Programmierter Zelltod in *Plasmodium* infizierten HbA/A und HbA/S Erythrozyten

Programmed Cell Death in *Plasmodium* Infected Normal and Sickle Trait Red Blood Cells

DISSERTATION

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Contents

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Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Lang F, Huber SM (2003). Dependence of intraerythrocytic *Plasmodium falciparum* development on extracellular Ca and Na. P 09-2. *Pfluegers Archiv.* European Journal of Physiology. Supplement to Volume 445, Feb.:S71

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List of figures and tables

List of abbreviations

AMA1	apical membrane antigen 1
ASM	acid sphingomyelinase
$[Ca^{2+}]_i$	(free) cytosolic ionic calcium concentration
CD 95, Apo-1	cluster of differentiation 95, Fas receptor
CFTR	cystic fibrosis transmembrane conductance regulator
Clag 9	member of the *clag* multi-gene family, apparently necessary for cytoadherence to CD36
COX	cyclooxygenase
CR1	complement receptor type 1
DAG	diacylglycerol
DC	dichloroisocoumarin
DETAPAC	diethylene triamine pentaacetic acid
Df	dilution factor
DIDS	4,4' - diisothiocyantostilbene – 2,2' – disulfonic acid
DMSO	dimethylsulfoxid
EBA 175	erythrocyte binding antigen; 175 kD
EDTA	ethylenediaminetetraacetic acid, [Ethylenedinitrilo]tetraacetic acid
EGTA	ethylene glycol- bis (β-aminoethylether) – N,N,N',N' – tetraacetic acid
EIA	enzyme immunoassay
EIPA	5-(N-ethyl-N-isopropyl) amiloride
FACS	fluorescence assisted cell sorting
FITC	fluorescein isothiocyanate
FP IX	ferri/ferroprotoporphyrin IX, free heme
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glycerinaldehyd-3-phosphat-dehydrogenase
Gardos channel	calcium activated potassium channel
G_{cat}	nonselective cation conductance
GSH	gluthathione
GSSG	oxidized gluthathione
Hb	hemoglobin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	histidine-rich protein

ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
ISC	irreversible sickled cells
KAHRP (HRP I)	knob-associated histidine-rich protein
LCPL	lysocholinephospholipids
MACS	magnetic assisted cell sorting
MAP	mitogen activated protein
MESA	mature-parasite-infected erythrocyte surface antigen
(= PfEMP 2)	(*Plasmodium falciparum* erythrocyte membrane protein 2)
MSP	merozoite surface protein
NMDG	N-methyl-D-glucamine
NPP	New Permeability Pathway
NPPB	5-Nitro-2- (3-phenylpropylamino) benzoic acid
NSC	nonselective cation
NSM	neutral sphingomyelinase
OH-PUFA	esterified monohydroxy derivatives of polyenoic fatty acids
P	permselectivity
p. i.	post invasion
PAF	platelet activation factor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PECAM	Platelet extracellular adhesion molecule
PerCP	peridinin chlorophyll a protein
RESA = Pf155	ring-infected erythrocyte membrane surface antigen
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
pfmdr	*Plasmodium* multi-drug resistance gene
PfSUB	*Plasmodium falciparum* subtilisin
PG	prostaglandin

PK	protein kinase
PL	phospholipase
PS	phosphatidylserine
P_{sickle}	deoxygenation - induced cation pathway, found in HbS/S erythrocytes
PVM - TVM	parasite vacuolar membrane – tubovesicular network
RAP	rhoptry - associated protein
RBC	red blood cell
RhopH	high molecular mass protein complex of merozoite rhoptries
Rifin / Rosettin	product of the *rif* (repetitive interspersed family) multi-gene family
RT	room temperature
SAGM	sodium chloride, adenine, glucose, mannitol solution (SAG-Mannitol)
SM	sphingomyelin
spp.	species (plural)
stevor	product of the *stevor* multi-gene family, may-be a subfamily of the *rif* genes
t-BHP	*tert*-butylhydroperoxide
TEA	tetraethylammonium
TH	T- helper cells
TNBS	2,4,6 – trinitrobenzene sulphonic acid
TNF	tumor necrosis factor
TRAP = PfSSP2	thrombospondin-related anonymous protein, *P. falciparum* sporozoite surface protein type 2
TRP	transient receptor potential
TSP	thrombospondin

1 Introduction

1.1 Impact and distribution of malaria

The term malaria designates the diseases produced by the infection with any parasite of the genus *Plasmodium.* Four of the more than 100 species infect humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae* [874]. Of these four, *P. falciparum* is the most widespread and dangerous because it can cause fatal cerebral malaria if left untreated. 300 – 500 million people are infected with malaria each year, predominantly in developing countries, such as Africa, Southeast Asia, South and Central America, where inadequate health care systems and poor socio-economic conditions further exacerbate the spread of this disease [163]. Between 1 and 3 million people die of malaria each year; 90 % thereof in Africa, with an annual death toll of around 1 million among children up to 5 years old [139,888]. This makes malaria one of the top three killers besides AIDS and tuberculosis among communicable diseases [727].

Attempts in the 1950's and 1960's to eradicate malaria globally were initially successful in many countries, but have been abandoned as malaria vectors have become more resistant to insecticides, especially DDT (discovered from Julius Wagner-Jauregg) [352,757]. Moreover, *Plasmodia* have become resistant to chloroquine and other anti-malarial drugs [67,138,354,777,792,881], making prevention and treatment increasingly more difficult and costly [757].

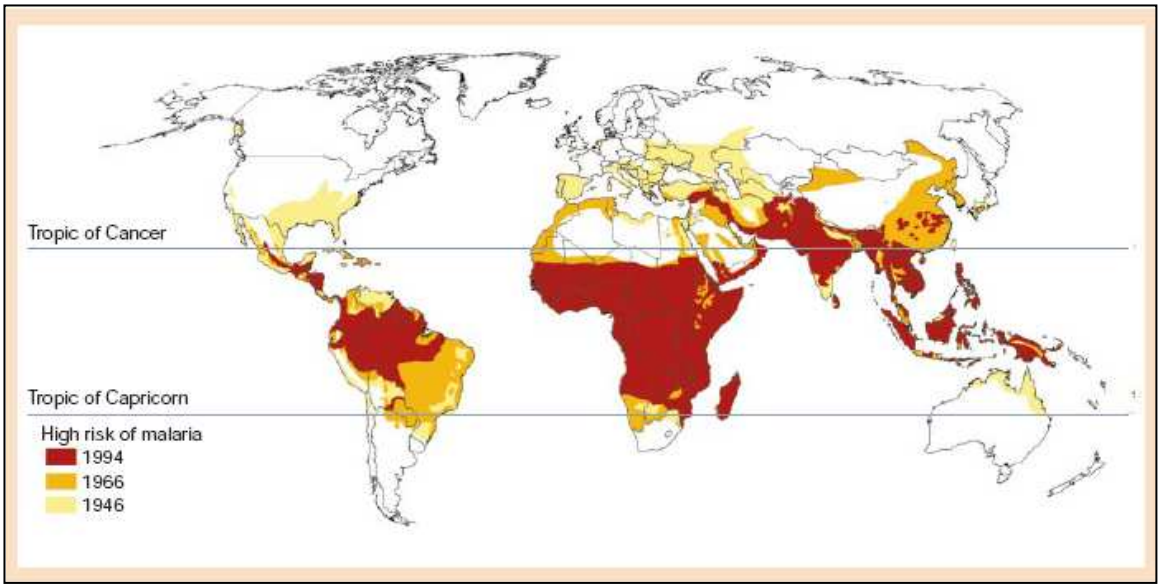


Figure 1.1. Global distribution of malaria.

The changing global distribution of malaria risk from 1946 to 1994 shows a disease burden increasingly confined to tropical regions [727]. In almost all temperate zones malaria has been eradicated due to changes in land use, environment, agricultural practices, house construction and some targeted vector control [352].

1.2 Discovery of *Plasmodium*

The malaria-like intermittent fevers and other symptoms such as enlarged spleen, periods of high fever, headaches, shaking chills, and weakness were already known more than 3000 years ago in China, Assyria and India. Hippocrates (460 - 370 B.C.) in Greece is credited with having accurately described the clinical symptoms in his medical writings [355,760]. Malaria was widespread in Europe during the Middle Ages, but malaria tropica was endemic only in the warmer South Europe. The name is derived from the Italian word *mala aria* (Latin: *malus* = bad, *aeris* = air) because the disease often appeared near swamps with their typical odor [632]. It was thought that "miasma", which literally means "bad air", caused the disease [916]. The "miasma" theory was proved wrong in the 19th century. In 1880 Charles Louis Alphonse Laveran identified the parasite in its blood stage in the act of exflagellation under the microscope [578,761]. In 1897 Sir Ronald Ross, whose teacher was Patrick Manson, who discovered the mosquito transmission of filariasis, demonstrated the transmission of malaria via mosquitoes [723,722]. However, it was only in 1948 that Short and Garnham described the liver stage of primate and human malaria (*P. vivax*) [760]. Finally, in 1966 the liver stage of *P. falciparum* was discovered [314].

