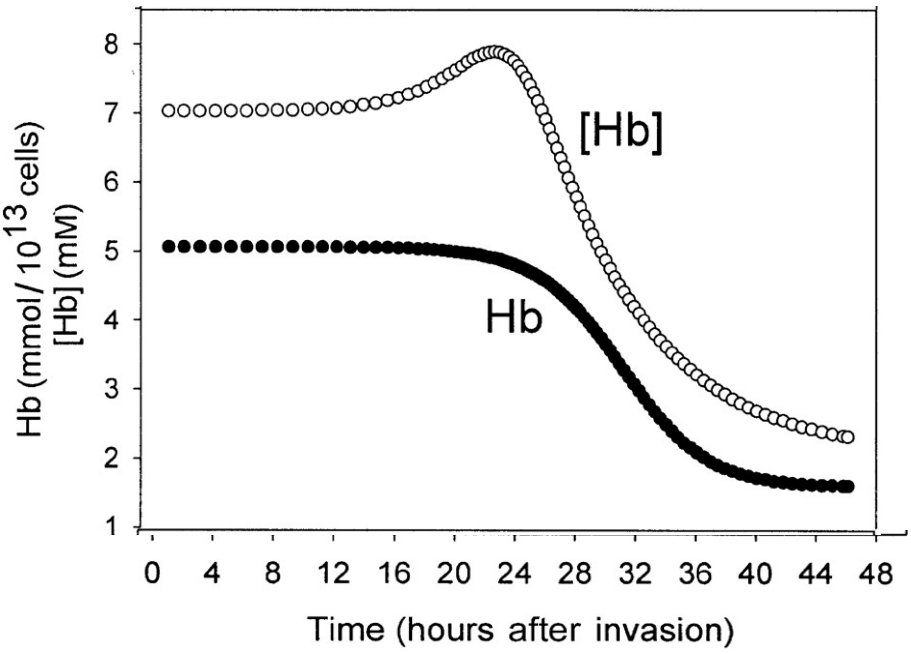


Fig. 8 from (Lew *et al.*, 2003): Hemoglobin (Hb) digestion by *Plasmodium falciparum* following invasion. The diagram shows the decrease in Hb content and concentration ([Hb]).

Oxidation of non-infected human RBCs by peroxides *in vitro* has been demonstrated to induce membrane permeabilities in the RBC membrane very similar to the infection-induced *NPPs.* (Wozencraft, 1986; Huber *et al.*, 2002). Accordingly hemolysis of infected and oxidized RBCs in isosmotic sorbitol solution is inhibited by similar channel/transport blockers. In addition, hemolysis experiments using isosmotic solutions of various neutral carbohydrates indicate the same permeability sequence for both, infected and oxidized human RBCs. Moreover, oxidation by peroxides and infection induce activation of identical channels types in the RBC membrane, as indicated by whole-cell recording. Finally, reduced glutathione (GSH) added to the pipette solution induced a run-down of the infection-induced anion channels in whole-cell experiments suggesting the dependence of these channels on an oxidized state of the RBC cytosol (Wozencraft, 1986; Huber *et al.*, 2002). These data indicate that oxidative processes are involved in the induction of the *NPPs* during the *P. falciparum* blood cycle. The data further demonstrate that *in vitro* oxidation of non-infected RBCs might provide a good model system to study the induction of the *NPPs* by infection.

5/ Purinoceptors in erythrocytes

A/ General features.

Extracellular purines and pyrimidines have important and diverse effects on many biological processes (smooth muscle contraction, neurotransmission, immune response, endocrine and exocrine secretion, platelet aggregation, etc.). Purines and pyrimidines mediate their effects by interactions with distinct cell-surface receptors. “Purinergic” receptors were first formally reported by Burnstock in 1978, he then proposed to classify the receptors in P1-purinoceptors at which adenosine is the principal natural ligand and P2-purinoceptors recognizing ATP and ADP (Burnstock, 1978). The second group is subdivided into two subfamilies based on their transduction characteristics: the ligand-gated ion channels (P2X) and G-protein coupled channels (P2Y). To date seven mammalian P2X receptors, $P2X_{1-7}$, and eight P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11-14}$ (Burnstock & Williams, 2000; Communi *et al.*, 2001; Lee *et al.*, 2003; Marteau *et al.*, 2003; Marteau *et al.*, 2004) have been cloned, characterized pharmacologically and accepted as valid members of the P2 receptor family. The pharmacological characterization of endogenous P2 receptors is often equivocal due to the lack of really specific agonists and antagonists and because of the widespread coexpression of different types of P2 receptors, which complicate the discrimination between the different subtypes. Table 3 is summarizing the different characteristics (distribution, agonists, antagonists) of each channel subtype found until now.

B/ P2X receptors

The P2X receptors are ligand-gated ion channels that gate extracellular cations in response to ATP. This family comprises seven receptors ($P2X_1$ through $P2X_7$) (North, 2002), and the possibility exists that these receptors form hetero-oligomers exhibiting pharmacological or regulatory properties distinct from those of the seven different homo-oligomeric forms. P2X receptors account for fast neurotransmission and largely, but not exclusively, are found in excitatory tissues. For example, the $P2X_7$ receptor acts as an ATP-activated ion channel but also forms a pore, gating passage of molecules up to 1000 Da in response to ATP (Nuttle and

Dubyak, 1994; Rassendren *et al.*, 1997). This unique receptor plays an important role in cells involved in immunological and inflammatory responses.

C/ P2Y receptors

The P2Y receptors are G protein-coupled receptors that are categorized into a subfamily of receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) that predominantly couple to Gs, and therefore activate phospholipase C-, and into a family of Gi-coupled receptors ($P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) that inhibit adenyl cyclase and regulate ion channels (Abbracchio *et al.*, 2003). The P2Y receptors are 7-membrane-spanning proteins, numbering from 328 to 379 amino acids, for a molecular mass of 41 to 53 kDa after glycosylation. The aminoterminal domain faces the extracellular environment, and the carboxyterminal is on the cytoplasmic side of the plasma membrane. Signal transduction occurs via the classical pathways triggered by most 7-membrane-spanning receptors: activation of phospholipase C and/or stimulation/inhibition of adenylate cyclase. All of the P2Y receptors are activated by ATP, but at 2 of them, $P2Y_4$ and $P2Y_6$, UTP is more potent and at $P2Y_2$ ATP and UTP are equipotent. At $P2Y_1$, UTP is inactive and ADP is reported to be equipotent or even more potent than ATP; at $P2Y_{11}$, ATP is more potent than ADP and UTP is inactive. With respect to the signal transduction pathway, $P2Y_1$ and $P2Y_2$ are coupled to stimulation of phospholipase C- and inhibition of adenylate cyclase via Gq/11 and Gi proteins, respectively. There are reports suggesting that $P2Y_2$ can also trigger stimulation of phospholipase D and breakdown of phosphatidylcholine, but the mechanism is unclear. $P2Y_4$ and $P2Y_6$ seem to only couple to phosphoinositide breakdown, whereas $P2Y_{11}$ stimulates activation of both the phosphoinositide and the adenylate cyclase pathways (Di Virgilio *et al.*, 2001). A widely used P2Y antagonist is suramin, a drug originally developed for the treatment of tripanosomiasis. However, suramin does not discriminate between P2Y and P2X and has been reported to inhibit other receptors such as the nicotinic, glutamate, GABA, and 5-hydroxytryptamine receptors as well as the activity of diverse growth factors. Reactive blue 2, trypan blue, pyridoxalphosphate-6-azophenyl 2',4'-disulfonic acid (PPADS) and reactive red have also been used as P2Y antagonists, but they also block P2X-dependent responses.

P2 Receptor Subtype	Tissue Distribution	Principle Agonists	Principle Antagonists (pA_2 or pK_B)
$P2X_1$	Wide including bones, smooth muscles, lymphocytes, platelets	2-MeSATP, ATP, Bz-ATP, α,β-methylene-ATP, Ap4A-Ap6A	Suramin (5.5), PPADS (5), TNP-ATP (≤8)
$P2X_2$	Wide including neuronal system, Pheochromocytoma cells, bones, smooth muscles, lymphocytes,	ATP, Bz-ATP, 2-MeSATP, ATPγS	Suramin (≥4), PPADS (≥4.5)
$P2X_3$	Restricted distribution: spinal cord neurons, a subset of sensory neurons, heart	2-MeSATP, ATP, α,β-methylene-ATP	Suramin (6), PPADS (6), TNP-ATP (6)
$P2X_4$	Wide including vascular endothelium brain neurons, pancreas, bones, smooth muscles, lymphocytes	ATP,2-MeSATP, ATPγS	TNP-ATP (5)
$P2X_5$	Skeletal muscle, smooth muscles, spinal cord neurons, bronchial epithelia, ovary	ATP,2-MeSATP, ADP	Suramin (≥4.5), PPADS (≥4.5), TNP-ATP (≥5)
$P2X_6$	Skeletal muscle, brain	ATP,2-MeSATP, ADP	TNP-ATP (4.5), iso-PPADS (6)
$P2X_7$	Wide including spinal cord neurons, glial cells, airway epithelium, bones, pancreas, smooth muscles, skin, macrophages, lymphocytes, erythrocytes	Bz-ATP, ATP, 2-MeS-ATP	Suramin (5), PPADS (5), calmidazolium (7), KN62 (5) , oxidized ATP, Brilliant blue G
$P2Y_1$	Wide including platelets, heart, skeletal muscle, neuronal tissue, digestive tract , prostate, ovary, brain.	2- MeSADP, ADP, ATP, ATPγS, 2-MeSATP	MRS2179 (6.75) Reactive blue 2 (6.1) Suramin (5.5) PPADS (5.4-4.9)
$P2Y_2$	Wide including lung, heart, skeletal muscle, spleen, kidney	UTP, ATP	Suramin (4.3)
$P2Y_4$	Placenta, lung, vascular smooth muscle	UTP, UTPγS, ATP, UDP	PPADS (IC50≥ 100 µM) Reactive blue 2 (IC50≥ 100 µM)
$P2Y_6$	Wide including lung, heart, aorta, spleen, placenta, thymus, intestine, brain	UDP, 5-Bromo-UDP, UTP, 2-MeSADP	Reactive blue 2 (6.0) PPADS (decrease by 69 % at 100 µM)
$P2Y_{11}$	Spleen, intestine, immunocytes	ATPγS, Bz-ATP, ATP, 2-MeSATP	Suramin (6.09) Reactive blue 2 (decrease by 80 % at 100 µM)
$P2Y_{12}$	Platelets, brain	2-MeSADP, ADP	AR-C69931MX, 2-MeSAMP, AR-C67085
$P2Y_{13}$	Platelets, brain	2-MeSADP, ADP, ADPβS, IDP	AR-C67085, AR-C69931MX
$P2Y_{14}$	Bone marrow-derived hematopoietic stem cells	UDP-Glucose	No data

Table 3 Characteristics of the human purinoceptors from (Fredholm *et al.*, 1994; Chessell *et al.*, 1998; Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000; North, 2002).

D/ Purinergic receptors in erythrocytes.

Effects of extracellular ATP on dog erythrocytes were initially reported in 1972 by Parker and Snow (Parker & Snow, 1972) who showed that this nucleotide caused Na^+ influx and K^+ efflux paralleled by an increase in water content. All other nucleotides tested were ineffective. An increase in plasma membrane permeability of erythrocytes was also reported by Trams (Trams *et al.*, 1980) who showed a dramatic accumulation of extracellular adenylates in the presence of extracellular ATP. These authors concluded that ATP caused a permeability change in erythrocyte plasma membrane that allowed for leakage of cytoplasmic ATP ("ATP-induced ATP release"). Their data suggested that erythrocytes expressed a $P2X_7$-like receptor, but no further characterization of this phenomenon was carried out. However the group of Wiley has shown very recently that $P2X_7$ (and at a lower level of expression, $P2X_2$) receptors were indeed expressed on human erythrocytes (Sluyter *et al.*, 2004). Similarly to the findings of Parker and Snow on canine RBCs, Wiley *et al.* showed that extracellular ATP induced a reversible permeabilization of erythrocytes to both K^+ and Na^+ ions mediated by the $P2X_7$ receptors.

Contrary to P2X receptor subtype, many studies were performed by the group of Harden since the early 90´s to characterize a $P2Y_1$ receptor found on turkey erythrocyte (Boyer *et al.*, 1989). Avian red blood cells indeed express a typical $P2Y_1$ receptor coupled to phospholipase β activation via a G protein of the Gs family (Boyer *et al.*, 1989; Vaziri & Downes, 1992). Finally, to my knowledge no publication reports the functional expression of P2Y purinoceptor subtypes on human erythrocytes until now.

Necturus RBCs reportedly release ATP during hypotonic swelling and extracellular ATP activates a P2 receptor presumably of the P2X subtype, which leads to a rise in cytosolic Ca^{2+}, thereby stimulating RVD by activating a K^+ conductance (Light *et al.*, 2001).

Moreover, human erythrocytes have been shown to release ATP under hypoxic conditions (Bergfeld & Forrester, 1992) and under shear stress by a mechanism dependent on CFTR activity (Sprague *et al.*, 1998). This release of ATP renders the cells sensitive to ATP released by other blood cells (e.g., platelets, endothelial cells) and endows them with the ability to modulate the function of circulating or endothelial cells by secreting large amounts of this nucleotide. It has

been proposed that ATP release from erythrocytes could contribute to regulation of local blood flow by acting at P2Y receptors on vascular endothelium. ATP induces NO release from endothelial cells; thus, under ischemic conditions, when the ATP release from erythrocytes is maximal, it could counteract the decreased blood flow by inducing vasodilatation (Sprague *et al.*, 2003). Furthermore recent studies from Olearczyk *et al.* (Olearczyk *et al.*, 2004a; Olearczyk *et al.*, 2004b) have shown that NO inhibits ATP release from erythrocytes via inactivation of the heterotrimeric G protein Gi therefore contributing to the regulation of vascular flow.

6/ Channel-induced "apoptosis" of Plasmodium falciparum infected erythrocytes.

Similar to other cell types, erythrocytes have to be eliminated after their physiological life span or when they are defective. In other cell types the primary mechanism of clearance is apoptosis. Until very recently, erythrocytes have been considered unable to undergo apoptosis as they lack mitochondria and nuclei, key organelles in the apoptotic machinery of other cells. However most recent observations have shown that similar to nucleated cells, human erythrocytes activate a non-selective cation channel upon osmotic cell shrinkage, energy depletion, extracellular removal of Cl^- (Huber *et al.*, 2001) and oxidative stress (Duranton *et al.*, 2002). This channel is permeable to Ca^{2+} and opening of the channel increases cytosolic [Ca^{2+}] subsequently leading to cell shrinkage, membrane blebbing and stimulating an intraerythrocytic scramblase (Zhou *et al.*, 2002) that catalyses bi-directional phospholipid migration across the lipid bilayer, resulting in breakdown of the phosphatidylserine asymmetry of the cell membrane. The exposure of phosphatidylserine at the outer membrane leaflet is apparent from annexin binding. The membrane changes and shrinkage are typical of apoptosis in other cell types. The phosphatidylserine exposure following osmotic shock is blunted by amiloride (Lang *et al.*, 2003a) and ethylisopropylamiloride (EIPA) (Lang *et al.*, 2003b), both putative inhibitors of the non selective cation channel. The exposure of phosphatidylserine at the outer surface of the cell membrane is presumably followed by binding to phosphatidylserine receptors on macrophages and subsequent phagocytosis and lysosomal degradation of the erythrocyte (Bratosin *et al.*, 1997).

The infection of erythrocytes by *Plasmodium falciparu*m also leads to activation of a non-selective, Ca^{2+}-permeable cation channel and several distinct anion channels. Activation of the non-selective cation channel allows entry of Ca^{2+} and Na^{+}, both of which are required for intracellular growth of the pathogen. Invasion of the merozoites (Wasserman *et al.*, 1982; Lew *et al.*, 2003) as well as parasite amplification within the erythrocyte (Wasserman *et al.*, 1982) are strongly dependent on the Ca^{2+} concentration in the medium. Elevated cytosolic Ca^{2+} concentrations, however, have been demonstrated to induce programmed cell death of non-infected RBCs (Bratosin *et al.*, 2001; Lang *et al.*, 2002). Therefore the enhanced activity of the infection-induced non-selective cation conductance could lead to erythrocyte apoptosis and play a dual role in pathogen survival, this hypothesis will be investigate in the present study. Absence of the channels is not compatible with pathogen growth; enhanced channel activity accelerates erythrocyte apoptosis that may represent a host defence mechanism serving to eliminate infected erythrocytes.

OBJECTIVES OF THE STUDY

The *NPPs* develop slowly in *Plasmodium*-parasitized or oxidized RBCs suggesting the involvement of complex signal transduction pathways in the reorganization of the RBC membrane permeability. Organic osmolytes and anion channels which resemble the *NPPs* of *Plasmodium*-infected or oxidized RBCs have been demonstrated in other cell types to be regulated by ATP release and autocrine purinoceptor signaling. Therefore, the present work has been performed to answer the following questions:

1. Is the induction of the *NPPs* accompanied by a release of ATP from the oxidized or *Plasmodium*-infected RBCs into the medium?