1.3 Evolution of *Plasmodium spp.*

Genetic sequence comparisons indicate that the genus *Plasmodium* may have originated some 150 million years ago around the time when birds and reptiles are thought to have split [263,265]. *P. vivax* and *P. malariae* have a low morbidity and lethality and are also found in chimpanzees and gorillas. They might therefore have infected humans the earliest [264]. Morphologically and genetically, *P. falciparum* was found to be related most closely to the bird parasites *P. gallinaceum* and *P. iophurae. P. falciparum* is therefore thought to have only recently switched from its avian host to humans [590,869,870]. Its affliction of humans may have started about 10.000 years ago, at the onset of agriculture. This is consistent with the hypothesis that relatively new pathogens are less well adapted to their hosts, producing more malignant and dangerous diseases. Recent analyses support more the hypothesis that *P. falciparum* emerged only recently through a population bottleneck in Africa [64,190,706,708,707]. The agricultural revolution and animal domestication probably facilitated the spread of *P. falciparum* in Africa when human agriculture practices brought about changes in speciation

and anthropophilic behavior of anopheline mosquito populations. This may have led to an increase in malaria transmission and perhaps selection of more aggressive strains of *P. falciparum* parasites [186]. According to haplotype analysis, it was in the same timeframe, *i.e.* between 3,000 and 11,000 years ago, that a major glucose-6-phosphate dehydrogenase (G6PDH) enzyme deficiency, (G6PDH A^-) was selected for as a malaria protective polymorphism in the Middle East and Northeast Africa [834]. In the Western hemisphere *P. falciparum* probably was introduced only by the colonization of the Americas by Europeans and the trade of African slaves [265].

1.4 Life cycle of *Plasmodium falciparum*

Just as other members of the infraphylum sporozoa, *Plasmodia* pass through a sequence of three different types of reproduction during their life cycle (Fig. 1.2. for *P. falciparum*):

(1) A single run of sexual reproduction, called the "sporogonic cycle" (sporogony), taking place in the *Anopheles* host that transmits sprorozoites to the human host,

(2) A single run of asexual reproduction, the "preerythrocytic hepatocytic cycle", in a liver cell of the human host, followed by an indefinite number of runs of asexual reproduction, the "erythrocytic cycle". Both these cycles produce merozoites, and the act of reproduction during this phase is therefore referred to as merogony.

(3) Alternatively, some parasites will undergo gametogony and differentiate into male and female gametocytes, which, if taken in the female *Anopheles* mosquito midgut with the blood meal, will initiate the sporogonic cycle again.

Plasmodium belongs to the subphylum apicomplexa (classification see appendix), because sporozoites and merozoites invade cells with the help of an “apical complex” (Fig 1.2.C. Invasive stages and 1.2.E.I.). The apical complex is a group of membrane-bound organelles located at the anterior, *i.e.* apical end (AP). The organelles consists of micronemes (Mn) (ovoid bodies, 100 nm in length [71], 40 - 60 nm in width), two prominent pear-shaped rhoptries (Rh) (400 - 570 nm in length, 200 - 330 nm in width), and spheroid, rounded dense granules (vesicles around 90 - 140 nm in diameter). Surrounded by two polar rings (Pr) at the apical end, the rhoptries extend posterior from the plasma membrane.

1.4.1 The arthropod vector

Humans contract malaria if bitten by a malaria-infected female mosquito of the genus *Anopheles*. The principal malaria mosquito is *Anopheles gambiae* [577]. There are about 380 species of *Anopheles*, but only about 60 are vectors of *Plasmodia* infecting humans, some 30 of which being of major importance. They are very selective of their vertebrate host, so that human malarias have no animal reservoir, *i.e.* there are no known zoonoses. All are highly active during the night, especially just before sunrise.

The habitat of the immature *Anopheles* is water, where eggs are laid on or on the edge of water. The eggs hatch after 2-3 days to release larvae (wrigglers) that develop through four larval and one pupal aquatic stage until they become adult flying mosquitoes. Together, the aquatic stages commonly last between 7 and 20 days, depending on the temperature. The male feeds only on vegetable juices whereas the female mosquito bites, as it requires blood for the maturation of the eggs. Mating that takes place only once, soon after emergence of the adult female. The female stores the spermatozoa in a deposit called spermatheca, from where they are released to fertilize successive egg batches. The adult female may live from a few days to well over a month, going through several 2-4-day cycles of blood feeds and egg laying (some 100-200 per batch). Survival and egg development are mainly dependent on temperature and relative humidity [632].

1.4.1.1 Sporogony

The mosquito's blood meal (1 - 2 µl) contains asexual and sexual stages (~ 1 to 10^5 gametocytes). (Fig. 1.2.A. and B.: Sexual development in the mosquito of *P. falciparum*). The asexual stages of the erythrocytic cycle are digested in the midgut. Microgametocytes (male) exflagellate (b) within 10 – 20 min. Each one forms eight haploid motile microgametes to quickly fertilize around 12 macrogametes (a) that has been formed from mature macrogametocytes (female). The zygotes, spherical in shape, 6 µm in diameter and non-motile (d), transform within 18- 24 h into motile palmate shaped ookinetes of approximately 15- 19 µm length and 1 – 2.7 µm width (e) that penetrate the stomach wall [308,778]. 5-6 ookinetes cross two barriers: the peritrophic matrix (PM) and the midgut epithelium, but successfully only in the appropriate vector [458]. They pass through up to 6 epithelial midgut cells and initiate programmed cell death by peroxidases to reach the extracellular space between the midgut epithelium and the overlying basal lamina (membrane) [486]. Around two of

the 5 - 6 ookinetes transform into oocysts 2 – 7 days later, surrounded by a 0.1 – 1 µm thick electron-dense capsule (f), which grows up to approximately 35 µm in diameter as the nucleus divides repeatedly into about 2000 - 8000 (2^{11} - 2^{13}) motile sporozoites [308,778]. (Fig. 1.2.B. Micrograph: Mature oocysts of *Plasmodium gallinaeceum* on the hemocoel surface of the midgut of a female *Aedes aegypti* [805].) The development within the oocysts takes 9 to 35 days (g) depending on the temperature. Temperature tolerances are as follows: (20 - 29 °C) for *P. falciparum*; (20 – 27 °C) for *P. malariae* and (16 -30 °C) for *P. vivax*. As *P. vivax* tolerates temperatures down to 16 °C, it was widespread in the Mediterranean region and even prevalent in the Netherlands, Germany, Poland and Scandinavia in former times [632]. The sporocysts (around 16000 from two oocysts) burst into the mosquito's body cavity, termed the hemocoel. Infection of the salivary gland is dependent on thrombospondin-related anonymous protein (TRAP = PfSSP2), which gradually increases during sporozoite maturation [713,196,716]. The sporozoites (11 µm in length and 1µm in diameter) invade the salivary glands, then to be transmitted (~ 10 – 20) via the saliva into the blood stream of a susceptible vertebrate host during each subsequent bite made by the infected mosquito (h) [308,778]. The enzyme apyrase, produced in the three lobes, is secreted during the bite, hindering the aggregation of platelets [423,833].

1.4.2 Merogony in the liver

Half an hour after entering the blood stream the sporozoites reach the liver tissue (Fig. 1.2.D. pre-erythrocytic, hepatocytic stages). They leave the sinusoid capillaries through the endothelium and reach the liver parenchyma cells through the perisinusoid lymph space (space of Disse) or through Kupffer cells (sessile macrophages in the sinusoids). Each successful sporozoite develops into a mature liver schizont and divides 11 – 15 times depending on the *Plasmodium* species (see appendix). Only the last division is an endodyogeny, producing 10,000 to 30,000 merozoites that are liberated into the circulation and invade the red blood cells (RBCs). *P. vivax* and *P. ovale* can remain dormant in the liver for months or even years in a form called hypnozoite. This form is responsible for true relapses. Development or persistence of the parasite in the liver does not produce any symptoms.

1.4.3 Erythrocytic cycle: Disease

After penetrating an RBC merozoites initiate the erythrocytic cycle by maturing into blood schizonts containing 8 - 32 new merozoites (Fig. 1.2.F.). The blood schizonts burst, causing fever in the human host. The merozoites rapidly invade new RBCs. The erythrocytic cycle is completed in 48 hours in the so-called tertian malarias (benign in the case of *P. vivax* and *P. ovale*; malignant in the case of *P. falciparum*) and in 72 hours in quartan malaria (*P. malariae*). (For the most important characteristics of human malaria see appendix.)

The manifestations of the disease are primarily the result of the parasitization and destruction of RBCs. Initial symptoms are quite variable, particularly in children, and may include irregular fever, malaise, headaches, muscular pains, sweats, chills, nausea, vomiting, and diarrhea. If left untreated the fever tends to occur in periodic bouts alternating with days with less or no fever. 50 % of all human malaria victims suffer from *P. falciparum* infection (malaria tropica), 43 % from *P. vivax* (malaria tertiana).

1.4.3.1 Invasion of erythrocytes by merozoites

Merozoite invasion is dependent on the presence of Ca^{2+} [866,868,867]. The erythrocytic merozoite (Fig. 1.2.E.I.) is an ovoid cell approximately 1.3-1.5 µm in length and 0.9 - 1µm in width with an apical complex at the apical end (Ap), and a nucleus (N). Its mitochondria (Mt) are located around an apicoplast (Pl). The apicoplast has four membranes, suggesting that it is an endosymbiont of prokaryotic origin and related to chloroplasts [592,594,593,699,698]. Extracellular merozoites are intrinsically short lived and rapidly invade new host erythrocytes within 30 s [308]. Malaria merozoite invasion can be divided into 4 phases (Fig. 1.2. E.II.):

(1) Initial recognition, reversible attachment, reorientation by projecting tufts of fine filaments [72]. They cover the plasma membrane (7.5 nm thick) like a surface coat (15 - 20 nm thick) [308] consisting of the MSP1-complex: MSP1, 2, 3, 4, 5, merozoite surface proteins 1, 2, 3, 4, 5; ABRA; S - antigen. The plasma membrane of the merozoite encases two closely fitting inner membranes (Im). Microtubules (M) extend subpellicularly (Fig. 1.2.E.II.(A)).

(2) Apical reorientation; irreversible attachment, junction formation between the apical end (Ap) of the merozoite and the erythrocyte (Fig. 1.2.E.II.(B - C): Release of microneme (Mn) contents, *e.g.* EBA175 (*P. falciparum*), erythrocyte binding antigen 175, that binds to the sialic acids of glycophorins [910]. Thus EBA175 mediates high affinity adhesion to the RBC membrane. Rhoptries start to discharge proteins: AMA1, apical membrane antigen 1; RAP1,

2, rhoptry - associated protein 1, 2; RhopH3. Proteins such as Rhop-H interact with vesicles of anionic PC and PI, which are preferentially localized on the inner leaflet of the RBC membrane. This might disrupt interactions of the RBC cytoskeleton with transmembrane proteins, thus initiating the formation of the parasitophorous vacuole membrane (PVM) [859]).

(3) Movement of the tight junction, inducing invagination of the erythrocyte membrane around the merozoite with concomitant removal of the merozoite's surface coat (Fig.1.2.E.II.(D - F)): The merozoite passes through the tight junction into a concurrently developing PVM. It discharges more rhoptries and micronemes and releases the contents of its dense granules (PfSUB-1 and 2) into the PVM. Its inward motion is driven by an actomyosin motor (protein myosin A). The surface coat of the merozoite is progressively stripped off as it moves through the tight junction by proteolytic shedding of the MSP-1 complex [786].

(4) Resealing of the PVM and erythrocyte membrane after completion of merozoite invasion (Fig.1.2.E.II.(F - G)). Daughter merozoites released upon schizont rupture develop into either all asexual-stage or all sexual-stage parasites. Each sexually committed merozoite produces progeny of only one sex: either micro- (male) or macro- (female) gametocytes [783].

1.4.3.2 Asexual replication: trophozoites and schizontes

After invasion, the merozoite becomes round due to rapid degradation of the inner membrane complex and subpellicular microtubules of the pellicular complex, and eventually tranforms into a trophozoite. Dense granules move towards the merozoite's pellicle and release their contents into the parasitophorous vacuole space [836]. The PVM serves as an interface between the parasite and host cell cytoplasm [550]. The morphology seen in Giemsa stained blood smears of a trophozoite until ~ 12 - 15 h post invasion (p.i.) is ring-shaped, 2 to 3.7 µm in diameter and discoidal, usually with a biconcave shape. This is referred to as the ring stage. The predominant position of its ribosomal RNA content is reflected in the basophilic methylene blue ring-like appearance in Giemsa-stained preparations. The trophozoite ingests host cell cytoplasm via its cytosome (a circular structure possessing a double-membrane, the outer (parasite plasma lemma) and inner membrane (PVM) [11]). It digests hemoglobin (Hb), producing in the process the insoluble waste product hemozoin, also referred to as malaria pigment. Methemoglobin and Hb is degraded to toxic free heme [ferri (Fe^{3+}) / ferro (Fe^{2+}) protoporphyrin IX (FP IX)]. Around 90 % of the heme is detoxified as polymerized β-hematin (crystallized Fe(III) protoporphyrin IX dimers) and stored in food

vacuoles (150 to 300 nm across in diameter). Hemozoin contains biomineralized heme, plasmodial proteins and DNA [70,252]. At around 24 – 36 h p.i. the trophozoite has mitochondria (Mt) with few or no cristae and a large number of ribosomes [308]. Trophozoites develop into schizonts, which release merozoites after 48 h of the asexual cycle.

In contrast to other human infective *Plasmodia*, only *P. falciparum* infected erythrocytes become progressively rigid during development. Moreover, mature trophozoites and schizonts of *P. falciparum* are usually not found in the peripheral blood as they are sequestered in the postcapillary venules. Sequestration mainly takes place through the formation of knobs, which are alterations in the erythrocyte surface membrane induced by the parasite. Mature trophozoite and schizont stages [763] express knobs, *i.e.* evenly distributed electron-dense, conical elevations of the erythrocyte surface [10,60,192,357,358,393,522,529,863,906,907]. They measure 30 – 40 nm in height and 100 nm in width as judged by electron microscopic observations [13,60]. The knob surface is positively charged and the remainder of the erythrocyte surface is negatively charged [12]. Parasite-encoded molecules on the knob surface, referred to as neoantigens, are responsible for cytoadhesion and resetting [308]. Neoantigens adhere to adjacent erythrocytes (rosetting) or to the vascular endothelium (cytoadherence) and form focal junctions with the endothelial cell membrane [191]. Thus these plasmodial proteins cause sequestration, hampering blood flow and inducing local hypoxia, in particular in cerebral capillaries. The parasite encodes histidine-rich protein (HRP) I and HRP II (= KAHRP) [529,636], *P. falciparum* erythrocyte membrane protein-1, 2 and 3 (PfEMP-1, 2 and 3) [78,863], ring-infected erythrocyte membrane surface antigen (Pf155/RESA), rifins, stevor [217], clag 9 [192,191]and others [192].The erythrocyte's adhesion to the vascular endothelium results in organ specific failure and lethal syndromes like cerebral malaria [9,307], the main factors responsible for the acute severity and mortality of *P. falciparum* infection. Cerebral, hepatic, renal, pulmonary, or gastrointestinal edemas are known symptoms of malaria tropica. In addition, *P. falciparum* multiplies more rapidly than other human *Plasmodium* species, causing massive destruction of erythrocytes with consequent severe anemia and hemoglobinuria or blackwater fever [573].