2. Does extracellular ATP promote the induction of the *NPPs* during oxidation or infection?

3. Which purinoceptor subtypes are involved in a putative ATP effect on the *NPPs* in oxidized or infected RBCs?

4. What is the functional significance of such purinoceptor signaling? Does pharmacological inhibition or gene targeting of purinoceptor subtypes impair acquisition of the *NPPs* by the parasitized RBC or the *in vitro* and *in vivo* amplification of the parasite?

5. The infection-induced *NPPs* reportedly comprise Ca^{2+}-permeable nonselective cation channels. Activation of these channels in non-infected RBCs by, e.g., oxidation triggers programmed death in aging RBCs. A further goal of this study is to monitor the phospholipids asymmetry of the RBC membrane during infection, as a measure of host RBC viability.

MATERIALS AND METHODS

1/ Preparation of human erythrocytes.

EDTA- blood was withdrawn from healthy volunteers (different blood groups; donors gave informed consent), washed three times in Ca^{2+}-free NaCl solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES/NaOH pH 7.4 (hematocrit of 5%), after each centrifugation "buffy coat" and upper 10-20% of red cells were removed. The washed RBCs were then resuspended at 10% hematocrit and stored at 4°C until use (3-5 days after).

2/ In vitro culture of *P. falciparum* infected human erythrocytes.

Plasmodium falciparum FCR3 strain was cultured according to the modified method of Trager and Jensen (Trager & Jensen, 1976; Jensen & Trager, 1978). Cultures were maintained by routine passage in fresh and banked human erythrocytes (1-2% hematocrit) at a parasitemia of 2-5% in an atmosphere of 90% N_2/5% O_2/5% CO_2 at 37°C in RPMI 1640 medium supplemented with 25 mM HEPES, 20 µg/ml gentamicin sulphate, 2 mM glutamine, 200 µM hypoxanthine and 0.5% albumax II™. All culture reagents were purchased by Gibco Invitrogen, Karlsruhe, Germany. Synchronization to ring-stage was achieved by sorbitol treatment (Lambros & Vanderberg, 1979).

3/ Hemolysis of oxidized human erythrocytes in isosmotic sorbitol solution.

For oxidation, the RBCs were re-suspended in Ca^{2+}-containing NaCl solution (in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES/NaOH pH 7.4; hematocrit of 2%) further containing *tert*-butylhydroperoxide (*t*-BHP; 1 mM final concentration). After 15 min of incubation at room temperature (see below) oxidation was stopped by spinning down and by resuspending the pelleted RBCs in an isosmotic sorbitol solution (290 mM sorbitol, 10 mM HEPES/NaOH pH 7.4; 5% hematocrit). The oxidized cells were incubated for 2.5 h at 37°C in isosmotic sorbitol solution in the presence and absence of purinoceptor agonists/antagonists (see below), of NPPB (100 µM; an inhibitor of the infection- and oxidation-induced sorbitol permeability), or

of apyrase (1 unit/ml). Incubation in sorbitol solution was stopped by centrifugation and the supernatants were harvested. As a measure of hemolysis the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 546 nm upon transforming the Hb into *cyanomet*Hb. Formation of *cyanomet*Hb was accomplished by adding 0.8 mM KCN, 0.61 mM $K_3[Fe(CN)_6]$, 1 mM KH_2PO_4, 0.5% Extran to the supernatants (550 µl / sample). In each experiment, non-oxidized cells were processed identically to the oxidized samples but in the absence of *t*-BHP as a negative control. The different conditions (control, various agonist- and/or antagonist concentrations) of an experimental series were studied in paired experiments in quadruplicate and repeated in at least three independent preparations. Individual values of these preparations were expressed as relative hemolysis and oxidation-induced relative hemolysis, respectively. Relative data of the individual samples were pooled between the different preparations and given as means ± SE.

4/ In vitro *P. falciparum* growth assay.

Ring stage-synchronized parasitized RBCs were aliquoted in 96-well plates (200 µl aliquots, 1% hematocrit, 1% parasitemia) and grown for 48 h in the presence or in the absence of suramin (1-500µM). The parasitemia was assessed at time 0 and after 48h of culture by flow cytometry (FACS-Calibur, Becton Dickinson, Heidelberg, Germany). Parasitemia was defined by the percentage of RBCs stained with the DNA/RNA specific fluorescence dye Syto16 (30 nM final concentration, Molecular Probes, Göttingen, Germany). In further experiments to estimate the DNA/RNA amplification, the culture was ring stage-synchronized, and re-synchronized after 6h of culture (to narrow the developmental parasite stage), aliquoted (200 µl aliquots, 2.5% hematocrit and 10% parasitemia) and cultured for further 16 h in the presence or absence of suramin (100 and 500 µM). Thereafter, the DNA/RNA amount of the parasitized RBCs was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

5/ Magnetic separation of *P. falciparum*-infected human erythrocytes.

The parasite culture was enriched in late-stage trophozoites and schizonts infected RBCs using their electromagnetic properties conferred by the digestion of hemoglobin into hemozoin (Uhlemann *et al.*, 2000). For that purpose, CS separation columns were mounted on high-gradient magnetic cell separator VarioMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and the flow velocity was adjusted to 1 drop per second. Parasite culture was spun down and resuspended at approx. 5.10^9 RBC per ml in PBS + 5mM glucose. The RBCs suspension was then loaded on the column, the flow-through contained uninfected RBCs or ring-stage whereas trophozoites and schizonts infected RBCs were retained in the column by the magnetic field. The column was subsequently washed with PBS + 5mM glucose until the flow-out was cleared from RBCs, then removed from the magnetic field and eluted in PBS + 5mM glucose. The parasitemia of the eluate assessed with Syto16 staining (1µM final concentration in PBS) by flow cytometry analysis was over 85 %.

6/ Hemolysis of *P. falciparum*-infected human erythrocytes in isosmotic sorbitol solution.

Pelleted late-trophozoite/schizont-stage-enriched infected RBCs were resuspended in isosmotic sorbitol solution (290 mM sorbitol, 10 mM HEPES/NaOH pH 7.4; 5% hematocrit) and incubated for 15 minutes at 37°C in the presence or absence of ATP (100µM) or different purinoceptor antagonists. Incubation was stopped by centrifugation and the supernatants were harvested. The hemoglobin concentration in the supernatant was estimated as described above. In further experiments, ring-stage-synchronized RBCs were cultured for 16 h, aliquoted (200µl, 5% hematocrit, 20-25% parasitemia, and post-cultured for 6h in the presence or absence of ATP (100 µM) or suramin (500 µM) or MRS2179 (10 µM), pelleted, and resuspended in isosmotic sorbitol solution. Hemolysis was determined after 15 min incubation at 37°C.

For the biotin experiments, late-trophozoite/shizont-stage enriched infected RBCs were resuspended in PBS / 5 mM glucose containing 1 mM of LC-sulfo-NHS-(+)-biotin (Molecular Biosciences, CO, USA) and incubated for 30 min at 37°C. The parasitized RBCs were then washed in PBS / 5mM glucose for 10 min, pelleted and

resuspended in isosmotic sorbitol solution (additionally containing 5 mM glucose) in the presence or absence of ATP (100µM) and/or suramin (100µM) as described above. For each series of experiments, a positive control was performed where the biotin pre-incubation step had been omitted.

7/ Purinoceptor agonists, antagonists and inhibitors.

ATP, Bz-ATP (2´ & 3´-O- (4-benzoylbenzoyl) adenosine 5´ triphosphate), suramin, ATP γ S, ADP, ADPβS, 2-MeSATP, UTP, reactive blue 2, MRS2179, D-sorbitol, grade VI apyrase, NPPB and PPADS were purchased from Sigma (Munich, Germany).

8/ In vivo proliferation of *P. berghei* ANKA.

Male and female C57BL/6 mice (8-9 weeks old) with $P2Y_1$ -/- (Leon *et al.*, 1999) or $P2Y_1$ +/+ genotype were used. These mice are viable with no apparent abnormalities affecting their development, survival, reproduction, or the morphology of their platelets. In addition the platelet, white blood cell, and RBC count in these animals as well as the hematocrit and the hemoglobin concentration are identical to those of the wildtype mice. However, platelets from $P2Y_1$ -/- mice are unable to aggregate in response to usual concentrations of ADP and display impaired aggregation to other agonists (Leon *et al.*, 1999).

18 mice from each genotype (9 male and 9 female) were infected by intraperitoneal injection of 10^6 *P. berghei* ANKA-parasitized mouse erythrocytes. From the seventh day of infection onwards, parasitemia was estimated daily by Syto16 DNA-RNA staining as described above.

9/ Isosmotic sorbitol hemolysis of oxidized and *P.Berghei* infected mouse erythrocytes.

RBCs (infected or not by *P.Berghei*) from $P2Y_1$ -/- mice and their sex-matched wildtype litter mates were washed three times in Ca^{2+}-free NaCl solution, resuspended to a hematocrit of 5% and stored for 1-4 days at 4°C. Similar to the experiments with human RBCs, the non-infected mouse RBCs were oxidized for 10-15 min with *t*-BHP (0.5 mM final concentration) at room temperature in $CaCl_2$-

containing NaCl solution (hematocrit 2%). Pelleted oxidized RBCs (5 µl aliquots) were then re-suspended in isosmotic sorbitol solution (290 mM sorbitol, 10 mM HEPES/NaOH pH 7.4) or for control in Ca^{2+}-containing NaCl solution (100 µl) and incubated for 1h at 37°C. Hemolysis was determined as described above for human erythrocytes. In sharp contrast to human RBCs, non-oxidized mouse RBCs express a sorbitol permeability. This permeability is sensitive to phloretin, inhibited upon oxidation but insensitive to NPPB (100 µM; own unpublished observation). Therefore, only the NPPB-sensitive fraction of hemolysis of oxidized RBCs in isosmotic sorbitol was analyzed.

For hemolysis of *P. berghei-parasitized mouse* RBCs, erythrocytes were retrieved from infected $P2Y_1$ -/- and wildtype mice (about 40% parasitemia), washed, pelleted and incubated (5µl aliquots of pelleted RBCs) in isosmotic sorbitol solution (100µl; see above) for 15 minutes at 37°C.

10/ Immunofluorescence staining and confocal microscopy.

Human RBCs (100 µl at 5 x 10^7 cells/ml) were smeared onto glass slides, air dried for 30 min and fixed with acetone/methanol for 2 min. The slides were briefly washed four times with PBS and then blocked for 20 min with 20% normal horse serum and 0.1% BSA in PBS before incubation with polyclonal sheep anti-$P2Y_1$ (1:100 dilution in PBS containing 0.2% normal horse serum) for 45 min at room temperature. The $P2Y_1$ antibody was raised against the residues 36-51 of rat $P2Y_1$ located in the extracellular N-terminal domain (Fong *et al.*, 2002); the corresponding human epitope varies by only two residues. The slides were washed three times for 30 min with PBS and incubated with a secondary anti-sheep IgG antibody conjugated to Cy3 (Jackson ImmunoResearch, West Grove, PA, USA) (1:200 dilution in PBS containing 0.2% normal horse serum) for 45 min at room temperature. The slides were washed as above before mounting in glycerol gelatin medium (Sigma). Cells were visualized with a Leica TCS NT UV laser confocal microscope system.

11/ Human RBC membrane preparation and Western-blot.

200 µl of the RBCs pelleted (prepared as described above) were hemolyzed in 20mM HEPES pH 7.4 containing a cocktail of protease inhibitors (Roche, Mannheim,

Germany) to remove the hemoglobin that may lead to non-specific binding during incubation with antibodies. Ghost membranes were pelleted (15000 g for 20 min at 4°C) and lysed in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% triton-X, 10 µl 2-mercaptoethanol. Lysates were separated by 8% SDS-PAGE (50 µg protein per lane), and transferred electrophoretically from gel to Protan BA83 nitrocellulose membranes (Schleicher and Schuell, Germany). After blocking with 5% non-fat milk, the blots were probed for 1 h at 21°C with a commercial polyclonal rabbit anti-$P2Y_1$ peptide antibody (Alomone Labs, Jerusalem, Israel; 1:400 dilution). The antibody was raised against the residues 242-258 of human $P2Y_1$ located in the 3^{rd} intracellular loop. A negative control was also performed by preincubation with the antigen peptide with the antibody for 1h. After thoroughly washing, the blot was incubated with a secondary anti-rabbit antibody (1:2000 dilution) conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) for 1h at 21°C. Antibody binding was detected with the enhanced chemoluminescence ECL kit (Amersham, Germany).

12/ Patch-clamp experiments.

Whole-cell currents were recorded at room temperature from late trophozoite-stage-infected erythrocytes selected optically as described previously (Huber *et al.*, 2002; Duranton *et al.*, 2004). Cells were preincubated for 6h with increasing concentrations of suramin (0-500 µM) by adding the purinoceptor agonist to the medium, Whole-cell currents were elicited by 11 square pulses (400 ms) clamping the voltage from the -30 mV holding potential to voltages between -100 mV and +100 mV applied in increments of +20 mV. In the original current traces the individual current sweeps are superimposed. Voltages are referring to the cytosolic membrane side in respect to the earthed extracellular side. Outward currents as defined as efflux of cations out of or influx of anions into the RBCs are positive currents and depicted as upward current deflections in the original current traces. Currents were analyzed by averaging the values between 350 and 375 ms of each square pulse. The liquid junction potentials between bath and pipette solutions and between the bath solution and the salt bridge (filled with NaCl bath solution) were calculated according to Barry (Barry & Lynch, 1991). Data were corrected for liquid junction potentials. The pipette solution contained (in mM) 115 Na-gluconate, 10 NaCl, 1

EGTA, 1 MgATP, 1 $MgCl_2$, 10 HEPES/NaOH (pH 7.4). The bath solution contained (in mM) 115 NaCl, 10 $MgCl_2$, 5 $CaCl_2$, 20 HEPES/NaOH (pH 7.4). A further bath solution contained (in mM): 140 Na-gluconate, 10 HEPES/NaOH (pH 7.4), 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.4).

13/ ATP release from oxidized and *P. falciparum* infected human RBCs.

To avoid ATP release upon mechanical deformation (Sprague *et al.*, 1996) the RBCs (100 µl) were resuspended in Ca^{2+}-containing NaCl solution (2 ml aliquots) and sedimentated overnight at 4°C. The experiment was started by adding *t*-BHP (0 and 0.2 mM final concentration, respectively) to the supernatant. The RBCs were incubated at 37°C without shaking and at time 0, 5, 60 and 120 min an aliquot (50 µl) of the supernatant was harvested.

To measure the ATP released by *P. falciparum infected* human RBCs, the culture was synchronized as described above and harvested at a hematocrit of 2% and an initial parasitemia of 4 to 7 % in a 96-well plate. After 24h under low O_2 culture conditions as previously described, an aliquot (50µl) of supernatant was harvested. A control condition was performed with the non-parasitized blood used for parasite culture following the same protocol than that of the infected RBCs.

In both experimental conditions (oxidized and *P. falciparum* infected), the extracellular ATP concentration of the aliquots was quantified by a luciferin-luciferase assay kit (Roche Diagnostics, Mannheim, Germany) using a luminometer (Berthold Biolumat LB9500 Bad Wildbad, Germany) according to the supplied experimental protocol. To estimate hemolysis-mediated contamination by intracellular ATP, the hemoglobin concentration of the supernatant was determined photometrically (see above).

14/ Annexin binding experiments.

Suspensions from non infected RBCs were stained with annexin V-FLUOS (Roche, Mannheim, Germany), suspensions of *Plasmodium falciparum* infected RBCs were stained with annexin V-568 (Roche, Germany) and/or with the DNA dye Syto16 (Molecular Probes) to assess phosphatidylserine exposure in the outer leaflet

of the RBC membrane and the ratio of infected RBCs, respectively. For annexin binding, RBCs were washed, resuspended in annexin-binding buffer (140 mM NaCl, 10 mM HEPES, 5 mM $CaCl_2$, pH 7.4), stained with annexin V-568 (dilution 1:50) or annexin V-FLUOS (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin binding buffer. Syto16 (final concentration of 20 nM) was either directly added to the diluted RBC suspension (and incubated for 30 min at 37°C) or co-incubated in the annexin binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur from Becton Dickinson) using FL-1 as an indicator of Syto16 or annexin V-FLUOS fluorescence intensity and with FL-2 as an indicator of annexin V-568 fluorescence intensity.

To induce annexin binding according to Lang *et al.* (Lang *et al.*, 2003a) non-infected RBCs were incubated for 1h at 37 °C with the Ca^{2+} ionophore ionomycin (0 and 1 µM, respectively) or oxidized by *t*-butylhydroperoxide (*t*-BHP, 1mM for 15 min followed by 24h h of post-incubation) in a modified NaCl test solution containing (in mM): 125 NaCl, 5 KCl, 5 D-glucose, 1 $CaCl_2$, 1 $MgSO_4$, 32 HEPES/NaOH, pH 7.4.

15/ Data analysis and statistics.

Differences between means were estimated by one-way ANOVA or two-tailed Student's *t*-test using InStat statistic program (GraphPad Software, San Diego, California, USA).

RESULTS

1/ Infection by *P. falciparum* induces an osmolyte permeability inhibitable by the *NPPs* blockers.

Hemolysis of infected erythrocytes in isosmotic sorbitol (10 min of incubation) is sensitive to NPPB, furosemide and glybenclamide (each 100 µM) Fig. 9A and B. These channel blockers have been reported to inhibit *NPPs* -assiociated hemolysis or tracer fluxes of infected RBCs (Kirk & Horner, 1995a). Furosemide and glybenclamide inhibit 87 ± 7% (n = 3) and 94 ± 1% (n = 3), respectively, of the sorbitol-induced hemolysis fraction that is inhibitable by NPPB (100 µM; Fig. 9B). Under the same conditions DIDS, another *NPPs* blocker, inhibits 37 ± 4% (n = 16) of the sorbitol-induced hemolysis fraction that is inhibitable by NPPB.

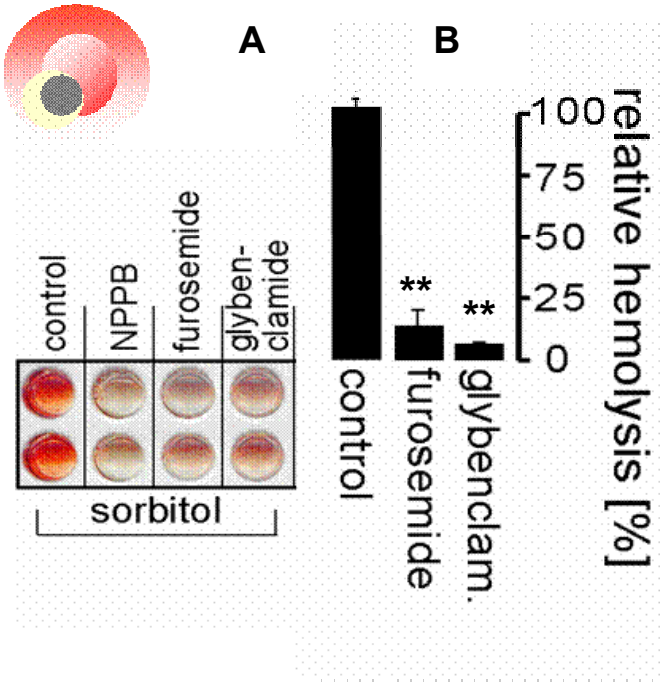


Fig. 9 Hemolysis of infected RBCs in isosmotic sorbitol solution. **A.** Enriched trophozoite-infected erythrocytes are suspended in isosmotic sorbitol in the presence or absence of NPPB, glybenclamide or furosemide (at 100µM each). Incubation is stopped by centrifugation and hemolysis is estimated by hemoglobin in the supernatant. Shown are the scanned supernatants from an individual experiment in duplicate. **B.** The hemoglobin concentration of the supernatants is determined photometrically and data are expressed as a percentage of that fraction of total hemolysis that could be inhibited by NPPB. Data are means ± SE; n = 3-16; **: $P \leq 0.01$, one-way ANOVA.

To test for the involvement of oxidative processes in the induction of the *NPPs*, oxidized cells (*t*-BHP/15 min) are suspended in isosmotic sorbitol or NaCl solution (Fig. 10A and B). Within 2.5 h of incubation almost 50 % of the RBCs hemolyze in sorbitol but only 5% in NaCl solution. In the absence of oxidation (Fig. 10A), the percentage of hemolysis is less than 5%.

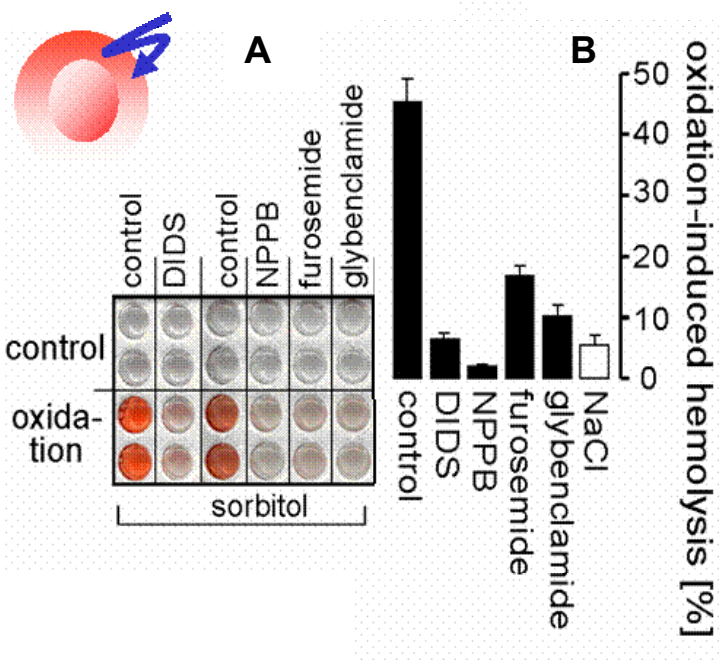


Fig. 10 Hemolysis of non-infected oxidized RBCs in isosmotic sorbitol solution.
A. Imaged supernatants of untreated (lines 1 and 2) and oxidized human erythrocytes (lines 3 and 4) after incubation in sorbitol in the absence (control) or presence of DIDS, NPPB, furosemide and glybenclamide (at 100µM each). Shown is an individual experiment in duplicate. **B.** Mean oxidation-induced sorbitol hemolysis in the absence (control) or presence of the blockers (at 100µM) as indicated. Data are mean percentage of hemolysis ± SE; n = 8-15; **: $P \leq 0.01$, one-way ANOVA.

Similar to hemolysis of infected RBCs, oxidation-induced hemolysis in sorbitol is inhibited by NPPB, furosemide and glybenclamide (all 100 µM) by 93 ± 5% (n = 21), 61 ± 6% (n = 23) and 71 ± 1% (n = 20), respectively. These values are similar to the inhibitory effect of these drugs on the infection-induced hemolysis in sorbitol. DIDS inhibited the oxidation-induced hemolysis by 48 ± 6% (n = 18). These experiments show that the induction of hemolysis in isosmotic sorbitol by *P. falciparum* is mimicked by oxidation of non-infected RBCs and that the osmolyte permeability induced by *P. falciparum* is mediated by endogenous channels.

2/ Oxidized and infected cells exhibit identical hemolysis in isosmotic carbohydrate solutions.

Hemolysis of infected (Fig. 11A) and non-infected oxidized (Fig. 11B) RBCs in isosmotic concentrations of different carbohydrates reveal the same permeability rank order of sorbitol>mannitol>myo-inositol ≈ lactose ≈ sucrose ≈ raffinose. These

data strongly suggest that the osmolyte permeability activated upon infection *by P. falciparum* and upon oxidation are identical.

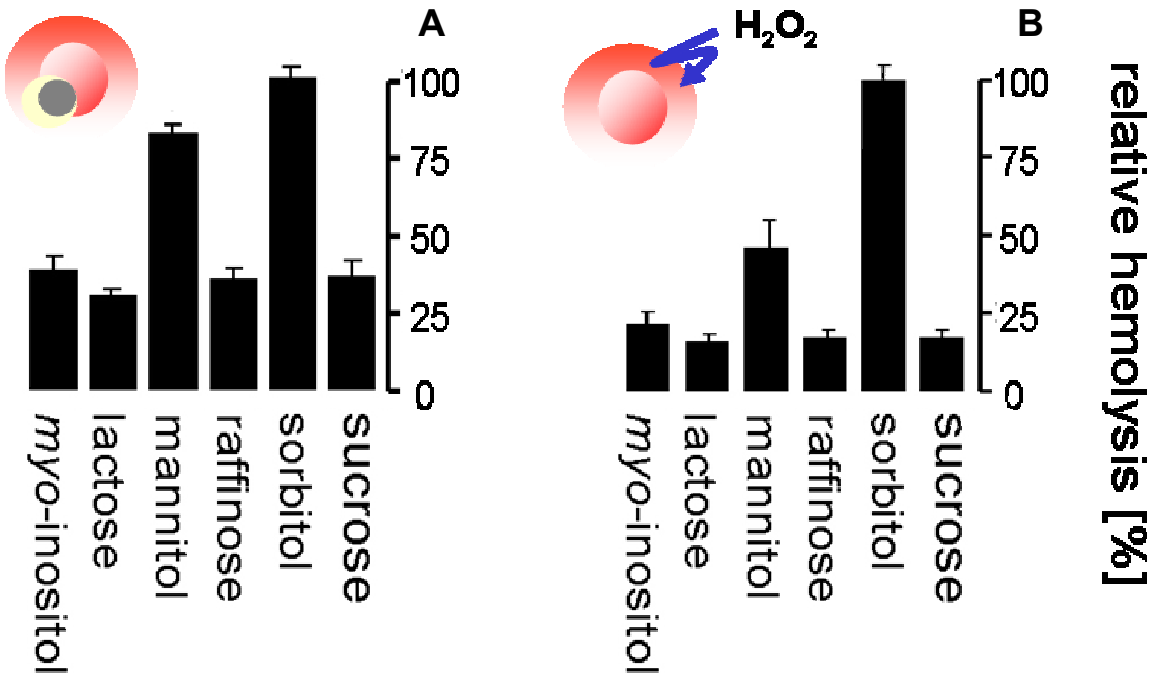


Fig. 11 Substrate dependence of the infection- and oxidation-induced isosmotic hemolysis. Mean relative hemolysis of (A) infected and (B) non-infected oxidized cells incubated in different isosmotic carbohydrate solutions as indicated.

3/ Purinoceptor antagonists suramin and MRS2179 inhibit the induction of organic osmolyte and anion permeability in *P. falciparum*-infected human RBCs.

Hemolysis of enriched late trophozoite/shizont-infected human RBCs in isosmotic sorbitol solution is not affected by either the non-specific purinoceptor antagonist suramin (100 µM) nor by the $P2Y_1$-specific inhibitor MRS2179 (10 µM; Fig. 12A) strongly suggesting that both antagonists do not inhibit directly the sorbitol permeation into the cell. However, the anion channel blocker NPPB inhibits the sorbitol hemolysis almost completely (Fig. 12A) indicating that hemolysis occurs

indeed by the uptake of sorbitol through a specific permeation pathway followed by colloidosmotic swelling of the host cell.

To test whether purinoceptor agonists/antagonists stimulate/inhibit the induction of the sorbitol pathway, ring-stage-synchronized *P. falciparum* were cultured for 16h under normal culture conditions and then post-cultured for 6h in the presence or absence of ATP (100µM), suramin (500 µM) or MRS2179 (10 µM) (Fig. 12B). Finally the parasitized RBCs were resuspended in agonist/antagonist-free sorbitol solution and hemolysis was estimated photometrically. Under these conditions where the purinoceptor agonists/antagonists are applied only during the induction of the osmolyte conductance, ATP stimulates while suramin (500 µM $suramin_{total}$) or MRS2179 (10 µM $MRS2179_{total}$) inhibits significantly hemolysis of parasitized human RBCs (Fig. 12B). These results strongly suggest that activation of purinoceptors is involved in the induction of the osmolyte permeability.

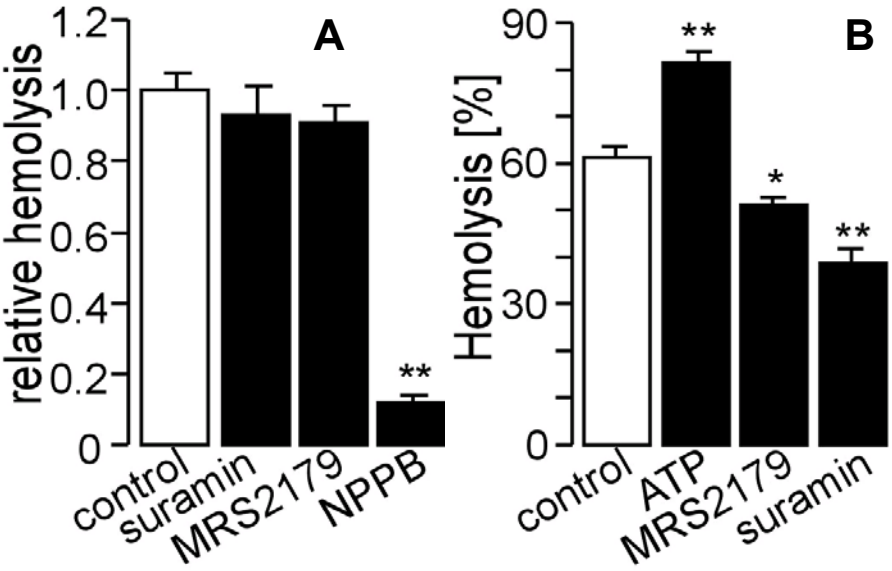


Fig. 12 Purinoceptor antagonists inhibit the induction of the organic osmolyte and anion permeability in *P. falciparum*-infected human RBCs

A. The infection-induced sorbitol permeability of the host RBC membrane induces swelling and eventually hemolysis when RBCs are bathed in isosmotic sorbitol solution. Shown is the relative hemolysis of enriched trophozoite-infected RBCs in isosmotic sorbitol solution (15 min / 37°C) as determined photometrically by the Hb concentration in the medium in the absence (control, open bar) and presence of the purinoceptor antagonists suramin (100 µM) or MRS2179 (10µM) and in the presence of the *NPP*s inhibitor NPPB (100 µM), respectively (data are means ± SE; n = 12-20; **: $P \leq 0.01$, one-way ANOVA). B. Hemolysis of parasitized RBCs (20-25 % parasitemia) in isosmotic sorbitol solution (15 min / 37°C) upon pre-treatment (6h / 37°C) of the RBCs with ATP (100µM), suramin (500 µM) or MRS2179 (10 µM) added to the culture medium (shown is the mean percentage of hemolysis normalized for 100% parasitemia ± SE; n = 8-10; *: $P \leq 0.05$, **: $P \leq 0.01$, one-way ANOVA).

Serum albumin is known to adsorb a wide range of substances (fatty acids, drugs, etc. (Schenkein *et al.*, 1971; Anfinsen *et al.*, 1974; Myers *et al.*, 1975)). To estimate the free, i.e. effective, suramin concentration in the culture medium the adsorbance of suramin to serum albumin was measured. Fig. 13 shows the decrease of free suramin concentration when co-incubated with increasing serum albumin in the medium. The free suramin concentration decreases to about 80 µM at 500 µM suramin total concentration ($suramin_{total}$) and about 2 µM at 100 µM $suramin_{total}$ when co-incubated in the presence of serum albumin (Albumax, 0.5%). This result indicates that the effective concentration of suramin is highly decreased in all experiments where suramin is co-incubated with albumin (Figs. 14C, 23, 24).

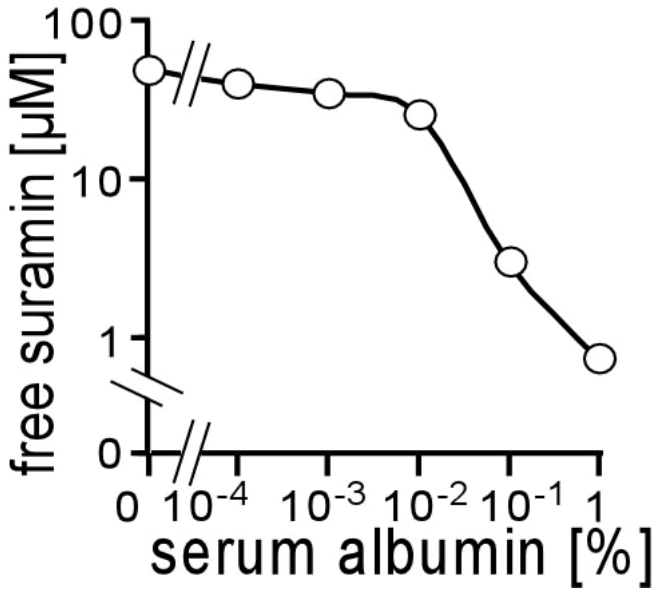


Fig. 13 Adsorption of suramin (50 µM) to increasing concentrations of serum albumin. Free suramin concentration (µM) is determined photometrically by absorbance at 320 nm upon separating free and bound suramin by spin columns (Ultrafree MC, Millipore, Schwalbach, Germany).

4/ Suramin pretreatment lowers the *P. falciparum* induced anion current.