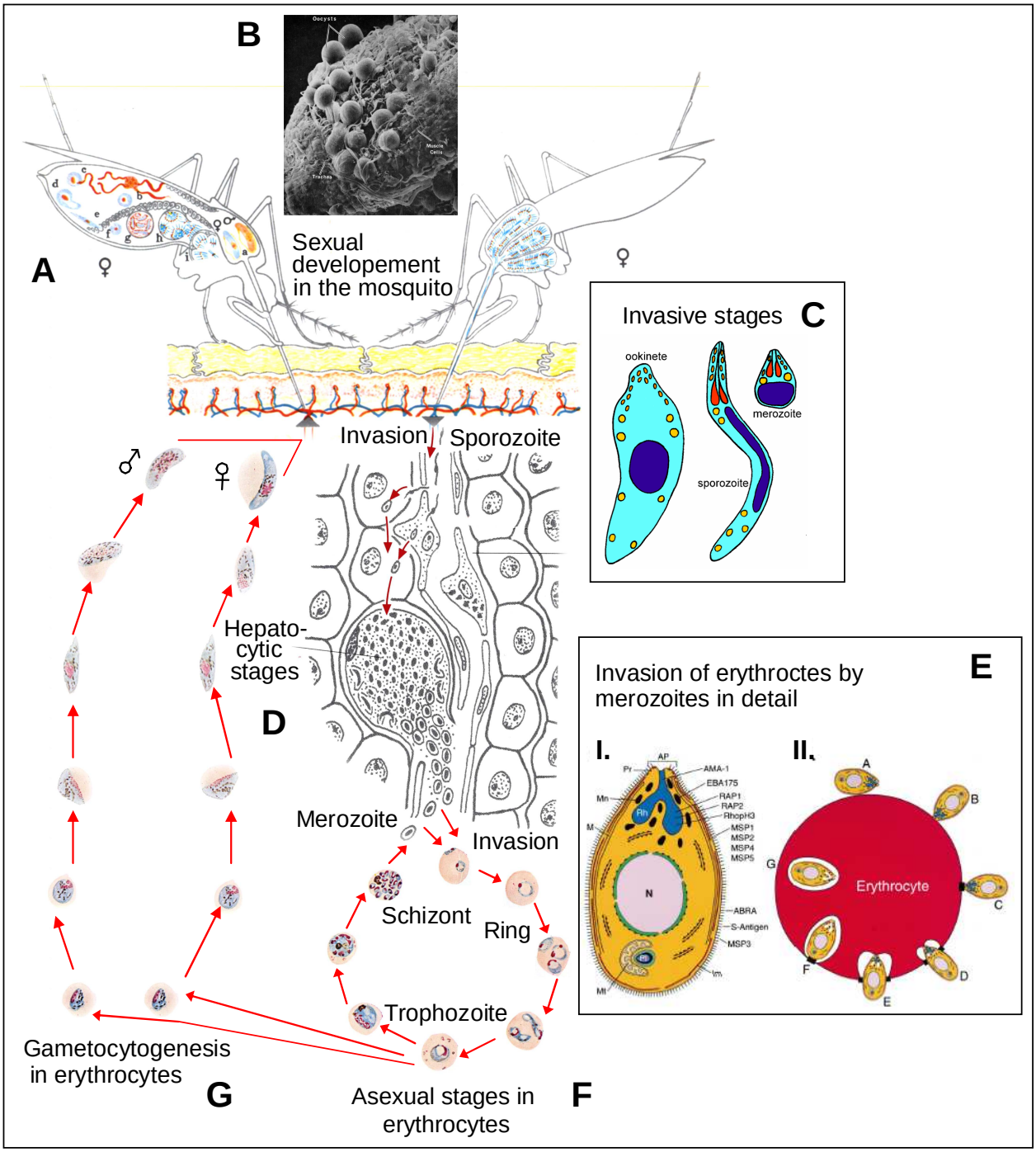


Figure 1.2. Life cycle of *P. falciparum*. Modified from [197,682,805,879,910] (**A**) Feeding of the female Anopheles mosquito (1-2 µl blood) with uptake of gametocytes (~ 1 – 10^5) and / or transmission of sporozoites (~ 10 – 20 from 16000 in the haemocoel) (**B**) Gametocytes develop sexually in the mosquito (details see text); Micrograph: Example of mature oocysts (of *P. gallinaeceum* on the haemocoel surface of the midgut of a female *Aedes aegypti*. Magnification x 300) [805]. (**C**) Invasive stages. (**D**) Sporozoites invade hepatocytes. (**E**) Merozoites invade erythrocytes (details see text). Merozoites develop asexually within erythrocytes (**F**) or switch to gametocytogenesis (**G**).

1.4.4 Gametocytogenesis

Genetically identical clonal haploid asexual-stage parasites transform into sexually dimorphic gametocytes, male microgametocytes and female macrogametocytes [70,209,783,810] (Fig. 1.2.G.). What triggers this alternative development pathway is poorly understood. High levels of asexual parasitemia, anemia, antiparasitic immune responses, chemotherapeutic agents and, possibly, other environmental stresses contribute to the induction of gametocytogenesis [810]. As a consequence, a minority of merozoites becomes sexually committed. While they still develop into trophozoites, instead of then developing into schizonts, these sexually committed parasites transform into gametocytes [70] first detectable through sexual-stage-specific gene expression [810,619]. In *P. falciparum*, gametocytogenesis takes place over approximately eight days, passing through five morphologically distinct substages until the final crescent shape is reached [209,810]. Unlike the cycling asexual-stage parasites that are adapted to maintaining and perpetuating erythrocytic infection [70], mature gametocytes are specialized escapists – arrested in cell-cycle development [458]. The gametocytes will die within their vertebrate host unless an encounter with a suitable vector permits their continued existence [79].

1.5 Development of resistance towards antimalarial drugs

During the last decades [673,881], there has been an increasingly rapid selection for and dispersal of *P. falciparum* parasites resistant to antimalarial drugs [49,112,354,601,637,871,878,887,886,914]. The reason for this is that these drugs are increasingly being used as prophylactics [880] and for self-medication, often in insufficient doses [883].

The mechanisms underlying *Plasmodium* drug resistance [288,772,852,864] remain poorly understood. In part, resistance is due to polymorphisms in a variety of genes including the *Plasmodium falciparum* chloroquine resistance transporter (pfcrt) [138,882] and the *Plasmodium falciparum* multi-drug resistance transporter pfmdr, a P-glycoprotein variant [235]. Pfcrt is a molecule that is thought to function as a transporter in the parasite's digestive (food) vacuole membrane [284]. It might thus counteract the effect of chloroquine by increased export of the drug.

Moreover, while polymorphisms can confer drug resistance, different strains of *P. falciparum* may also rely on alternative mechanisms of gene regulation to overcome the selective pressures of anti-malarial drugs. These mechanisms may include induction of

alternative pathways to bypass the effect of the compounds, repression of genes that aggravate the drug effects, or overexpression of transporters in response to drug pressure [558]. For example, mefloquine and sulfadoxin-pyrimethamine resistance might directly be modulated by an increased copy number of pfmdr1, resulting in an amplification in the number of cellular transcripts [691,771,911]. Therefore, regulatory responses may act alone or in concert with previously identified polymorphisms in favoring parasite survival under drug exposure [558].

1.6 Erythrocyte ion composition and regulation

Blood cells are suspended in human plasma with an electrolyte composition as shown below (Table 1.1)

Cations	mM	total: 150 mM
Na^+	142	
K^+	4.5	
Ca^{2+}	2.5	
Mg	1	
Anions	mM	total: 150 mM
Cl^-	104	
HCO_3^-	24	
HPO_4^{2-}	2	
Proteins	14	(66 – 83 g/l)
Other	6	

Table 1.1. Normal values of the major electrolytes of human plasma [110].

Erythrocytes, or RBCs, make up the largest population of blood cells, numbering from 4.5 million to 6 million per cubic millimeter of blood. A typical erythrocyte has a flattened, biconcave shape and measures about 6 - 8 µm in diameter. In human adults, erythropoiesis usually occurs within the bone marrow of flat bones. These release immature erythrocytes, referred to as reticulocytes. 1 % of the RBCs of a normal healthy adult are reticulocytes.

Normal erythrocytes contain a 5 mM solution of hemoglobin (Figure 1.3. A), constituting 97.5 % of the total cell protein by weight [378]. The hemoglobin molecule is a tetramer consisting of four polypeptide chains:

- 2 α chains, each 141 amino acids long
- 2 β chains, each 146 amino acids long

Within each chain is an iron containing molecule known as heme (Fig. 1.3. B) that binds oxygen. The other erythrocytic proteins support and regulate the integrity of the cell as well as the function of hemoglobin [215].

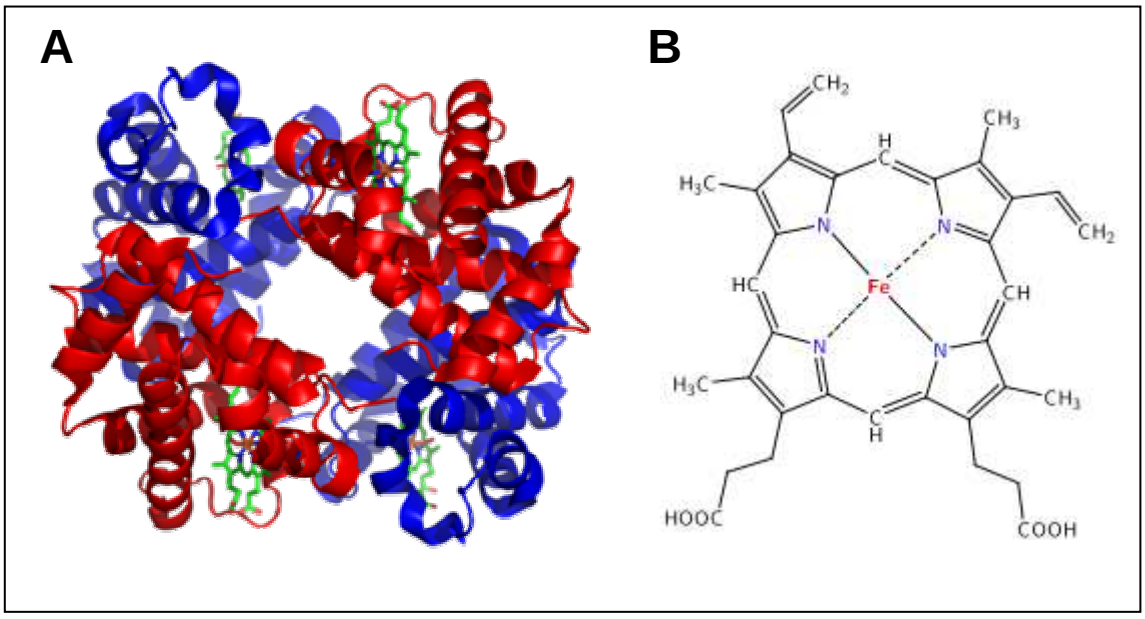


Figure 1.3.A. Structure of hemoglobin. The protein subunits are in red and blue, and the iron-containing heme groups in green [664]. **B. Heme group.**

1.6.1 Active ion transport

Most proteins associated with the erythrocyte membrane are involved in active and passive ion transport, *i.e.* processes to maintain erythrocyte homeostasis [542,538].

1.6.2 Na^+/K^+ pump-leak balance in non-infected erythrocytes

The Na^+/K^+ pump-leak mechanism of human RBCs is well understood. It builds up and maintains a high intracellular K^+ ($[K^+]_i$) and a low intracellular Na^+ ($[Na^+]_i$) concentration [839,687]. The ouabain-sensitive Na^+/K^+-ATPase in the RBC membrane pumps 2 K^+ ions into versus 3 Na^+ out of the cell, thereby generating opposing chemical gradients for the two ions. The pumping helps to build up the membrane potential and counterbalances the "leak" of the Na^+ and K^+ ions down their respective concentration gradients via various cotransporters, exchangers, and channels that adjust a steady-state cytoplasmic $[Na^+]$-to-$[K^+]$ ratio of 0.12-0.16 in normal human erythrocytes [98,528]. Intraerythrocytic Na^+ and K^+ concentrations have

been determined as approximately 10 to 20 mM and 140 mM, respectively [442,475,331,214]. Several cation channels that may contribute to the cation leak have been identified electrophysiologically.