Suramin (0-500 µM $suramin_{total}$) preincubated for 6h in the culture medium modulates dose-dependently the whole cell currents of late-trophozoite-infected human RBCs (Fig. 14A-C) as assessed by patch-clamp recording. Currents were recorded in the absence of suramin. Preincubation of suramin decreases the principal whole-cell current fraction of parasitized cells. This fraction is anion-

selective since (i) replacement of NaCl in the bath by Na-gluconate inhibits the outward current almost completely (Fig. 14A) and (ii) the current-voltage relationship of the whole-cell current when recorded with K-gluconate pipette and NaCl bath solution exhibits a reversal potential close to Cl^- equilibrium potential (Fig. 14B). In addition the principal current fraction is outwardly rectifying (Fig. 14B) indicating that suramin pre-treatment results in a lower activity of the infection induced outwardly rectifying anion channels (Fig. 14C). As described previously, the pretreatment with suramin was performed under normal culture condition i.e. in the presence of albumin in the medium.

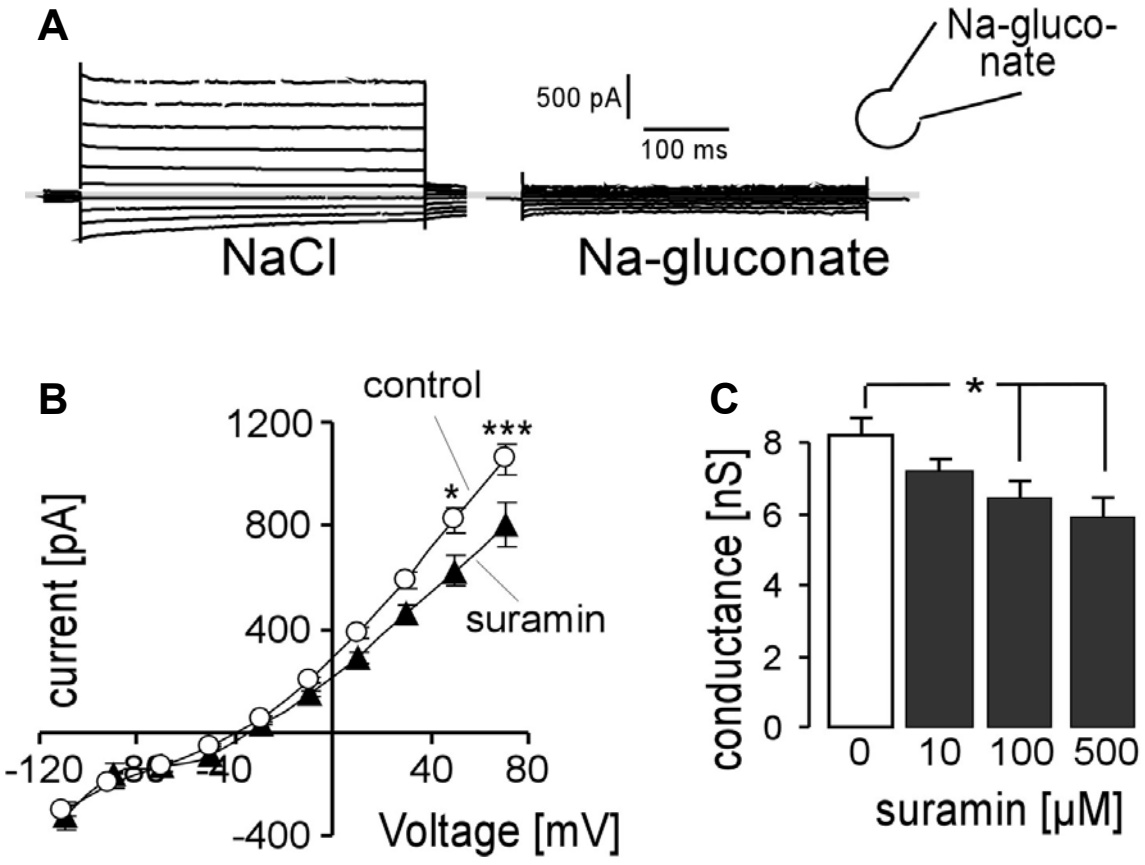


Fig. 14 A. Current traces recorded in whole-cell voltage-clamp mode from a parasitized RBC (late trophozoite-stage) with Na-gluconate pipette solution combined with NaCl (left traces) and Na-gluconate bath solution (right traces). Currents are elicited by square pulses from the -40 mV holding potential to voltages between -110 and +70 mV (currents of the individual voltage squares are superimposed). Replacing the Cl^- anions in the bath solution by gluconate extinguishes the outward currents (as shown as upward deflections of the current traces) indicating the anion selectivity of the whole-cell current. **B.** Mean current-voltage relationships (± SE; n = 10-28) of non-treated (open circles, control) and suramin–pretreated (100 µM added for 6h to the culture medium) trophozoite-parasitized RBCs (closed triangles) as recorded in C, left (* and ***: $P \leq 0.05$ and 0.001, respectively, one-way ANOVA). **C.** Mean conductance (± SE; n = 10-28) of trophozoite-parasitized RBCs pre-treated with different concentrations of suramin (0-500 µM added for 6h to the culture medium) as calculated by the slope of the current-voltage relation between -30 and +10 mV voltage (*: $P \leq 0.05$, one-way ANOVA)

5/ Purinoceptor agonists stimulate the infection and oxidation-induced hemolysis of human RBCs in isosmotic sorbitol solution.

Covalent binding of LC-sulfo-NHS- (+)-biotin (1 mM for 30 min at 37°C) to lysine residues inhibits the isosmotic sorbitol hemolysis of enriched late trophozoite/shizont-infected human RBCs (Fig. 15). Hemolysis of the biotin-treated RBCs is performed upon washing (PBS containing 5 mM D-glucose), pelleting, and incubating the cells (30 min / 37°C) in sorbitol solution (supplemented with 5 mM glucose) in the absence or in the presence of ATP and / or suramin or NPPB (100 µM each). Co-incubation with ATP stimulates the hemolysis of biotin-treated parasitized cells in a suramin-sensitive manner (Fig. 15, open bar). The hemolysis of biotin-treated parasitized human RBCs in sorbitol solution is similar to that of non-treated cells (see Fig. 12A). The hemolysis is inhibited by NPPB, which reportedly is not directly acting on purinoceptors (Mitchell *et al.*, 1998), again indicating that hemolysis results from specific uptake of sorbitol by the parasitized cells. Taken together, the data of Figs. 12-15 strongly support the involvement of purinoceptor signaling in the (re-) induction of the organic osmolyte and anion permeability of parasitized human RBCs.

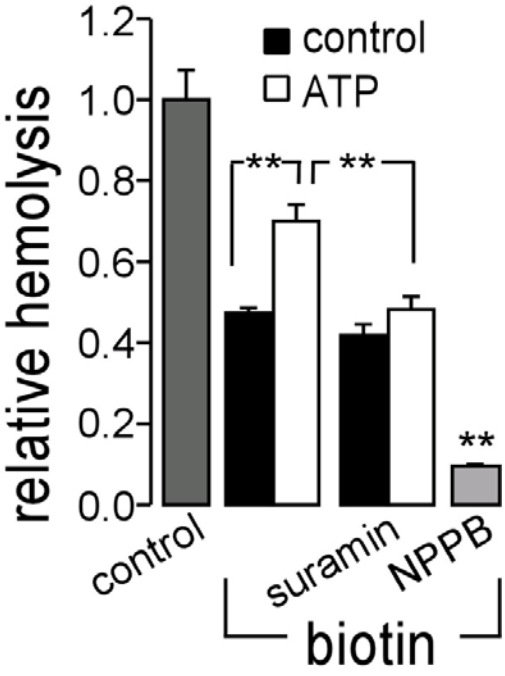


Fig. 15 Re-induction of the organic osmolyte and anion permeability in lysin-targeted parasitized RBCs. Depicted is the relative sorbitol hemolysis of enriched trophozoite-infected RBCs preincubated in the absence (control, grey bar) or presence of LC-sulfo-NHS- (+)-biotin (1 mM for 30 min at 37°C), rinsed, and post-incubated (30 min at 37°C) in isosmotic sorbitol solution. Postincubation is performed in the absence (closed bars) or presence of ATP (100 µM, open bars) and / or suramin (100 µM) or NPPB (100 µM).

The oxidation-induced organic osmolyte and anion permeability is also dependent on purinoceptor signaling as shown by the following experiments. Oxidation of non-infected human RBCs with *t*-BHP (1mM for 15 min) induces hemolysis in isosmotic sorbitol solution (Fig. 16A). On average, 17 ± 0.9 % (n=112) of the total oxidized cells hemolyze within 2.5 h of incubation in isosmotic sorbitol. The anion channel inhibitor NPPB (100 µM) abrogates the oxidation-induced hemolysis (Fig. 16A) pointing to oxidation-induced activation of a NPPB-sensitive sorbitol permeability in the RBC membrane identical to that induced by the infection with *P. falciparum*. Suramin (100 µM) blunts the oxidation-induced hemolysis (Fig. 16A) suggesting the involvement of purinoceptor signaling in the induction of the sorbitol permeability by oxidation. Conversely, ATP (100 µM) enhances the oxidation-induced hemolysis by a factor of 2-3. Similar to the oxidation-induced hemolysis, the ATP-stimulated oxidation-induced hemolysis is inhibited by NPPB and suramin (Fig. 16A). Most importantly, ATP does not induce hemolysis in non-oxidized cells (Fig. 16A), indicating that purinoceptor activation is not sufficient to activate the osmolyte conductance but that oxidation is a prerequisite to the induction of the sorbitol permeability. The insert in Fig. 16A shows the scanned images of RBC suspensions oxidized for 0, 15, and 20 min with *t*-BHP. Only the RBCs oxidized moderately (15 min, middle well) as monitored by the RBCs colour change were used for the hemolysis experiments.

Since purinoceptor signaling in human RBCs requires extracellular ATP, the effect of apyrase that degrades ATP and ADP in the incubation medium into AMP is tested. Addition of apyrase (1 U/ml) to the sorbitol solution abolishes the oxidation-induced hemolysis (Fig. 16B) indicating that oxidation-induced hemolysis, indeed, requires extracellular ATP and/or ADP.

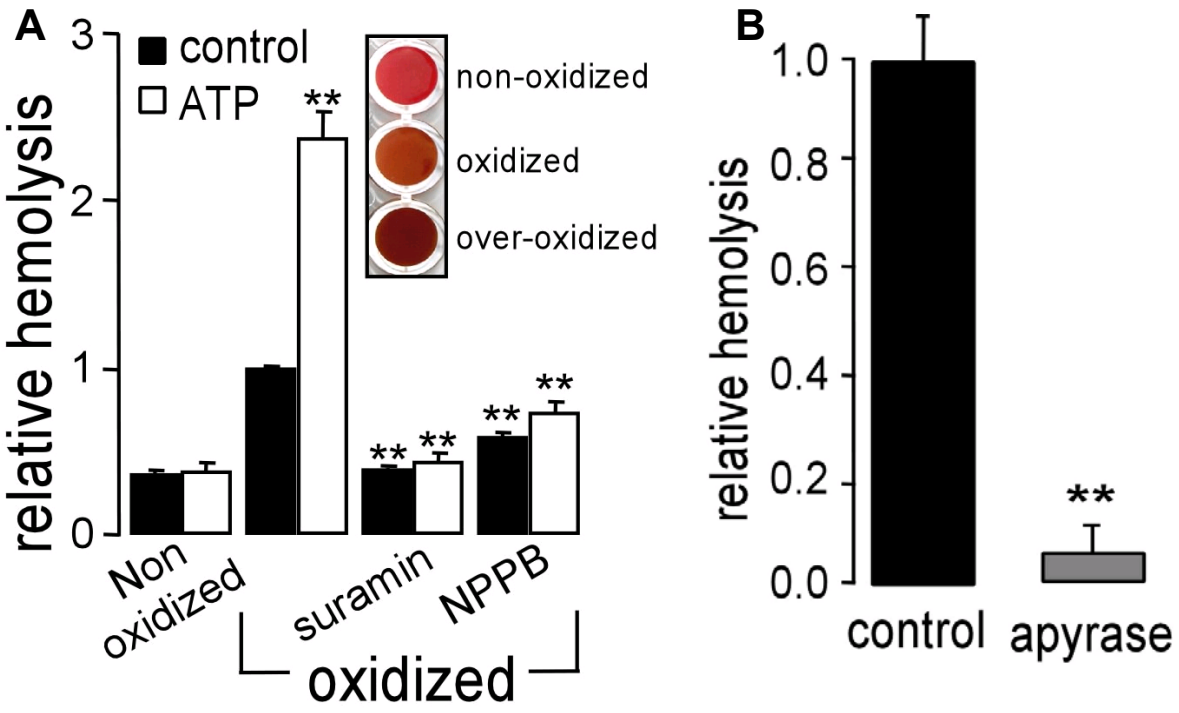


Fig. 16 Purinoceptor agonists stimulate the induction of the organic osmolyte and anion permeability in oxidized non-infected human RBCs. A. Hemolysis of non-oxidized (open bars) and oxidized RBCs (closed bars) in isosmotic sorbitol solution (2.5 h / 37°C). Incubations are performed either in the absence or in the presence of ATP (100 µM), suramin (100 µM) and/or NPPB (100µM). Data are means ± SE; n = 12-112; **: P ≤ 0.01, one-way ANOVA. The insert shows scanned images of a non-oxidized (upper well) RBC suspension, and RBC suspension oxidized for 15 (middle well) and 20 min (lower well) with *t*-BHP (1 mM). Hemolysis experiments were performed with moderately oxidized RBCs as indicated by the dark-red colour (insert, middle well) which develop the organic osmolyte and anion permeability. **B.** Degradation of extracellular ATP abolishes oxidation-induced sorbitol hemolysis. Mean relative hemolysis (± SE; n= 12) of oxidized human RBCs incubated for 2.5 h at 37°C in isosmotic sorbitol solution in the absence (control; closed bar) or presence of apyrase (1 U/ml; grey bar; **: P≤ 0.01; two-tailed Student's *t* test).

6/ P2Y purinoceptor agonists mimic the ATP effect on oxidation-induced hemolysis.

To investigate the concentration dependence of the stimulatory ATP effect, oxidation-induced hemolysis was performed in the presence of increasing concentrations of ATP (1 µM -1 mM; Fig. 17). ATP stimulates the oxidation-induced sorbitol-permeability in a dose-dependent manner with an apparent EC_{50} of about 30 µM further pointing to a purinoceptor-triggered signal transduction pathway. The insert of Fig. 17 shows in an individual experiment the hemolysates by the scanned image of the RBC supernatants.

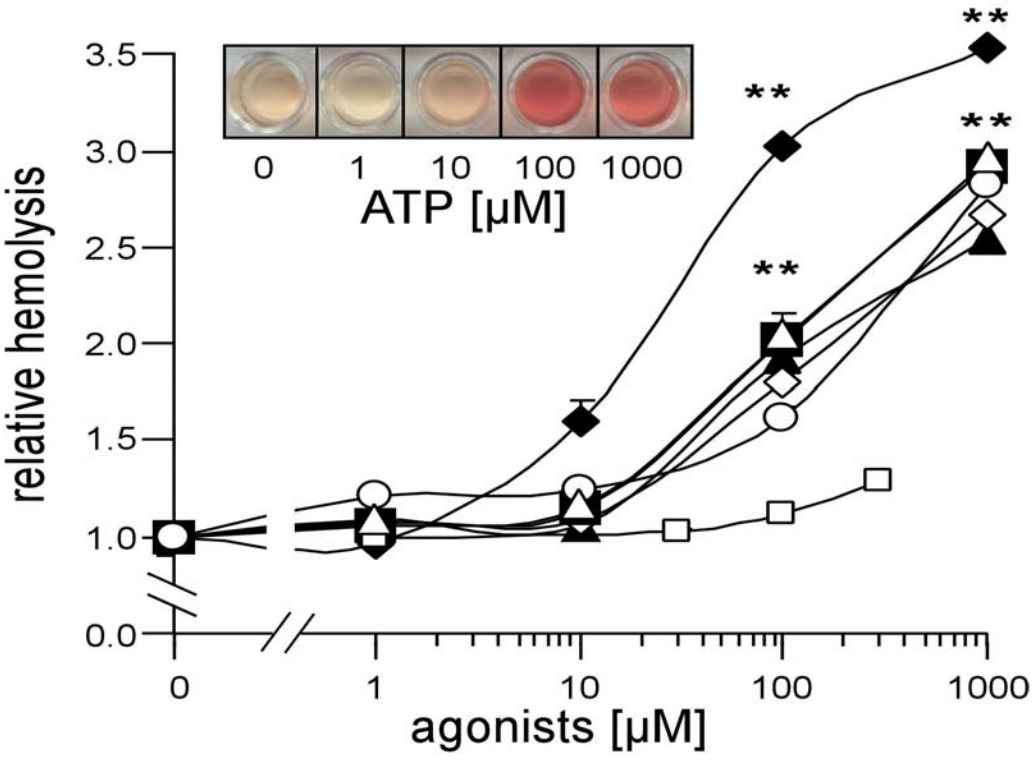


Fig. 17 Effect of different purinoceptor agonists on the induction of the organic osmolyte and anion permeability in oxidized non-infected human RBCs. The relative oxidation-induced sorbitol hemolysis (2.5h at 37°C) is shown in the absence or in the presence of increasing concentrations of ATP (♦), of the non-hydrolysable form of ATP, ATPγS (▲), as well as increasing concentrations of 2-MeSATP (◊), Bz-ATP (□), ADP (Δ), UTP (■) or ADPβS (○). Data are means ± SE; n = 12-20; **: P ≤ 0.01, ANOVA. The insert shows the scanned supernatants from human RBCs incubated for 2.5h at 37°C in isosmotic sorbitol solution. Hemolysis is indicated by the Hb concentration of the supernatant (individual experiment).