1.6.3 Ca^{2+} homeostasis in non-infected erythrocytes

Human RBCs have a low amount of total Ca^{2+} as compared to nucleated cell types [117,262,379]. This results from a low cytoplasmic Ca^{2+} buffering capacity [280,828], the absence of Ca^{2+}-sequestering endoplasmic reticulum and mitochondria. Sickle (HbS/S) RBCs contain a minimum of endocytotic Ca^{2+}-sequestering vesicles [541,901]. Due to the powerful Ca^{2+} extrusion by the Ca^{2+} ATPase pump [547,740] and the low Ca^{2+} permeability of non-stressed RBCs, physiological ${}_{free}[Ca^{2+}]_i$ remains below 100 nM [208,262,547].

1.6.4 Nonselective cation channels in non-infected erythrocytes

Whole-state and nystatin-perforated patch-clamp single-channel recordings have led to the discovery of ~20 pS nonselective cation (NSC) channels in the human RBC membrane that are activated by strong depolarization of the membrane potential [177,715]. These voltage-gated NSC channels [438] are stimulated through nicotinic acetylcholine [89] and PGE_2 receptors [437]which can be directly activated by clotrimazole and its analogues [74]. They are permeable to divalent cations such as Ca^{2+}, Ba^{2+}, and Mg^{2+} [439,437], and exhibit a hysteresis-like voltage-dependent gating (*i.e.* half-maximal voltages of activation and deactivation curves differ by ~25 mV) [90,437]. In addition, voltage-independent NSC channels have been discovered. Experiments with simultaneous activity of both channel types suggest their differential regulation [517].

Activation of the voltage-independent NSC cation channels occurs within minutes upon replacement of extracellular Cl^- by gluconate [507,512,511,517,626], similar when extracellular Cl^- is replaced by NO_3^-, Br^-, or SCN^-. Furthermore, voltage-independent NSC channels are activated when the ionic strength of the bath solution is lowered by isosmotic substitution of NaCl with sorbitol, indicating that the underlying process is probably identical with that of the Na^+ and K^+ permeability increase upon incubation of human erythrocytes in low ionic strength medium [97,433,494]. Decrease of the extracellular Cl^- concentration to 27 mM half-maximally activates these cation channels [244]. Remarkably, the anion channel/transporter inhibitor DIDS directly inhibits K^+ efflux in low ionic strength medium paralleled by

inhibition of Cl^- exchange [433]. Moreover, whole-cell recordings show that upon extracellular Cl^- removal DIDS prevents activation of the NSC channel, while having no effect on the activated cation channels [244]. Furthermore, cation channel activity strongly depends on the cytosolic Cl^- concentration [408], suggesting that a DIDS-sensitive pathway equilibrates the Cl^- concentrations between the cytoplasmatic and extracellular membrane face and that intracellular rather than extracellular Cl^- ions modulate the cation channel activity. A similar dependence of fetal and epithelial nonselective channels [585,835] and epithelial Na^+ channels [230] on intracellular Cl^- concentration has been demonstrated. In the latter study, the effect of intracellular Cl^- concentration was mediated via a pertussis toxin-sensitive G protein. The activation of the NSC channel is inhibited by intracellular or extracellular Cl^- [244,408]. During continuous whole-cell recordings the voltage-independent cation channels activate reversibly within some minutes upon hyperosmotic shrinkage [408,507,512,511] and oxidative stress (1 mM *tert*-butylhydroperoxide; *t*-BHP) [244]. The voltage-independent channels hardly discriminate between monovalent cation channels (cation permselectivity in the rank order of $Cs^+ > K^+ > Na^+ = Li^+ >> NMDG^+$) and have a Ca^{2+}-permeability similar to that of voltage-gated NSC channels (Duranton et al., 2002). Amiloride, $GdCl_3$ and EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride) inhibit the voltage-independent NSC channels with increasing potency [244,408,511], while tetraethylammonium (TEA) (1 mM), 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (100 µM), or quinidine (500 µM) have no effects on channel activity.

Thus, human RBCs most probably express at least two types of Ca^{2+} permeable NSC channels. Moreover, there are mechanosensitive Ca^{2+}- channels [131] in aged RBCs, upregulated endogenous stretch-activated Ca^{2+}-channels in *Plasmodium gallinaceum* infected chicken erythrocytes [825], and the deoxygenation-induced cation pathway P_{sickle} in HbS/S erythrocytes [457,539,538,701].

1.6.5 Ca^{2+} activated Gardos K^+ channels

The erythrocyte uses at least four well-characterized K^+ transport mechanisms: the K^+Cl^- cotransporter, a NaK_2Cl cotransporter, a Na^+/K^+ ATPase (a sodium pump) and a Ca^{2+} activated Gardos K^+ channel [313]. The gene for the Gardos channel in erythrocytes is the 4th member of the potassium intermediate / small conductance calcium-activated channel subfamily N (KCNN4; aliases: hsK4 (human small conductance potassium channel 4), hIK1 (human intermediate conductance potassium channel)) [395,515]. Gardos channels have been characterized by single channel recordings [176,359,532]. They play an important role in the

regulation of cell volume [143,144,313,544,674,768] and are major factors in HbS/S RBC dehydration [121,537,542,538].

Gardos channel activity is dependent on the free Ca^{2+} concentration at the cytoplasmatic membrane face. Concomitant with an increase in free $[Ca^{2+}]_i$ from 500 nM to 60 µM its open probability increases from 0.1 to 0.9. Ca^{2+} acts via binding to calmodulin constitutively associated with the Gardos channels [219]. In addition to Ca^{2+}, channel activity is dependent on intracellular K^+, and inhibited by extracellular Na^+, K^+, and intracellular Na^+. Gardos channels are highly K^+ over Na^+-selective (P_K/P_{Na} > 100) and exhibit an inwardly rectifying current-voltage relationship (when recorded with symmetrical K^+ solutions) with a conductance of ~25 pS (at 0 mV voltage). In addition, protein kinase A (PKA) reportedly induces a dramatic enhancement of Gardos channel activity, possibly by modulating the Ca^{2+} sensitivity [674,717].

In non-stressed and unstimulated human RBCs the fractional Gardos K^+ channel activity is low. Moreover, human erythrocytes have high intracellular Cl^- concentrations (around 80 mM [501,929]) resulting in a very low membrane potential (≤ -10 mV) [167] close to the Cl- equilibrium (zero current, reversal, Nernst) potential [929]. Activation of Gardos channels by increased intraerythrocytic Ca^{2+} levels (≥ 150 nM) [106,507,521] leads to hyperpolarization of the RBC membrane potential close to the K^+ equilibrium potential. This imposes an outwardly directed electrochemical Cl^- gradient across the RBC membrane, driving Cl^- and K^+ into the extracellular space, followed by osmotically obliged water efflux. The Gardos channel inhibitors clotrimazole (IC_{50} ~50 nM) [145] and charybdotoxin (IC_{50} ~5 nM) [144,142], or Cl^- channel blocker NPPB blunt phosphatidylserine (PS) exposure, hyperosmotic and Ca^{2+}-stimulated isosmotic cell shrinkage [521,627], confirming the contribution of Gardos and anion channels to KCl and water efflux during RBC shrinkage.

Increase in free $[Ca^{2+}]_i$ therefore favors RBC shrinkage (Gardos effect) [507] subsequently decreased RBC deformability under isosmostic conditions, measured as decreased cell size or filterability [23,440,517,548]. The Gardos effect is dependent on the extracellular Ca^{2+} concentration and is sensitive to amiloride [106,507,521]. During complement activation a rising cytosolic calcium triggers the Gardos effect, thus limiting the colloidosmotic swelling and lysis of erythrocytes [369]. Cell swelling tends to favor hyperpolarization over depolarization, since the resting RBC membrane potential (≤ - 10 mV) is more positive than the expected equilibrium potential for the slightly K^+ over Na^+-permeable NSC cation channel.

1.7 Functional significance of the nonselective cation channels, Ca^{2+} signaling, and Gardos channel activity for the volume and programmed death of erythrocytes

Programmed cell death is a cell suicide mechanism enabling metazoans to control cell number in tissues and to eliminate individual cells that threaten the animal's survival [347]. This suicide mechanism is initiated by unique sensors on the cell membrane surface, termed death receptors , an example of which is the Fas receptor (Apo-1, CD95) [287,506,504,361]. Death receptors detect the presence of extracellular death signals (stressors) such as oxidants, radiation [721], chemotherapeutics [153,890], energy depletion [689] or osmotic shock [125]. This triggers the cell's intrinsic apoptosis machinery. Because erythrocytes lack mitochondria and nuclei, key organelles in the apoptotic machinery of other cells, they have been considered unable to undergo programmed cell death. Moreover they might be even taken as post-apoptotic, because transient mitochondrion-triggered caspase-activation probably leads to enucleation during their development [207,925].