To further characterize the stimulatory ATP-effect on the sorbitol permeability in oxidized human RBCs different analogues of nucleoside di- and triphophates were tested (Fig. 17). The non-hydrolysable form of ATP, ATPγS increases the hemolysis in a dose-dependent manner with an apparent EC_{50} of ≥ 100 µM. Moreover, further P2 receptor agonists such as 2-MeSATP, UTP, ADP, ADPβS stimulate the oxidation-induced sorbitol hemolysis with EC_{50}s in the range of ≥ 100 µM (2-MeSATP, UTP, ADP) or higher (ADPβS). In contrast, 3´-O- (4-benzoyl)benzoyl ATP (Bz-ATP), an effective agonist of the $P2X_7$, $P2X_1$ and $P2Y_{11}$ receptor subtypes has no significant effect on the oxidation-induced sorbitol hemolysis. Taken together, these data indicate that ATP and (with lower efficiency) its hydrolysis product ADP are effective.

The potency of the nucleoside di- and triphosphate follows the sequence of:

ATP > 2-MeSATP = UTP = ADP ≥ ADPβS >> Bz-ATP.

7/ Antagonists of purinoceptor subtypes inhibit oxidation-induced hemolysis.

To identify candidates of involved purinoceptor subtypes, oxidation-induced and ATP-stimulated hemolysis was performed in the presence of increasing concentrations of P2 receptor antagonists. Suramin, a non-specific P2 receptor antagonist inhibits about half of the oxidation-induced hemolysis in a dose dependent manner with an apparent IC_{50} of about 10 µM (Fig. 18A, closed bars). In addition, suramin blocks the oxidation-induced ATP-stimulated hemolysis with an IC_{50} of about 1-10 µM (Fig. 18A, open bars). Reactive blue 2, a non-selective P2 purinoceptor antagonist inhibits about 2/3 of oxidation-induced hemolysis with IC_{50}s of below 1 µM in the presence and absence of ATP (Fig. 18B). PPADS, another non-selective purinoceptor antagonist inhibits half of both oxidation induced hemolysis and oxidation-induced ATP-stimulated hemolysis at a concentration of ≥ 10µM (Fig. 18C). Moreover, the $P2Y_1$-specific antagonist MRS2179 inhibits about half of the oxidation-induced hemolysis (Fig. 18D, closed bars) and of the oxidation-induced ATP-stimulated hemolysis (Fig. 18D, open bars) with IC_{50}s of about 1 µM and 0.1 µM, respectively, suggesting involvement of $P2Y_1$ in oxidation-induced hemolysis with or without additional stimulation by ATP. Since ATP still has an effect on oxidation-induced hemolysis at high MRS2179 and reactive blue 2 concentrations, oxidation-induced hemolysis of human RBCs most probably employs further purinoceptor subtypes.

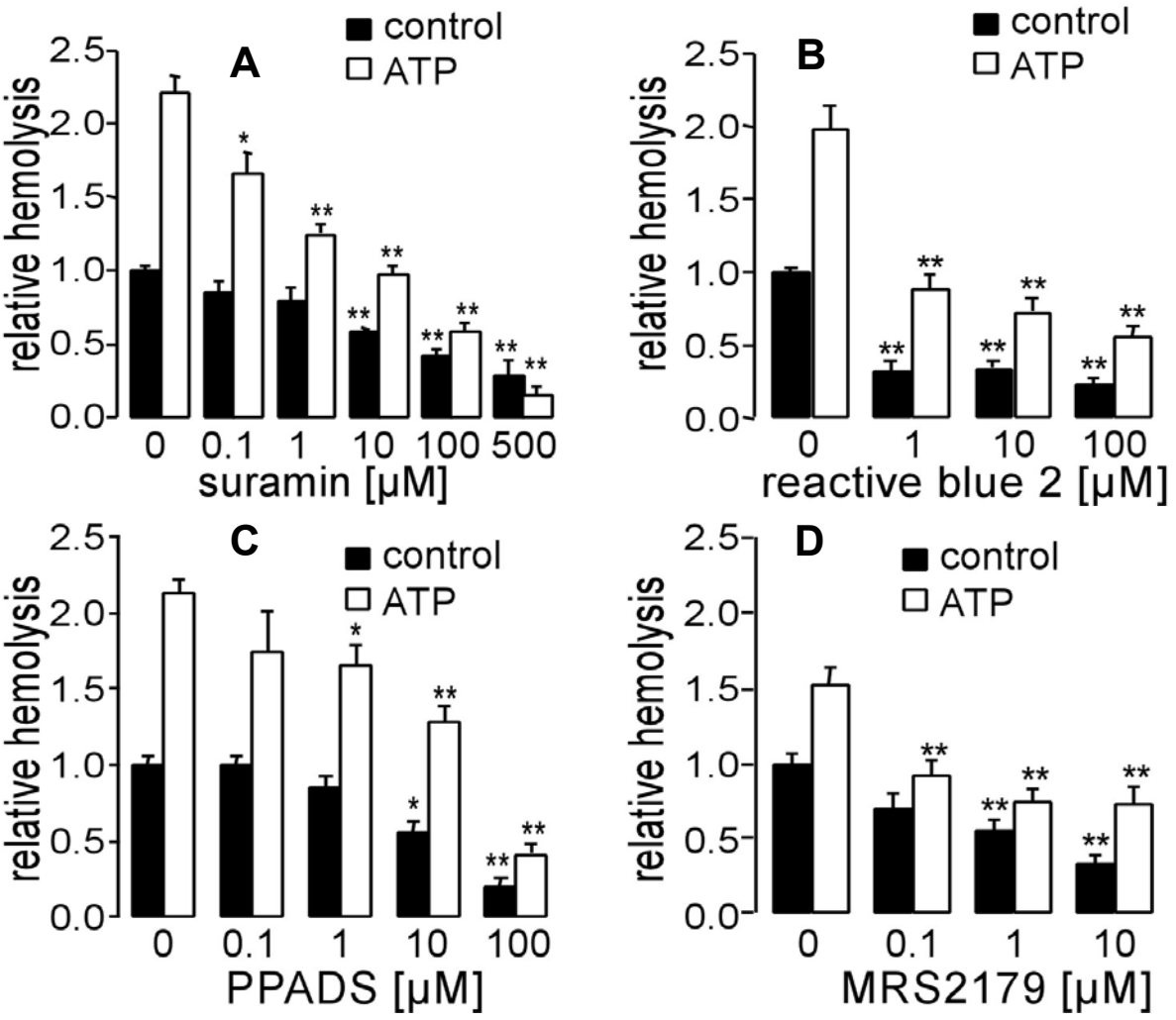


Fig. 18 Effect of purinoceptor antagonists on the oxidation-induced sorbitol hemolysis in the presence or absence of ATP. Oxidation-induced sorbitol hemolysis without (closed bars) or with additional stimulation by ATP (100 μM) (open bars) were performed in the absence or presence of increasing concentrations of (**A)** suramin, (**B**) reactive blue 2, (**C**) PPADS or (**D**) MRS2179. Shown is the mean relative oxidation-induced hemolysis ± SE; n = 12-20; *: $P \leq 0.05$; **: $P \leq 0.01$, one-way ANOVA.

8/ Oxidation- and *P. falciparum* infection-induced ATP release from human RBCs.

To test for oxidation-induced release of ATP, the time-dependent changes of the extracellular ATP concentration in suspension of control and oxidized human RBCs were analyzed (Fig. 19A). Extracellular ATP is detectable in nanomolar concentrations in suspensions of non-oxidized control RBCs. Oxidation by *t*-BHP (0.2 mM) induces a continuous increase of extracellular ATP (Fig. 19A, open triangle) indicating a release of ATP from the RBCs. The supernatant of oxidized RBCs

reaches an about 10-fold ATP concentration within 2h of incubation as compared to that of non-oxidized cells (Fig. 19B). Suspensions of non-oxidized control RBCs, in contrast, do not show any time-dependent change in extracellular ATP-concentration (Fig. 19A, closed square). This indicates that the applied experimental protocol does not stimulate ATP release by, e.g., mechanical deformation. In addition, the extracellular Hb concentration of the suspensions is below detection threshold further indicating that the observed accumulation of extracellular ATP is not due to RBCs hemolysis. Similarly, *P. falciparum* infected RBCs exhibit a time-dependent release of ATP (Fig. 19C, closed bar). Non-infected control RBCs cultured under identical experimental conditions show only little ATP release (Fig. 19C, open bar).

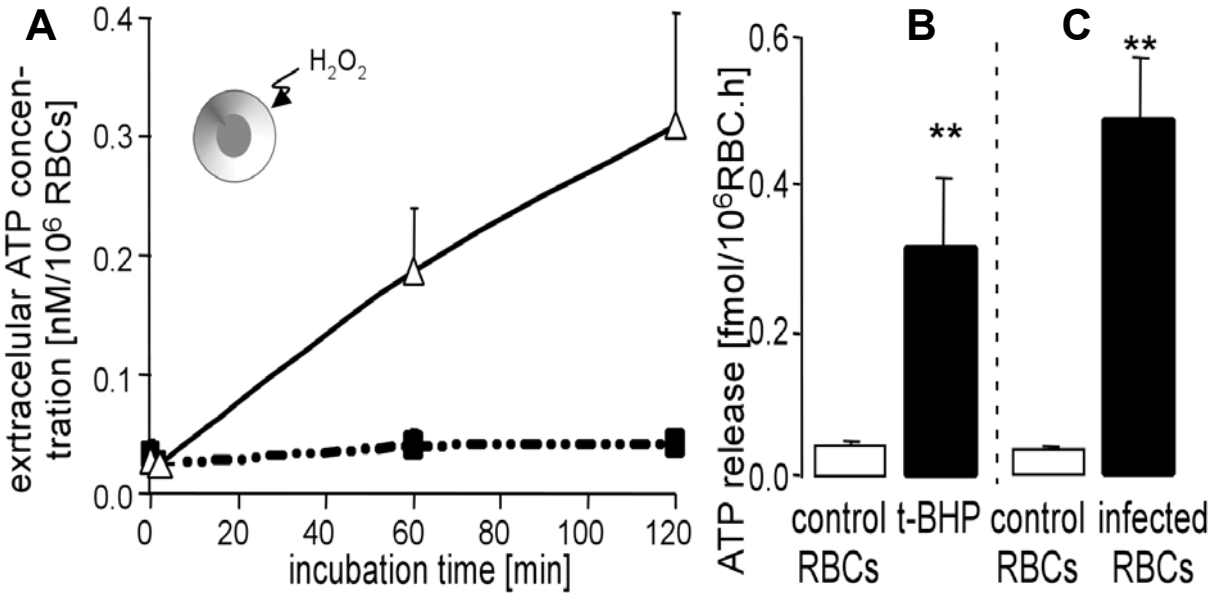


Fig. 19 Oxidized and *P. falciparum* infected human RBCs release ATP. A. Time course of ATP concentration changes in the Ca^{2+}-containing NaCl incubation medium of non-oxidized (closed squares) and oxidized human RBCs (open triangles). The oxidant *t*-BHP (0.2 mM) is added at time zero. **B-C**. Mean ATP release (± SE); n = 9-12; of non-oxidized and oxidized human RBCs (**B**) and of non-infected and *P. falciparum* infected human RBCs (**C**; **: $P \leq 0.01$, two-tailed Student's *t* test).

9/ Protein and functional expression of P2Y purinoceptor subtypes in RBCs.

Immunofluorescence staining with a polyclonal anti-$P2Y_1$ antibody and confocal microscopy demonstrates that $P2Y_1$ is expressed on human RBCs, with the levels of expression varying between subjects. $P2Y_1$ is present at low to moderate

levels on RBCs from six subjects (Fig. 20A). $P2Y_1$ was not detected on RBCs from two other subjects. The $P2Y_1$ receptors are localized at the cell surface of RBCs. Pre-immune serum is routinely included as a negative control and demonstrates no staining (Fig. 20B).

Immuno-blots of human RBC membrane preparations were also performed using another polyclonal anti-$P2Y_1$ antibody. This antibody picks two bands at 42 ± 1 and 59 ± 0.5 kDa (n = 3; Fig. 20C, left lane). Pre- and co-incubation of the antibody with the $P2Y_1$-specific peptide used to raise the antibody abolished the detection of the 42 kDa band and largely decreased the intensity of the 59 kDa band indicating specificity of the antibody (Fig. 20C, right lane). The larger protein corresponds to the size of the glycosylated human $P2Y_1$ subtype.

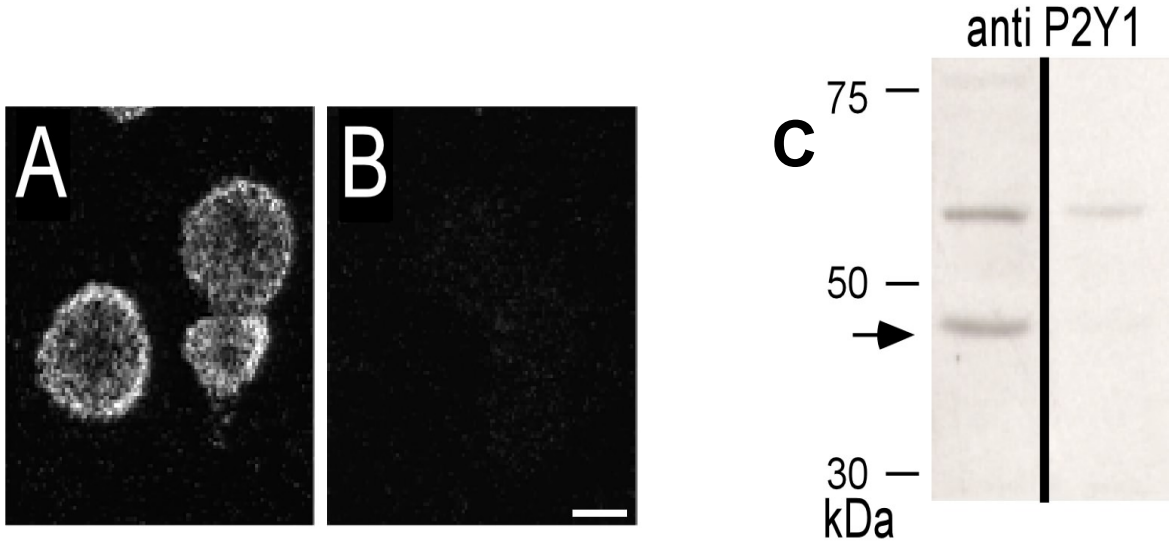


Fig. 20 Protein-expression of P2Y purinoceptor subtypes in human RBCs: **A-B.** Human RBCs were incubated with polyclonal sheep anti-$P2Y_1$ (A) antibody and subsequently with Cy3-conjugated anti-sheep IgG antibody before examination by confocal microscopy. Pre-immune serum (B) was routinely included as a negative control and demonstrated no staining. The calibration bar is 5 µm. **C.** Membrane proteins prepared from human RBCs were electrophoretically separated on 8% acrylamide gel, transferred on a nitrocellulose membrane and incubated with a commercial polyclonal rabbit anti-$P2Y_1$ antibody (1:400 dilution). The blot was either probed with antibody alone (first lane) or with antibody pre-adsorbed and coincubated with $P2Y_1$-specific peptide (15 µg/ml) against which the antibody had been raised (second lane).

To study the functional expression of $P2Y_1$, RBCs from $P2Y_1^{-/-}$ mice were compared in hemolysis experiments with those from sex- and age-matched wildtype controls (Fig. 21A). Within 1h of incubation in isosmotic sorbitol solution, oxidation induces a NPPB (100 µM)-sensitive hemolysis in 20.7 ± 3 % of the RBCs from wildtype mice. In RBCs from $P2Y_1^{-/-}$ mice, in contrast, NPPB-sensitive hemolysis is delayed resulting in significantly less oxidation-induced hemolysis (Fig. 21A). ATP

(100 µM) has a significant but less stimulating effect on the oxidation-induced and NPPB-sensitive hemolysis in mouse RBCs as compared to human RBCs (compare Fig. 21A with Fig. 15). In $P2Y_1^{-/-}$ mouse RBCs, however, ATP does not exhibit any stimulatory effect on the oxidation-induced hemolysis (Fig. 21A) within a 1 h-incubation period. In summary, these experiments indicate functional expression of $P2Y_1$ purinoceptors in RBCs and involvement of this receptor subtype in activation of the sorbitol permeability. In addition, sorbitol hemolysis (15 min/37°C) is performed with *P. berghei* ANKA-infected RBCs retrieved from $P2Y_1^{-/-}$ and $P2Y_1^{+/+}$ mice with similar mean parasitemia (43 and 45 %, respectively). Infected $P2Y_1$ -/- mice RBCs exhibit a significantly lower NPPB (100µM)-sensitive hemolysis in isosmotic sorbitol solution as compared to that of the wild type mouse RBCs (Fig. 21B)

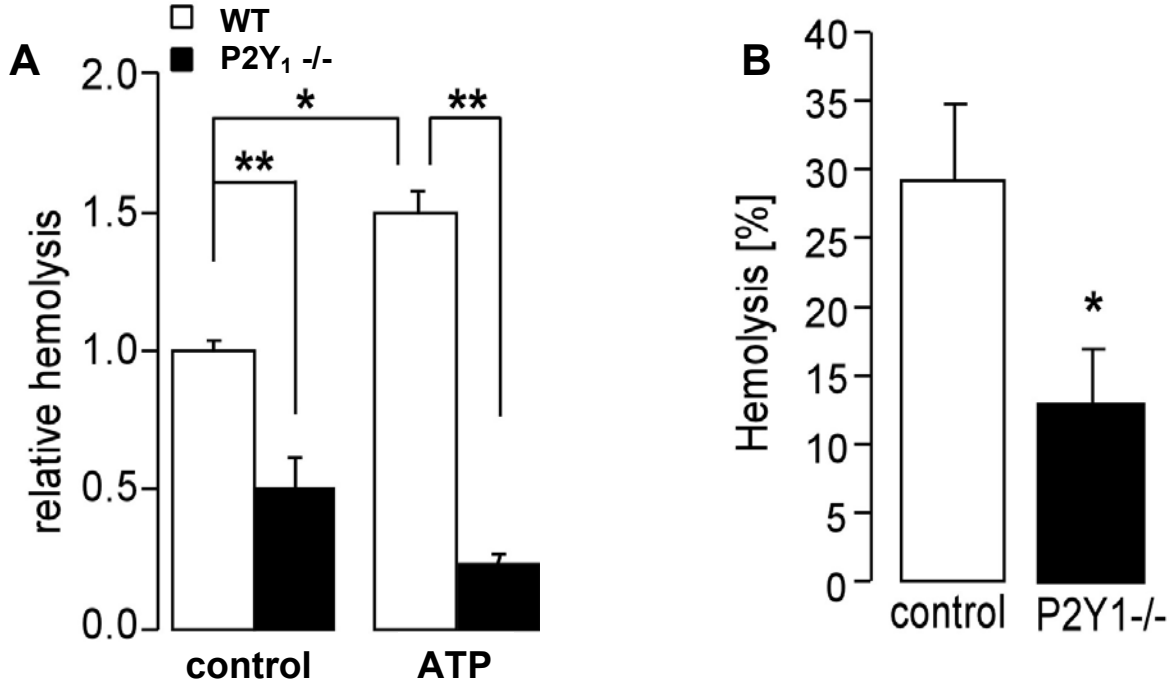


Fig. 21 Functional expression of P2Y purinoceptor subtypes in mouse RBCs A. Oxidation-induced-sorbitol hemolysis with or without additional stimulation by ATP of RBCs from $P2Y_1$ +/+ (open bars) and $P2Y_1$ -/- mice (closed bars). Shown is the mean NPPB (100 µM)-sensitive hemolysis (± SE); n = 12-24; $P \leq 0.05$; **: $P \leq 0.01$, ANOVA. **B.** *P. berghei* ANKA infection-induced sorbitol hemolysis (15 min/37°C) of $P2Y_1$ +/+ (open bars) and $P2Y_1$ -/- mice (closed bars) mouse erythrocytes. Shown is the percentage of hemolysis calculated for 100% parasitemia; at the time of experiment parasitemia measured by Syto16 staining was between 40 and 48 %.

10/ Delayed in vivo growth of *P. berghei* in $P2Y_1$-deficient mice.

$P2Y_1^{-/-}$ and sex- and age-matched wild-type mice with identical genetic background were infected with *P.berghei* ANKA (two independent experiments/18

mice in each group). The increase in parasitemia was measured every day from day 7 after infection to day 25 (Fig. 22A). Reportedly, parasitemia of *P. berghei*-infected wildtype mice increases slowly during the first days of infection, followed by an exponential increase up to 60-70% infected cells and reaches a plateau thereafter (Otten-Kuipers *et al.*, 1997; Kisilevsky *et al.*, 2002; Huber *et al.*, 2004). The parasitemia of *P. berghei*-infected wildtype mice ($P2Y_1^{+/+}$) develops with the same sigmoid curve (Fig. 22A, open squares). In sharp contrast, parasitemia of $P2Y_1$-deficient mice ($P2Y_1^{-/-}$) develops almost linearly showing no exponential increase (Fig. 22A, closed triangle). From day 16 onwards the maximal slope corresponding to the delayed parasitemia increase was calculated. This slope is significantly lower between days 16 and 21 after infection in the $P2Y_1^{-/-}$ mice (2.22 ± 0.81; n=10) as compared to that of the wild-type mice (6.45 ± 1.03; n=13; Fig. 22B). The delayed development of *P. berghei* ANKA shown in Fig. 22B did not result in a better survival of these mice when compared to the wild type controls. From 18 mice in both groups, only 1 (wildtype) and 4 ($P2Y_1^{-/-}$) mice survived day 28 of infection. To test for a different immune response in infected $P2Y_1^{-/-}$ and wildtype mice, white blood cell counts were performed on day 27 of infection. Similar numbers of white blood cells were found in both mouse groups therefore suggesting no gross differences in the immune response between both genotypes.

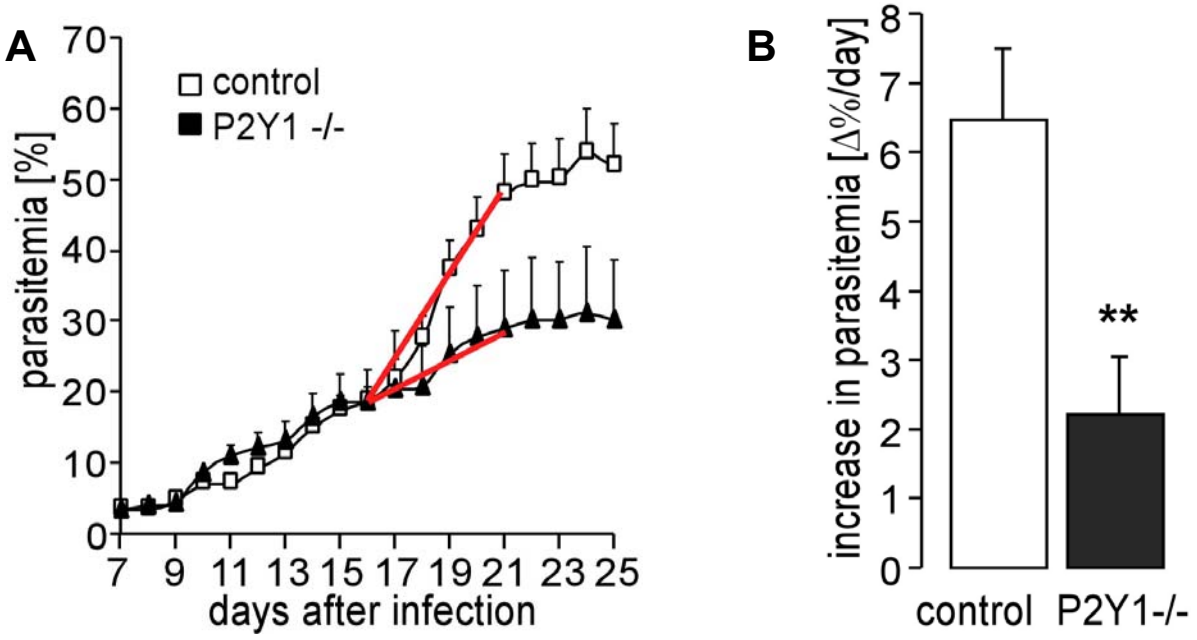


Fig. 22 The lack of $P2Y_1$ receptors delays infection by *P. berghei*. **A.** Time course of parasitemia increase in $P2Y_1$ +/+ (open circles) and $P2Y_1$ -/- mice (closed circles) intraperitoneally infected with 10^6 *P. berghei* ANKA-parasitized mouse erythrocytes at day 0 (means ± SE; n = 10-13).

B. Mean slope of parasitemia increase (± SE; n = 10-13) in $P2Y_1$ +/+ (open bar) and $P2Y_1$ -/- mice (closed bar). Slopes were calculated from the data of Fig. 22A by linear regression between days 16 and 21 (**: $P \leq 0.01$, two-tailed Student's *t* test).

11/ Suramin inhibits *P. falciparum* growth in vitro.

To study the functional significance of the purinoceptor signaling for *P. falciparum* development *in vitro*, 48-h growth assays were performed in the presence of increasing concentrations of suramin (from 1 to 500 µM; Fig. 23). Growth of ring-stage-synchronized parasites is dose-dependently decreased by suramin with an apparent IC_{50} of around 100µM which corresponds to an effective free suramin concentration of about 2 µM (see Fig. 13), a similar concentration range which inhibited the induction of the organic osmolyte and anion permeability in oxidized cells (see Fig. 18).

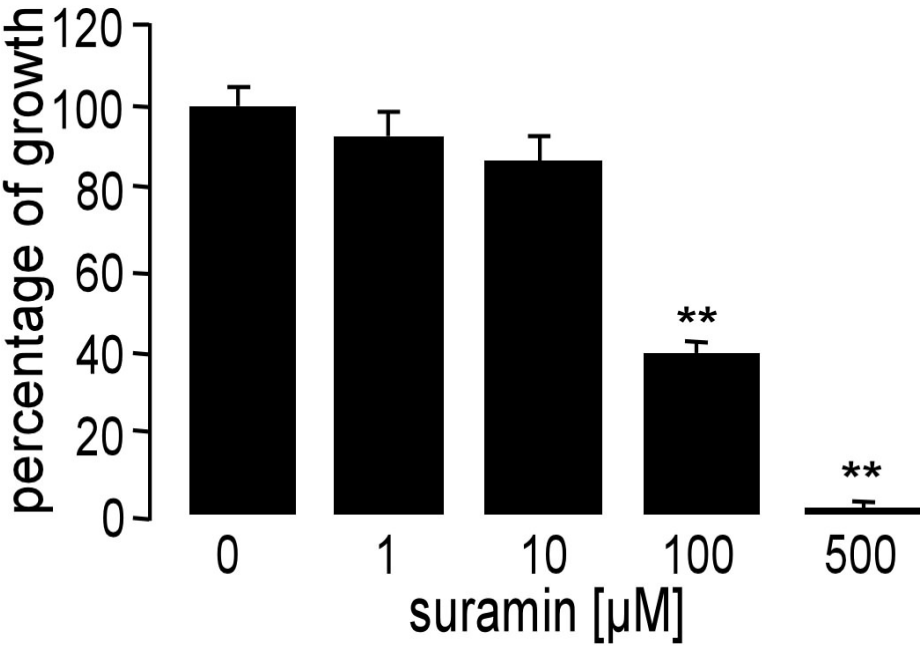


Fig. 23 Suramin inhibits *P. falciparum* growth *in vitro*. Growth of ring stage-synchronized parasites cultured for 48h in medium containing increasing concentrations of suramin. Parasite growth is given relative to growth in the absence of suramin (n=11-12; **: $P \leq 0.01$; one-way ANOVA).

12/ Suramin inhibits *P. falciparum* intraerythrocytic development.

The applied growth assay protocol did not distinguish between a suramin effect on intraerythrocytic parasite amplification and an effect on re-invasion of the merozoites. However a recent study demonstrated that suramin inhibits merozoite invasion into the human RBCs (Fleck *et al.*, 2003) In order to demonstrate that suramin, in addition, impaired intraerythrocytic parasite proliferation, growth assays where the time frame of parasite development was redueced to that of the intraerythrocytic amplification were performed. This was achieved by synchronizing twice (at 6 hours interval) parasitized RBCs at the ring stage so that the parasite are at maximal 15 h after post-invasion and cultured for 16h (so that they are in the late trophozoite/schizonts stage when they increase their DNA/RNA amount and no merozoite is released) in normal culture medium in the presence or absence of suramin. Applying this growth protocol does not result in an increase in parasitemia within the 16h of culture indicating negligible release and re-invasion of merozoites as well as negligible death of parasitized cells. Intraerythrocytic amplification is deduced from the DNA/RNA amount of the parasitized RBCs as assessed by Syto16 fluorescence in FACS analysis. After 16 h culture, a shift of fluorescence toward higher Syto16 fluorescence intensity is observed. Fig. 24A shows the Initial peak obtained at time 0 of culture and the Control peak obtained after 16 hours culture with the same FACS settings; this observed shift is corresponding to DNA/RNA amplification. Interestingly, Fig. 24A shows a lower Syto16 fluorescence intensity of parasitized RBCs cultured in the presence of suramin (500µM) as compared to those cultured in its absence. In Fig. 24B is the relative increase in Syto16 fluorescence intensity induced by parasite development (n=14-18) indicating significant inhibition of the DNA/RNA increase of parasitized RBCs by 100 µM and 500 µM suramin. In summary, these data demonstrate an inhibition of intraerythrocytic P. falciparum amplification *in vitro* by suramin.

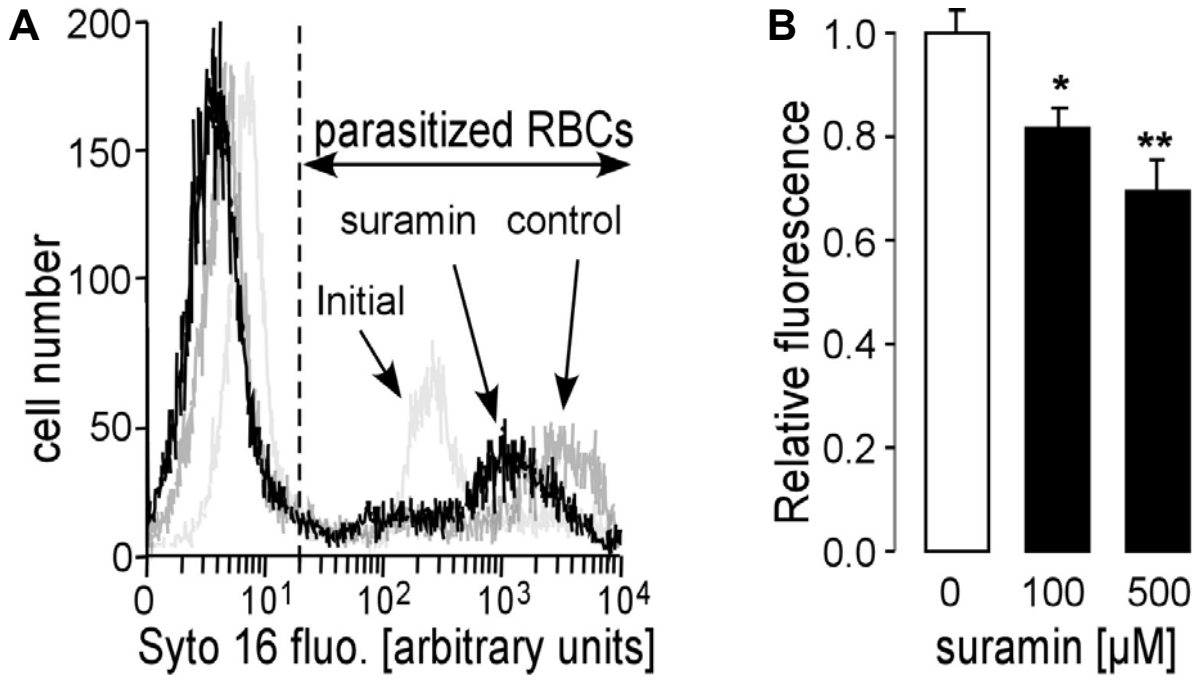


Fig. 24 Suramin inhibits the intraerythrocytic amplification of *P. falciparum*. A. Histogram plot overlay showing the DNA/RNA-specific Syto16 fluorescence of ring-stage-synchronized parasite cultures grown for 16 h in the absence (control) or presence of suramin (500µM). **B.** Mean Syto16 fluorescence intensity of RBCs grown under control conditions (open bar) or in the presence of suramin (100 µM, grey bar, or 500 µM, closed bar). The infection-induced fluorescence is defined as the fluorescence intensity of the Syto16-positive RBC population (geometrical mean) minus the background fluorescence of the non-infected RBC population; data are means ± SE; n = 8; * and ** indicate $p \leq 0.05$ and 0.001, respectively; one-way ANOVA).

13/ Extracellular calcium removal inhibits oxidation-induced sorbitol hemolysis.