Senescent erythrocytes have to be eliminated after their physiological life span of 120 days, or when they are defective, similar to other cell types in which the primary mechanism of clearance is programmed cell death or apoptosis. There is evidence that sialic acid residues of membrane glycoconjugates control the life span of erythrocytes, and that desialylation of glycans is responsible for the clearance of senescent erythrocytes. Their capture is mediated by a β-galactolectin present in the membrane of macrophages [135]. These and other observations support the theory that erythrocyte ageing is a form of apoptosis [127,128].

When human erythrocytes are injured through stressors such as osmotic shock, oxidative stress, ligation of cell membrane antigens and energy depletion they undergo a terminal form of apoptosis [207], even though it lacks the morphological alterations and DNA fragmentation that characterize apoptosis also in single-celled organisms [40,247,285,286]. Therefore, this process is termed "eryptosis" [207,499].

1.7.1 Erythrocyte death signaling pathways

To date two erythrocyte death signaling pathways have been identifed which are activated by oxidative stress and energy depletion (glucose or ATP) and merge to trigger eryptosis [507]. Both weaken the antioxidative defense of the erythrocyte. Energy depletion

presumably impairs the replenishment of gluthathione (GSH) [103,507,588]. Activation of phospholipase A_2 (PLA_2) leads to release of lyso-platelet activation factor (lyso-PAF) [509,519] or / and arachidonate from membrane phospholipids (Fig. 1.16.).

(1) Loss of cell volume accompanied by break-down of membrane phospholipids asymmetry [94,133,256,268,501,511,935] is a prerequisite and an early and fundamental feature of programmed cell death [232,337,336,338,347,503,506,505,508,498,497,704,721,802]. Released lyso-PAF upon hyper- or isosmotic erythrocyte shrinkage is acetylated to PAF by acetyl-CoA and lyso-PAF-acetyltransferase [353]. This leads subsequently to sphingomyelinase activation [148,380,411,335,616,615,510,511,519,807,930]. Sphingomyelinase cleaves the phosphorylcholine unit of sphingomyelin and releases the proapoptotic sphingolipid ceramide, which consists of the sphingosine backbone and the fatty acid unit of sphingomyelin (SM). Appearance of ceramide at the erythrocyte membrane or increase of cytosolic calcium results in scramblase activation and translocase inhibition. This enhances PS migration to the outer leaflet of the bilayer membrane [507].

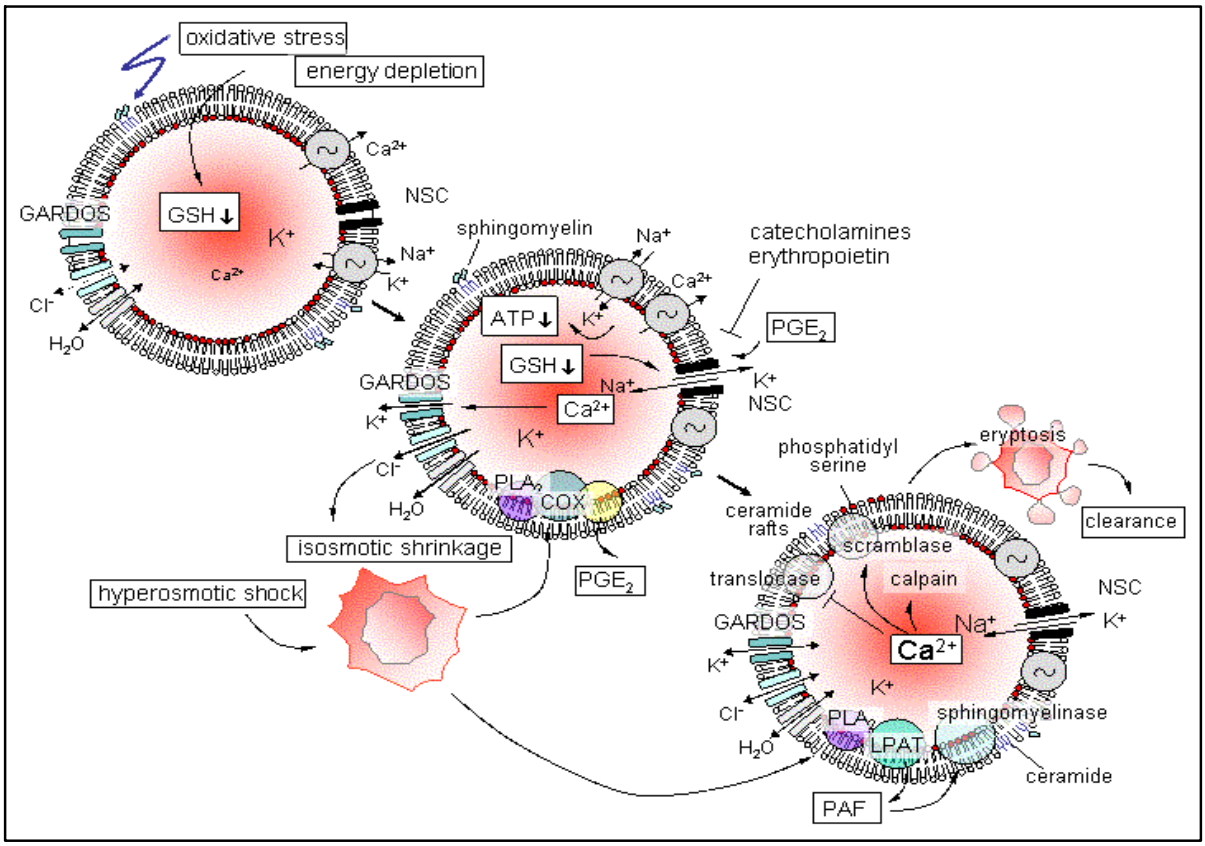


Figure 1.4. Synopsis of eryptosis signaling [499,889].

(2) Release of arachidonate and cyclooxygenase (COX) activation increases PGE_2 formation. PGE_2 stimulates NSC channels permeable for Ca^{2+}. In particular, increase in

cytosolic Ca^{2+}, $[Ca^{2+}]_i$ induces eryptosis [22,45,94,133,207,218,517,502,501,521,513,913]. Besides the Gardos effect (at free $[Ca^{2+}]_i \geq 150$ nM), Ca^{2+} activates a membrane-bound polyphosphoinositide phosphodiesterase (at free $[Ca^{2+}]_i \approx 2$ µM) [22], an endogenous transamidase [561], and the neutral endopeptidase calpain (at free $[Ca^{2+}]_i \geq 10$ µM) [94,276,368,499,517,514,773,791,934], fostering proteolysis, polypeptide breakdown and aggregation of membrane proteins [19,22,561]. The phosphodiesterase influences association of protein components of the membrane skeleton through loss of polyphosphoinosites [22,43], increasing phosphatidate (phosphorylated diacylglycerol) in dense cells [22,702]. The transamidase cross-links membrane components, thus increasing the amount of high molecular weight polypeptides [561]. Calpain is a Ca^{2+} - dependent cysteine protease of the papainase family [276] that degrades the cytoskeleton in particular by ankyrin R, fodrin (spectrin) and polypeptide 4.1 cleavage [94,499]. These processes lead to membrane budding [19,20,22,94,276,784,791,499,517,514,934] and decreased cellular flexibility [875]. Especially in the dense fractions of HbS/S and HbA/A RBCs microvesiculation, *i.e.* loss of up to 20% of the membrane lipid in form of microvesicles devoid of membrane skeletal proteins, leads to echinocytic and spherocytic deterioration of cellular shape [22,240,282,702,884,885]. Most importantly, increase in intracellular free $[Ca^{2+}]_i$ results in breakdown of PS asymmetry by downregulation of the ATP-dependent amino-phospholipid translocase and activation of the non-specific lipid scramblase $[Ca^{2+}]_i$ (see below).

1.7.1.1 The role of nonselective cation channels in eryptosis upon PGE_2 formation

Even at physiological concentrations (*i.e.* 0.1 nM) PGE_2 raises free $[Ca^{2+}]_i$ via calcium influx through a NSC channel in human RBCs [440]. Whereas experiments with the dye Fura-2 were contradictory [484], results obtained with Fluo-4 confirmed Ca^{2+}-influx upon stimulation with PGE_2 [441]. (Fluo-4, in contrast to Fura 2 and Indo-1, is more appropriate to measure Ca^{2+}-levels in erythrocytes, as its excitation and emission properties are the least influenced by hemoglobin. Its excitation light does not lead to significant auto-fluorescence of the erythrocytes.) Stimulation with PGE_2 induces voltage-independent cation channel activity even in the presence of high extracellular Cl^- concentrations [517]. Recent observations revealed a pivotal role of PLA_2 and PGE_2 in the regulation of PS exposure of human erythrocytes [517]. Low ionic strength (*i.e.* low Cl^- concentration), oxidative stress or hyperosmotic shrinkage that activate the RBC NSC channels in human RBCs during patch-clamp recording reportedly increase the RBC cation permeability of intact RBCs, as evident from tracer flux studies [433,494,507]. Activation of the voltage-independent cation channels in human RBCs by

hyperosmotic stress or Cl^- removal is paralleled by PGE_2 formation, obviously an autocrine signal to stimulate the NSC channels. Hence, PLA_2 inhibitors, palmitoyl-trifluoromethylketone, an inhibitor of the Ca^{2+}-dependent PLA_2, and quinacrine, as well as COX inhibitors, acetylsalicylic acid and diclofenac, prevent channel activation by hyperosmotic stress or Cl^- removal [517]. Furthermore, they reduce PS exposure [499]. Erythropoietin, too, inhibits both stress-induced cation channel activation and PS exposure, thus extending the life span of circulating erythrocytes [626].