Addition of the calcium chelator, EGTA (1mM) to the isosmotic sorbitol significantly reduced the oxidation-induced hemolysis (Fig. 25). The stimulating effect of ATP is abolished under this condition suggesting that calcium signaling is involved in the induction of the osmolyte conductance upon activation of the purinoceptor by ATP. It is generally recognized that all P2 purinoceptors bind ATP^{4-}, uncomplexed to cations (Ralevic & Burnstock, 1998;Di Virgilio et al., 2001). However, the effect of divalent cations removal is complicated by the concomitant inhibition of ecto-ATPase/ectonucleotidase activity, because these enzymes require the presence of divalent cations.

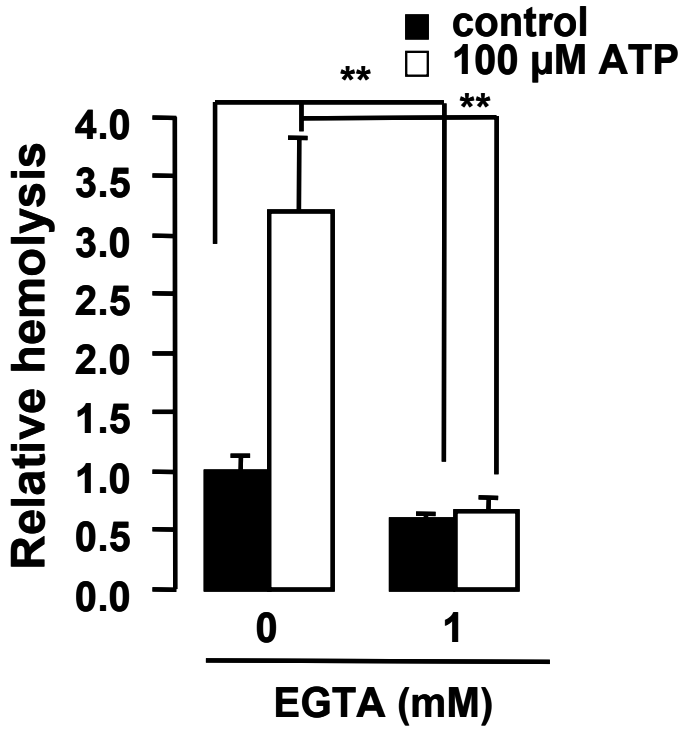


Fig. 25 Extracellular calcium removal inhibits oxidation-induced sorbitol hemolysis. Oxidation-induced sorbitol hemolysis without (closed bars) or with additional stimulation by ATP (100 µM) (open bars) performed in the absence or presence of 1 mM of EGTA. Shown is the mean relative oxidation-induced hemolysis ± SE; n = 7-12; **: $P \leq 0.01$, one-way ANOVA.

14/ Ca^{2+} permeabilization and oxidative stress induce break down of phosphatidyl serine asymmetry in human erythrocyte membrane.

In addition to the anion and organic osmolyte permeability, *Plasmodium* infection induces the activation of a non-selective cation channel. This channel can be activated upon oxidation. To test for the role of this non-selective cation channel in programmed cell death of human RBCs, control human RBCs were treated with the oxidant *tert*-butylhydroperoxide (*t*-BHP; 1 mM for 15 min; Fig. 26 A, B), post-incubated for 24h in NaCl test solution and then measured by annexin binding (reflecting cell membrane phosphatidylserine asymmetry) in flow cytometry. Following oxidation, almost all RBCs become annexin positive.

The channel is permeable to Ca^{2+} and opening of the channel increases cytosolic [Ca^{2+}] which is another feature of apoptosis in nucleated cells. Therefore, increasing cytosolic [Ca^{2+}] could induce a similar shift of fluorescence. To test for this hypothesis, non-infected RBCs were treated with the Ca^{2+} ionophore 1µM ionomycin

for 1h and annexin binding was measured. Increasing cytosolic [Ca^{2+}] mimics the stimulatory effect of oxidation on annexin binding (Fig. 26B) suggesting that oxidation-induced activation of the Ca^{2+}-permeable NSC conductance and the resulting increased Ca^{2+} permeability of the RBC membrane contribute to the observed annexin binding of RBCs.

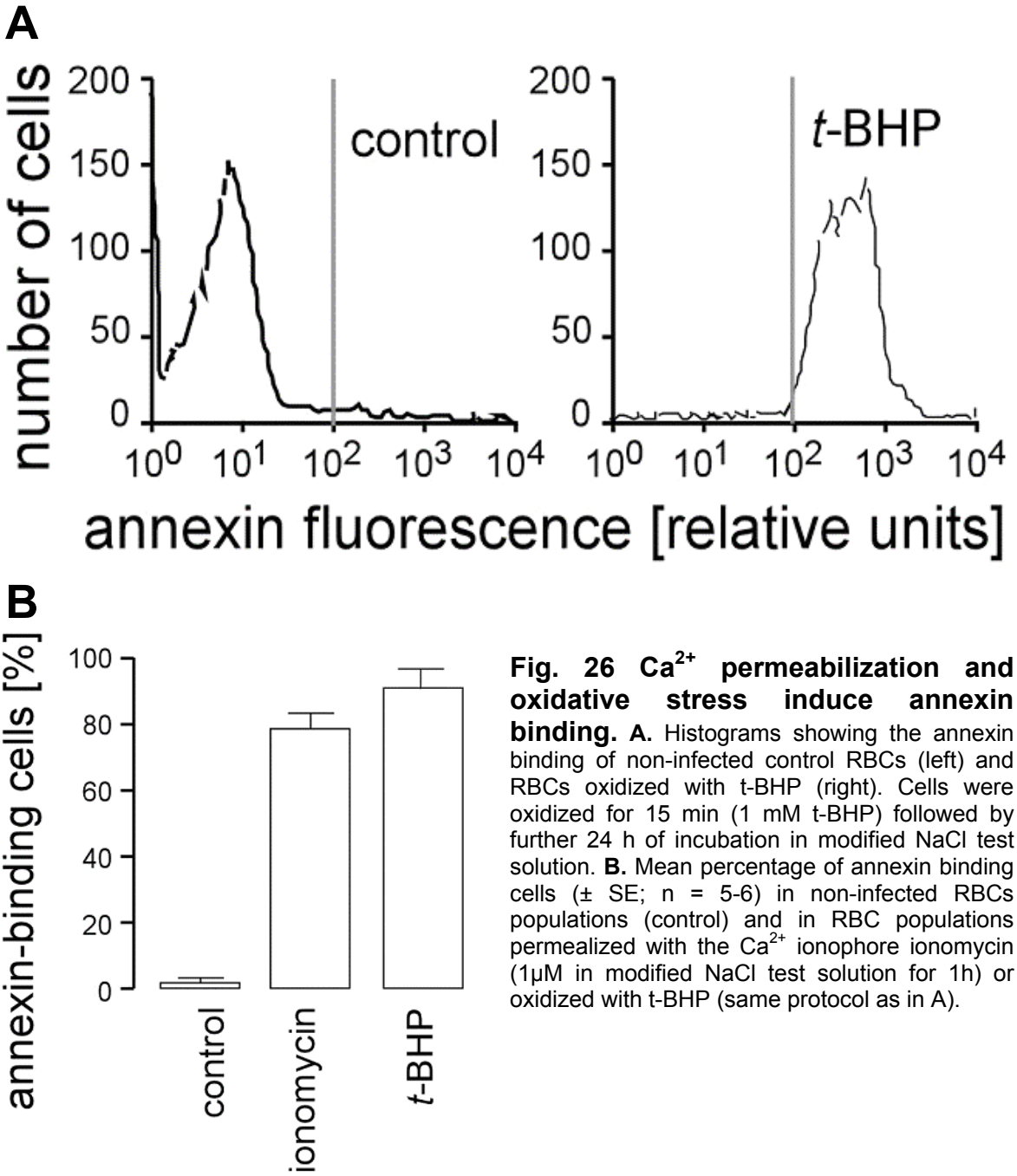
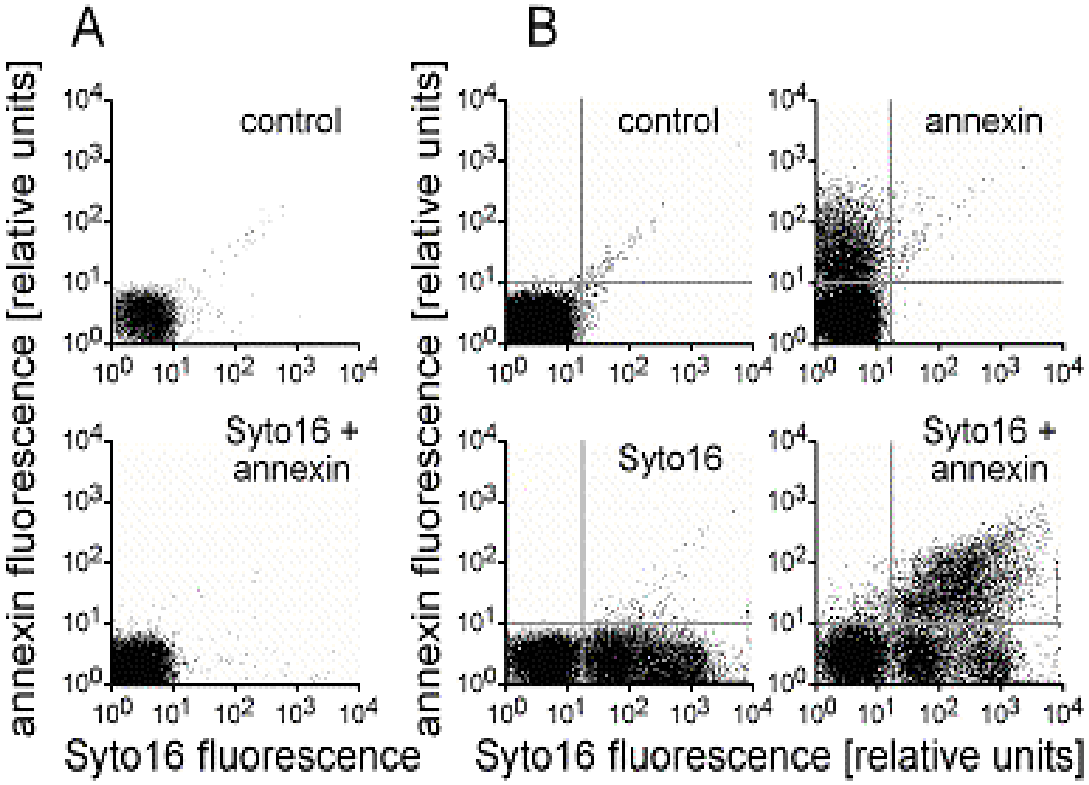


Fig. 26 Ca^{2+} permeabilization and oxidative stress induce annexin binding. A. Histograms showing the annexin binding of non-infected control RBCs (left) and RBCs oxidized with t-BHP (right). Cells were oxidized for 15 min (1 mM t-BHP) followed by further 24 h of incubation in modified NaCl test solution. **B.** Mean percentage of annexin binding cells (± SE; n = 5-6) in non-infected RBCs populations (control) and in RBC populations permealized with the Ca^{2+} ionophore ionomycin (1µM in modified NaCl test solution for 1h) or oxidized with t-BHP (same protocol as in A).

15/ *Plasmodium falciparum* infection induces break down of phosphatidyl serine asymmetry in the host erythrocyte membrane.

To test whether the infection-induced NSC conductance similarly induces a loss of the phospholipid asymmetry, phosphatidylserine exposure at the outer membrane leaflet of *Plasmodium falciparum*-infected RBCs was measured by annexin binding in a flow cytometry. For this experiment, the parasite culture was grown to high parasitemia as monitored by Syto16 fluorescence staining. Fig. 27A and 27B show individual dot plots of control RBC (A) and RBCs from such high parasitemia cultures. The fluorescence intensities recorded at two wavelengths (corresponding to the emission spectra of both dyes) are given for non-stained, Syto16-stained, annexin-stained and Syto16 and annexin-stained samples clearly indicating at least four distinct RBC populations. Analysis of these populations indicates that only a very small portion of control RBCs bound annexin (Fig. 27A and 27C, control). Syto16-negative (i.e., non-infected) RBCs from the parasite culture shows a slightly elevated annexin binding while Syto16-positive (i.e., infected) cells exhibits a high percentage of annexin binding cells (Fig. 27C, closed bars). This observation points to break down of the phosphatidylserine asymmetry of the cell membrane in infected RBC that increases with the percentage of parasitemia in the *in vitro* culture (Fig. 27C).



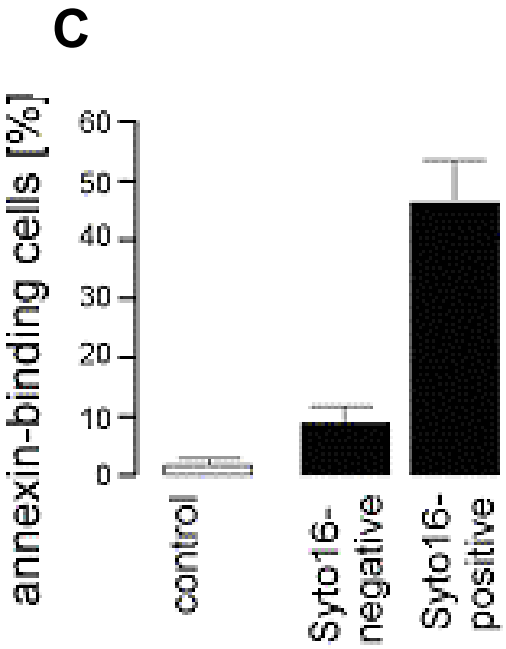


Fig. 27 *P. falciparum* infection induces annexin binding. A-B. Dot plots recorded by flow cytometry in non-infected (**A**) and *P. falciparum*-infected RBCs (**B**). The blots are obtained from non-stained RBCs (control), and from cells stained with annexin V568 fluorescence dye (annexin), with Syto16 fluorescence dye (Syto16), or with both fluorescence dyes (Syto16 + annexin). Annexin specifically binds to phosphatidylserine in the outer membrane leaflet and to DNA, thus assessing break down of phospholipid asymmetry and parasite infection, respectively. Shown are individual samples, upon counting of 100,000 RBCs. **C.** Mean percentage of annexin binding cells (±SE; n = 3-5) in populations of control RBC (open bar, control) and *Plasmodium falciparum*-infected RBC cultures (closed bars). Syto16-positive and Syto16-negative cells are the infected and non-infected cell populations in the *P. falciparum*-infected culture, respectively.

DISCUSSION

The present study demonstrates the stimulation of an organic osmolyte and anion permeability in the human RBC membrane by extracellular ATP.

Oxidative stress induces complex alterations of membrane ion permeabilities in human RBCs. Non-infected human RBCs have very little membrane conductance in whole-cell recordings. Upon *Plasmodium* infection, New Permeation Pathways (*NPPs*) are up-regulated to meet the requirements of intraerythrocytic parasite development (parasite nutrition, waste disposal, cation leakage, maintenance of host-cell constancy). Tracer flux measurements and isosmotic hemolysis experiments describe these *NPPs* as organic osmolyte and anion channels with additional low but significant cation permeability. Recent patch-clamp recordings have characterised inwardly rectifying and outwardly rectifying anion conductances as well as a non-selective cation channel (Duranton *et al.*, 2002; Huber *et al.*, 2002). The outwardly rectifying conductance was shown to be permeable to organic osmolytes (Duranton *et al.*, 2004). Virtually identical inwardly and outwardly rectifying anion channels are observed in non-infected RBCs following oxidation. As *P. falciparum* is known to confer oxidative stress to the host cell, we can conclude that the oxidation and/or the *Plasmodium* infection activate endogenous quiescent (i.e. non active under resting conditions) conductances (Huber *et al.*, 2002).

ATP stimulated the osmolyte permeability both in *P. falciparum* infected (by about 30-40 %) and in non-infected oxidized RBCs leading to an increased hemolysis in isosmotic sorbitol. The enhanced osmolyte conductance in the presence of ATP was inhibited by the chloride channel and *NPPs* inhibitor, NPPB. Moreover, degradation of extracellular ATP and ADP by apyrase almost abolished the induction of the osmolyte permeability in oxidized human RBCs pointing to a pivotal role of extracellular ATP and/or ADP.

However, ATP failed to activate directly the osmolyte permeability in non-oxidized and non-infected RBCs. Similarly, non-oxidized and non-infected RBCs incubated in isosmotic sorbitol do not hemolyse. Therefore, activation of the osmolyte permeability through a *Plasmodium*-dependent or/and oxidative process seems a prerequisite to the stimulating effect of ATP.

Moreover, ATP has been demonstrated to be released by parasitized and oxidized RBCs suggesting a positive feed back of the ATP-induced signaling cascade. ATP (ADP)-induced ATP release has been demonstrated in human RBCs (Trams *et al.*, 1980; Knofler *et al.*, 1997). The mechanism of the release from erythrocytes remains unclear. Elevated plasma ATP concentration in patients with acute malaria infection has previously been described (Essien & White, 1998). However, the authors attributed this rise in ATP concentrations to the rupture of parasitized RBCs. They further hypothesized that the ATP released may activate platelets, which has been demonstrated during acute malaria infection (Essien & Ebhota, 1981).

In addition to *P. falciparum* infection or oxidation, decreased oxygen tension, reduced pH, or mechanical deformations have been shown to stimulate ATP release from uninfected mature human RBCs. The released ATP has in turn been demonstrated to induce relaxation of blood vessels via its link to P2Y receptors on endothelial cells and subsequent NO formation (Kennedy *et al.*, 1985; Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995; Sprague *et al.*, 1996; Sprague *et al.*, 1998; Dietrich *et al.*, 2000; Gonzalez-Alonso *et al.*, 2002). Induction of ATP release by, e.g., mechanical deformation, involves heteromeric Gs protein (Olearczyk *et al.*, 2001) and cAMP formation (Sprague *et al.*, 2001), and is dependent on CFTR (Sprague *et al.*, 1998). The infection-induced inwardly rectifying anion channel of human RBCs is similarly regulated by protein kinase A (Egee *et al.*, 2002) and dependent on CFTR (Verloo *et al.*, 2004). It might be speculated that CFTR-dependent ATP release links activity of CFTR to that of the infection-induced inward rectifier as reported for other anion channel types in nucleated cells. Braunstein *et al.* (Braunstein *et al.*, 2001) indeed showed that CFTR enhances ATP release stimulated by hypotonic challenge.

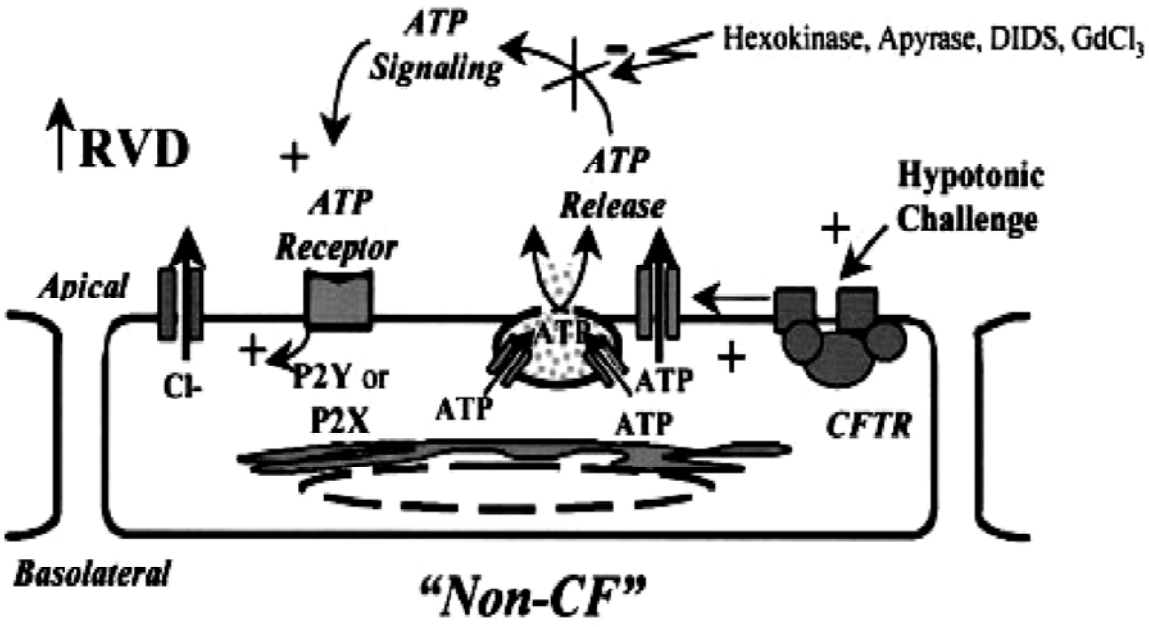


Fig. 28 Model proposed by Braunstein *et al.* (Braunstein *et al.*, 2001) of autocrine effect of ATP on RVD.

Fig. 28 shows the model proposed by Braunstein *et al.* Blockade of ATP release by ion channel blocking drugs, gadolinium chloride and DIDS attenuated the effects of CFTR on acceleration and potentiation of RVD. Therefore their results demonstrated that extracellular ATP and autocrine/paracrine purinergic signaling plays a key role in the regulation of membrane ion permeability. CFTR potentiates ATP release by stimulating a distinct ATP channel to exert an autocrine control of cell volume regulation. Infected human RBCs, in sharp contrast, do not employ organic osmolyte and anion channels for RVD (Brugnara, 1997). Induction of organic osmolyte permeability via (CFTR-dependent or –independent) ATP release - as demonstrated by the present study - suggests that similar processes as in nucleated cells are (re-) activated in mature human RBCs by Plasmodium infection or/and oxidation.

In addition, the present study shows that suramin-sensitive purinoceptors are involved in the induction of the osmolyte and anion permeability. Addition of suramin to the culture medium downregulated sorbitol permeability and anion currents of *P. falciparum*-infected human RBCs by some 25 %. Assuming that the osmolyte permeability develops linearly within 24h, the applied 6h-treatment is expected to decrease the permeability by some 25%. In contrast, whole cell patch-clamp

recordings and sorbitol hemolysis of late-trophozoite *P. falciparum* infected human RBCs showed that suramin applied acutely had no inhibitory effect indicating that the effect of suramin was upstream the osmolyte conductance.

Furthermore, suramin as well as other purinoceptor antagonists inhibited the induction of the sorbitol permeability in oxidized- or ATP-stimulated oxidized human RBCs. In particular, MRS2179, a specific $P2Y_1$ antagonist (Boyer *et al.*, 1998; von Kugelgen & Wetter, 2000), inhibited the permeability induction in oxidized- or ATP-stimulated oxidized in human RBCs suggesting that the suramin-sensitive $P2Y_1$ purinoceptor subtype contributed to the induction of the osmolyte permeability. In addition, $P2Y_1$ deficiency of mouse RBCs resulted in a decreased organic osmolyte permeability induced by both, oxidation- and *P. berghei*-infection. It remains controversial whether $P2Y_1$ receptors are ADP rather than ATP receptors which seems to be dependent on different factors like assay conditions or receptor density (Leon *et al.*, 1997; Palmer *et al.*, 1998; Ralevic & Burnstock, 1998).

Moreover, the pharmacology of the oxidation-induced sorbitol hemolysis of human RBCs clearly demonstrated involvement of further subtypes of purinoceptors such as UTP-stimulated $P2Y_2$-like receptors that are suramin-sensitive but reactive blue 2- and MRS2179-insensitive. There are several reports of reactive blue 2-insensitive UTP effects in other cell types (Norenberg *et al.*, 1997; Ralevic & Burnstock, 1998; Rubino *et al.*, 1999). In addition to metabotropic purinoceptors, the functional expression of ionotropic P2X receptor subtypes in mature human RBCs has been recently demonstrated ($P2X_7$, $P2X_2$) (Sluyter *et al.*, 2004) suggesting that similar to nucleated cells, mature human RBCs express a set of different purinoceptor subtypes.

The present study further reveals the functional significance of purinoceptor signaling for the intraerythrocytic development of Plasmodium *in vivo* and *in vitro*. *P. berghei* ANKA-infected $P2Y_1$-deficient mice presented a delayed increase in parasitemia compared to their wildtype littermates. However the lack in $P2Y_1$ receptor does not inhibit completely but slows down parasite development. This phenomenon suggests that the parasite might recruit other receptor subtypes to compensate the lack of $P2Y_1$. The inhibition of *P. falciparum in vitro* growth by suramin provides additional strong evidence to the functional significance of purinoceptor signaling for the intraerythrocytic development of *Plasmodium*. The effective (i.e., free) suramin

concentration in the culture medium was evaluated and the concentrations required for the inhibition of parasite growth met those needed for inhibiting the induction of the organic osmolyte and anion permeability in oxidized human RBCs (i.e. between 1-10 µM).

However, a recent study has demonstrated that suramin inhibits merozoite invasion into the human RBCs (Fleck *et al.*, 2003), therefore additional impaired intraerythrocytic parasite proliferation by suramin was demonstrated. In growth experiments, by reducing the time frame to the stages of intraerythrocytic DNA/RNA amplification of the parasite, we observed by Syto 16 staining that this amplification was significantly reduced in the presence of 100 and 500 µM of suramin (concentrations corresponding in fact to 2 and 80 µM). This strongly suggests that suramin and $P2Y_1$-deficiency exert their inhibitory effects on intraerythrocytic *Plasmodium* development through an impaired induction of the organic osmolyte permeability.

Interestingly, the calcium chelator EGTA inhibited oxidation-induced and ATP-stimulated oxidation-induced sorbitol hemolysis. Several studies have shown that nucleated cells release ATP following a hypotonic challenge (Wang *et al.*, 1996; Junankar *et al.*, 2002) concomitant with an increase in $[Ca^{2+}]_I$. Released ATP has been shown to activate P2Y receptors coupled to G-protein which in turn leads to activation of phospholipase C, formation of inositol 1,4,5 triphosphate (IP_3) and release of Ca^{2+} from the endoplasmic reticulum. This transient increase in $[Ca^{2+}]_I$ may contribute to RVD by direct activation or modulation of K^+, Cl^- or organic osmolyte channels. On *Necturus* RBCs, Light *et al.* (Light *et al.*, 2003) demonstrated that hypotonic swelling induced a rise in $[Ca^{2+}]_i$ which was inhibited by the presence of EGTA, suramin or hexokinase, an ATP scavenger in the medium. In addition, EGTA (5mM), suramin or hexokinase added to the extracellular medium reduced swelling-induced whole-cell currents. The inhibiting effect of suramin and hexokinase was also prevented by using a calcium ionophore indicating that the calcium-dependent processes are downstream the site of action of hexokinase and suramin.

Previous studies have reported an increased calcium permeability in *P. falciparum* infected RBCs (Staines *et al.*, 1999). In addition, Wasserman *et al.* (Wasserman *et al.*, 1982) showed that calcium was indispensable for normal *P. falciparum* growth *in vitro*. Calcium blocked merozoite re-invasion as it was impeded

in the presence of EGTA (1mM). Most importantly, they showed that parasite intraerythrocytic development was blocked when EGTA (1mM) was added to the culture in the first 24h following invasion (i.e. during the induction of the *NPPs*) whereas parasites grew normally when EGTA was added between 24 and 30h post-invasion. However patch-clamp experiments performed in our laboratory have shown that the osmolyte and anion conductance was not directly influenced by calcium. Moreover, a recent study showed that extracellular ATP induces cations fluxes by activation of the $P2X_7$ receptor (Sluyter *et al.*, 2004). However, it seems unlikely that this pathway is used in the induction of the osmolyte permeability upon oxidative stress as in the isosmotic sorbitol hemolysis upon oxidation, Bz-ATP had no effect and the EC50 for ATP was much lower than that described to activate $P2X_7$ receptors (around 100 µM). Finally, we can hypothesize that in human RBCs under oxidative stress, calcium might be a signal transducing the activation of the P2Y receptors to the osmolyte permeability.

In addition, the present study investigated erythrocyte apoptosis upon oxidative stress and *Plasmodium* infection. The infection of erythrocytes by *Plasmodium falciparu*m leads to activation of a non-selective, Ca^{2+}-permeable cation channel as described by patch-clamp techniques (Christophersen & Bennekou, 1991; Bennekou, 1993; Kaestner *et al.*, 1999; Kaestner *et al.*, 2000). These channels can be activated in non-infected RBCs by oxidation (Duranton *et al.*, 2002) and have recently been shown to promote apoptosis following energy depletion (Duranton *et al.*, 2002). The present study shows that *Plasmodium* infection triggers breakdown of membrane phospholipid asymmetry, a typical feature for apoptosis. In addition, both oxidative stress and intracellular calcium concentration have been demonstrated here to induce apoptosis suggesting a pivotal role of the non-selective cation-channel in erythrocyte apoptosis. It has recently been shown that the nominal absence of extracellular Ca^{2+} blunts but does not fully prevent erythrocyte phosphatidylserine exposure following osmotic shock, an observation pointing to additional mechanisms inducing apoptosis in osmotically shocked erythrocytes (Lang *et al.*, 2003a).

In sharp contrast, activation of the non-selective cation channel allows entry of Ca^{2+} and Na^{+}, both of which are required for intracellular growth of the pathogen. Invasion of the merozoites (Wasserman *et al.*, 1982; Lew *et al.*, 2003) as well as parasite amplification within the erythrocyte (Wasserman *et al.*, 1982; Brand *et al.*,

2003) is strongly dependent on the Ca^{2+} concentration in the medium. Therefore calcium seems to play a dual role in pathogen survival. Absence of calcium is not compatible with pathogen growth and increased cytosolic calcium concentration enhances erythrocyte apoptosis that may represent a host defence mechanism serving to eliminate infected erythrocytes.

In conclusion, the malaria infected RBC is a complex system in which membrane channels and transporters play a central role in a wide rage of physiological processes, including the uptake of nutrients, the export of metabolic waste products and the maintenance or dissipation of electrochemical gradients. The present study provides some insight into the transport mechanisms activated by the parasite. The data suggest a model showing that human RBCs respond to malaria infection or/and oxidative stress by ATP release (Fig. 29). Extracellular ATP may activate purinergic receptors in order to induce an organic osmolyte and anion permeability. This signaling is, at least in part mediated by the metabotropic purinoceptor subtype $P2Y_1$. Calcium may also play a key role in the signaling pathway between the purinoceptors and the osmolyte channels.

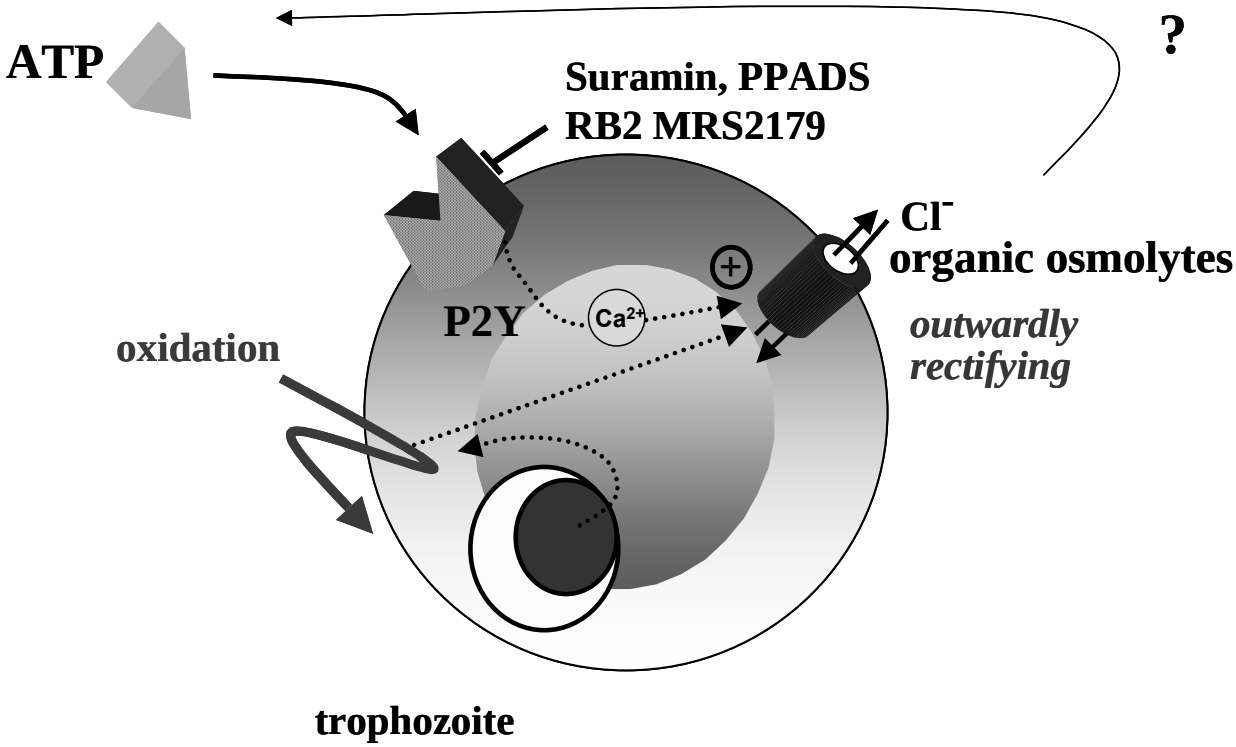


Fig. 29 Proposed model for the autocrine action of ATP on the activation of the organic osmolyte conductance in response to oxidative stress.

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List of publications

- **Tanneur V., Duranton C., Brand V., Sandu CD., Akkaya C., Gachet C., Sluyter R., Barden JA., Wiley JS., Lang F.** Purinoceptors are involved in the induction of an osmolyte permeability in malaria-infected and oxidized human erythrocytes. Faseb j. (2005, in press).

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