1.7.1.1.1 Activation of lipid transporters involved in phosphatidylserine movement

Lipids are usually asymmetrically distributed within the transversal plane of biological membranes. In human erythrocytes, choline-containing phospholipids, *i.e.* phosphatidylcholine and sphingomyelin, are enriched in the outer leaflet. (PC = 76% and SM = 82%), whereas aminophospholipids are either mostly (PE = 80 %) or exclusively (PS = 100 %) confined to the cytoplasmic inner membrane leaflet [652,858]. Any change of this composition, for example through migration of the anionic lipids or degradation of SM, can have grave physiological and pathophysiological consequences [115,858]. A case in point is the Ca^{2+}-dependent PS exposure at the outer RBC membrane leaflet that leads to erythrocyte-endothelium adhesion [179]. PS is present only in small amounts in the outer leaflet of the plasma membrane of younger RBCs, but increases with erythrocyte age, as assessed by increased cell density [137,188,229,386,421,809] and storage time [50,317,351,564,756]. In patients with sickle cell anemia [297,491,554,756,934], the amount of PS in the outer leaflet can increase 40-fold [763], from 300 sites per cell in fresh HbA/A to 12000-13000 sites per cell in HbS/S RBCs [114,309,562,809]. This effect is found in particular in dense, irreversibly sickled cells [173,172,496,661,809] as a result of translocase impairment [114,211,212] and / or scramblase activation [211,212,450]. Hence, trapping of PS-exposing erythrocytes and concomitant impairment of microcirculation has been observed in sickle cell disease [920], thalassemia [492], and diabetes [861]. Treatment of RBCs with ionomycin, a Ca^{2+} ionophore, leads to exposure of up to 300000 PS sites per cell [309].

Micromolar concentrations of $[Ca^{2+}]_i$ reversibly inhibit the aminophospholipid translocase (or flipase) [108,780,832,923], whose Mg^{2+}-ATPase activity is responsible for active translocation of aminophospholipids from the outer to the inner leaflet of the membrane [61,101,184,233,913,922]. The outside-inside translocation rate of PS at 37 ° C has been determined in human erythrocytes. Within 15 min, more than 90% of a phosphatidylserine analogue was found in the inner monolayer [620]. Thus, it is the aminophospholipid translocase that builds up

the phospholipid asymmetry of the RBC membrane [189].

An ATP-dependent floppase transports both aminophospholipids and cholinephospholipids from the inner to the outer leaflet, but it has the least influence on the composition of phospholipids in the membrane. Its transport rate is about 10 times slower than the rates that have been shown for the inward movement of PS and phosphatidylethanolamine (PE) [46,107,187].

Increase in free $[Ca^{2+}]_i$ to 63 - 88 µM half-maximally activates the phospholipid scramblase [94,218,900,913,926]. Elevated ceramide levels stimulate scramblase further [927]. Scramblase catalyzes bi-directional migration (flip-flop) of all major phospholipid classes at comparable rates across the lipid bilayer in a largely non-selective manner [780,900,899] - only the reorientation rates of SM are appreciably lower [780]. In platelets, scramblase activity is more rapid than in other cells (RBCs), leading within minutes to a loss of phospholipid asymmetry [99,100]. Although the scramblase does not require hydrolysable ATP, its activation partially decreases during prolonged ATP depletion [808,582].

Although PS-exposing subpopulations of HbS/S RBCs do not show sustained high cytosolic Ca^{2+} levels, PS exposure occurs at different stages in the life of the HbS/S RBC and correlates with the loss of ATP-dependent aminophospholipid translocase activity, the only common characteristic of PS-exposing sickle cells. Therefore, the additional requirement of scramblase activation for PS externalization may only apply to the time interval of transient increases in cytosolic Ca^{2+} [212,210].

Taken together, these data strongly suggest that the activity of the NSC channels does regulate free $[Ca^{2+}]_i$ in intact RBCs. A central role for the voltage-insensitive NSC in scramblase activation and translocase inhibition can be deduced from the following observations: All stress stimuli (oxidation, hyperosmotic shrinkage, removal of extracellular Cl^-, externally added PGE_2) that induce cation channel activation, probably via PGE_2 formation and autocrine PGE_2 signaling, also stimulate PS exposure. Further, the induced PS exposure is inhibited by blockage of the cell volume- and oxidant-sensitive non selective cation channels, for example by the putative inhibitors ethylisopropylamiloride (EIPA), amiloride and flufenamic acid [444,507,512,517]. Therefore calpain, transamidase, phosphodiesterase and scramblase probably execute an erythrocyte death program [133], thereby fostering phagocytic clearance. It can be concluded that PGE_2-stimulated cation channel activity senses RBC age or injury *in vivo* and prevents colloidosmotic hemolysis of the dying RBC by triggering RBC shrinkage, leading to PS exposure [15,16,369,384,455,454,453,516,514,635].

Cell shrinkage facilitates phagocytosis of the dying cells [115,250,169] that are eliminated

by this mechanism of apoptosis without releasing intracellular proteins that could cause inflammation [361,843]. Survival of the shrunken RBC may be extended until its encounter with a phagocyte. As a matter of fact, one of the earliest manifestations of cell death in erythrocytes and even nucleated cells is the exposure of PS at the cell's outer surface [271,583,856,927]. PS externalization even precedes DNA fragmentation, loss of membrane integrity, and cell lysis [271].

1.7.2 Recognition of phosphatidylserine-exposing erythrocytes by macrophages

PS exposure stimulated RBC clearance has been suggested to contribute to the dramatically decreased survival of thalassemia and sickle cells *in vivo* [212,210]. Removal of cells is mediated by a complex interaction of surface molecules exposed on apoptotic cells with their cognate receptors on macrophages or tissue cells [737,738,933]. The externalization of PS through calcium-quasi-lipoxygenase activity [459,420] is recognized by macrophages equipped with CD36, a primary receptor for PS [272,268,269,270,383,598], and leads to subsequent phagocytosis [272,269] in a phosphatidyl-L-serine dose-dependant manner [745]. RBCs exposing about 1 mol% PS are rapidly removed from the circulation, accumulating in splenic macrophages and liver Kupffer cells [115,745]. PS-containing liposomes bind to macrophages *in vitro* [744,703] and *in vivo* [688]. In addition, serum-opsonized autologous anti-band 3 (AE1) IgG antibodies covalently bound to C3b fragments [570,566,567,569,565] as well as loss of CD47 and sialic acids enable macrophages to quickly recognize, engulf and degrade senescent or injured RBCs in their lysosomes [115,137,250,268,269,499,509]. *In vitro*, these cells eventually hemolyze.

1.8 *P. falciparum* infection of human red blood cells (RBCs)

By invading host cells, pathogens protect themselves from the host's immune responses. Erythrocytes seem to be somehow ideal for the protection and survival of the parasite *Plasmodium*. As the erythrocyte has no nucleus or organelles, it is not capable of endocytosis, thus the parasite is residing inside the PVM. The erythrocyte has neither endolysosomes, not does it present molecules on the surface in the context of MHC I or II that would lead to easy detection of a foreign organism inside the RBCs. On invading an erythrocyte *Plasmodium* enters a cell with very limited capabilities which lacks the machinery for *de novo* lipid and protein biosynthesis [308]. In addition, in the host cell the pathogen faces a

completely different environment in terms of redox state, electrolyte concentrations and signaling molecules as compared to before. Moreover, the adequate disposal of potentially hazardous metabolic end products in its new intracellular environment can become critical for the parasite's own survival [824]. In general, pathogens either adapt themselves to the new situation or they modify the host cell compartment to suit their needs. Finally, the increase in host cell volume associated with the pathogen's intracellular amplification must be counteracted by an appropriately modified volume regulation of the host cell. Host cell volume regulation, pathogen nutrition, and adaptation of the host cytosol require an increased solute transport through the plasma membrane of the host cell [407]. The RBC membrane is naturally endowed with a variety of transporters, mainly geared to optimize oxygen transport and maintain cell homeostasis at minimal metabolic cost. If the transport rates of the host cell prove insufficient to accomplish these needs, the pathogens have to alter the permeability of the host's membrane.

1.8.1 RBC membrane modifications by *P. falciparum* to meet its environmental needs

In vitro growth of *P. falciparum* in culture requires a hexose sugar, a purine source [758,764], seven amino acids (cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine) [686] and panthotenate [231]. Dependent as it is on glycolysis as energy source for its intense metabolic and biosynthetic activity, the malaria parasite consumes glucose and produces lactic acid at a rate 40 - 100 times higher than that of the non-infected RBC. This results in intracellular acidification of the host cytosol which has then a pH of ~7.1 [126,917]. Inside *P. falciparum* the intracellular pH has been estimated to be within the range 7.2 - 7.4. *In vivo*, malaria infection often leads to acidosis of the patient. While the endogenous transport capacity of the RBC is probably sufficient to feed the parasite with glucose, the capacity to extrude lactic acid by the RBC monocarboxylate transporter may be not. In addition, the RBC membrane lacks appropriate transporters for essential nutrients of the parasite such as panthotenic acid.

The parasite completes its transition from the largely inert 'ring stage' form to the metabolically and biosynthetically active trophozoite stage between 15 and 20 h p.i.. The *New Permeability Pathways* (*NPP*s) become apparent about 12 – 15 h p.i. of the merozoite into the RBC and increase markedly up to a plateau at about 36 h p.i. [259,796]. During this time, *Plasmodium falciparum* permeabilizes the host erythrocytes for a wide variety of low-

molecular weight solutes with a substantial permeability for Cl^-, other monovalent inorganic anions [202,327,468,466,796], but also for ions with negative and positive charge such as choline [469,797], amino acids [259,329,466,470], small peptides [58,257,329,543,730], vitamins, various monocarboxylates [80,182,202,466,730], electroneutral solutes (such as nucleosides [322,329,466,759,849], and neutral carbohydrates, *i.e.* monosaccharide sugars and other small polyols [329,466,467]. In addition, the NPPs represent a low, but significant permeability for cations [119,243,471,462,463,465,800,796], including small quaternary ammonium compounds [800] and Ca^{2+} [119,224,477,482,813]. Thus, incubation of late-stage infected erythrocytes in isosmotic sorbitol leads to cell swelling due to the sorbitol uptake and eventually colloidosmotic hemolysis [328,495], which can be quantified photometrically. Isosmotic hemolysis and tracer flux experiments (done mainly by Hagai Ginsburg in Jerusalem and Kiaran Kirk in Oxford) have permitted the characterization of the substrate specificities of the *NPP*s [328,330,466,461] and their inhibition [149,468,466,468,464,489]. Four patch-clamping groups have worked on their electrophysiological characterization [407,799,793]: Desai [18,223,224,220,222,221]; Huber / Lang [244,243,245,242,246,410,409,406,407]; Thomas / Staines [130,253,254,797,800,798,799,794,793,826] and DeJonge [857].

Similarly to the infection-induced NSC currents, transport of monovalent cations in tracer flux experiments (Na^+, Rb^+, choline$^+$, and organic quaternary ammonium ions) and hemolysis in different isosmotic salt solutions of monovalent cations are strongly dependent on the nature of the anion in the suspending medium. The colloid osmotic hemolysis of infected RBCs in isosmotic salt solutions is rate-limited by the uptake of the cations, since anion permeability exceeds that for cations by several orders of magnitude [465,800,796]. In particular, replacement of extracellular Cl^- by NO_3^- increases monovalent cation fluxes by factors of 2 - 6 while having no effect on fluxes of sorbitol and lactate [465].

1.8.1.1 Molecular nature of the *New Permeability Pathway*s (*NPP*s)

It is still a matter of discussion and research whether the increased membrane permeability is due to an increase in the activity of (1) endogenous RBC proteins [216,254,330,410,409,815,857] by oxidation [244,405,410] or if *NPP*s are generated by (2) parasite-encoded xenoproteins exported and trafficked to the RBC membrane [18,81,223,220,222]. At present, neither the molecular identity of the *NPP*s nor their origin is defined. However, activation and participation of host (3) inwardly rectifying ClC-2 channels [406] and (4) cystic fibrosis transmembrane conductance regulator (CFTR) [857] in the altered permeability of *Plasmodium*-infected erythrocytes has been shown. Both ClC-2 and CFTR seem not to be essential for

parasite growth, as *Plasmodium* develops well in knockouts [406,857]. Furthermore, the mechanism of activation of the *NPP*s involves (4) phosphorylation steps by PKA for an inwardly rectifying anion channel (IRAC / IRCC) permeable for Cl^- [253,254] and (5) autocrine purinoreceptor signaling for an outwardly rectifying anion channel permeable for Cl^- and a range of organic osmolytes [815] (ORAC / ORCC [85,245,246,798]). Moreover, conductances with similar properties as the outwardly rectifying osmolyte channel (ORCC) were activated in non-infected RBC upon oxidation by peroxides [410]. Treatment of infected RBCs by reducing agents diminishes these conductances as well as the rate of hemolysis in isosmotic sorbitol. This might indicate that *NPPs* are endogenous human host RBC channels activated upon infection by *Plasmodium* by oxidative stress [244,324,330,405,410].

1.8.1.2.1 Oxidation-induced channel activation

Plasmodium falciparum trophozoite-infected human RBCs produce about twice as much H_2O_2 and OH radicals as non-infected erythrocytes due to the high metabolic and biosynthetic rate of the blood stage *P. falciparum* parasite. Digestion of host hemoglobin (Hb) provides a major source of amino acids required for the parasite's protein synthesis as well as a source of iron, which is required for the synthesis of iron-containing proteins such as ribonucleotide reductase or superoxide dismutase. At the same time Hb digestion leads to FP IX, redox active by-products, and H_2O_2 [56,325]. Most of the free heme (about 90% [252]) is neutralized by sequestration into a golden-brown crystalline form, the malarial pigment termed hemozoin. An appreciable amount of the free heme is bound by proteins such as histidin rich protein. About 0.5% of the heme, free, non-neutralized causes redox damage to the host erythrocyte membrane and proteins [831]. Oxidative stress [86,412] is counter-regulated by the parasite's and host's hexose monophosphate shunts [59,57], the thioredoxin redox cycle [622], which replenish the NADPH pool for the reduction of oxidized gluthathione (GSSG) to glutathione (GSH). Moreover, having a high capacity for GSH *de novo*-synthesis, the parasite actively exports GSSG to replenish the glutathione pool in the erythrocyte, whose own GSH synthesis is impaired as the combined result of cessation of GSH *de novo* synthesis and the leaks of Glu(-Cys) and GSH [58]. In spite of the concerted oxidative defense of the intraerythrocytic parasite and the host RBC, GSH/GSSG ratios decrease in the host RBC (but not parasite) compartment, indicating a forceful oxidant challenge [58]. In addition to the decreased GSH/GSSG ratios, oxidative stress is further indicated in *Plasmodium*-infected RBCs by the oxidative alteration of membrane proteins and lipids [332]. Remarkably, in

Plasmodium cultures *in vitro* oxidative injury such as membrane lipid peroxidation [552,775] and alterations in membrane protein and lipid composition is not restricted to the infected RBCs, but is also evident in non-infected cells [649], suggesting possible diffusion of reactive oxygen species out of the infected RBC into the blood. Reported oxidative alterations of RBCs membrane proteins such as anion exchanger 1 (AE1, band 3) include clustering [237,403]), methyl-esterification [419], and tyrosine phosphorylation [932], indicating a complex oxidative modification of RBC membrane proteins and signaling molecules.

1.9 Physiological roles of the *NPP*s

1.9.1 Ca^{2+} influx

Non-infected erythrocytes have exceedingly low levels of intracellular Ca^{2+} (below 100 nM). As the parasite matures, the Ca^{2+} content of *Plasmodium*-infected erythrocytes increases [126,481]. Extracellular Ca^{2+} passes the membrane of infected erythrocytes, but Ca^{2+} is almost exclusively localized in the parasite while the Ca^{2+} concentration only changes little in the cytosol of the host cell [579,813,811]. Up to the mature, late trophozoite stage the RBC Ca^{2+} pump in infected cells is largely intact [830]. In addition, the parasite avails itself of the host cytosol Ca^{2+} pool, thereby buffering the free $[Ca^{2+}]_i$ in the host. The bulk of the infection-stimulated Ca^{2+} uptake accumulates within the parasite where a low cytosolic $_{free}[Ca^{2+}]_i$ is maintained by Ca^{2+} sequestration in organelles [312,311], and the cytosolic free Ca^{2+} concentration of the host rises only transiently during the trophozoite/schizont stage [6,311].

1.9.2 Perturbation of the Na^+/K^+ pump-leak balance in *P. falciparum* infected RBCs

The interior of the host erythrocyte represents a highly unusual extracellular environment for the parasite, as it initially has high K^+ concentrations, low levels of Na^+ and only submicromolar levels of Ca^{2+}. In erythrocytes infected with *Plasmodium* there is a marked perturbation of the normal Na^+/K^+ ratios [238,326,766,796]. However, in the hours following invasion (*i.e.* the ring stage) the $[Na^+]/[K^+]$ ratio in the erythrocyte cytosol is maintained low [329,326,528]. The changes in parasite morphology and metabolism after exit of the ring stage are paralleled by an increase in the cation permeability of the host RBC membrane. This cation permeability exhibits a permselectivity of $Cs^+ > Rb^+ > K^+ > Na^+$, with a K^+-to-Na^+

permeability ratio of about 2 as determined by tracer flux studies in trophozoite stage infected erythrocytes [462,796] (Fig. 1.5.A).

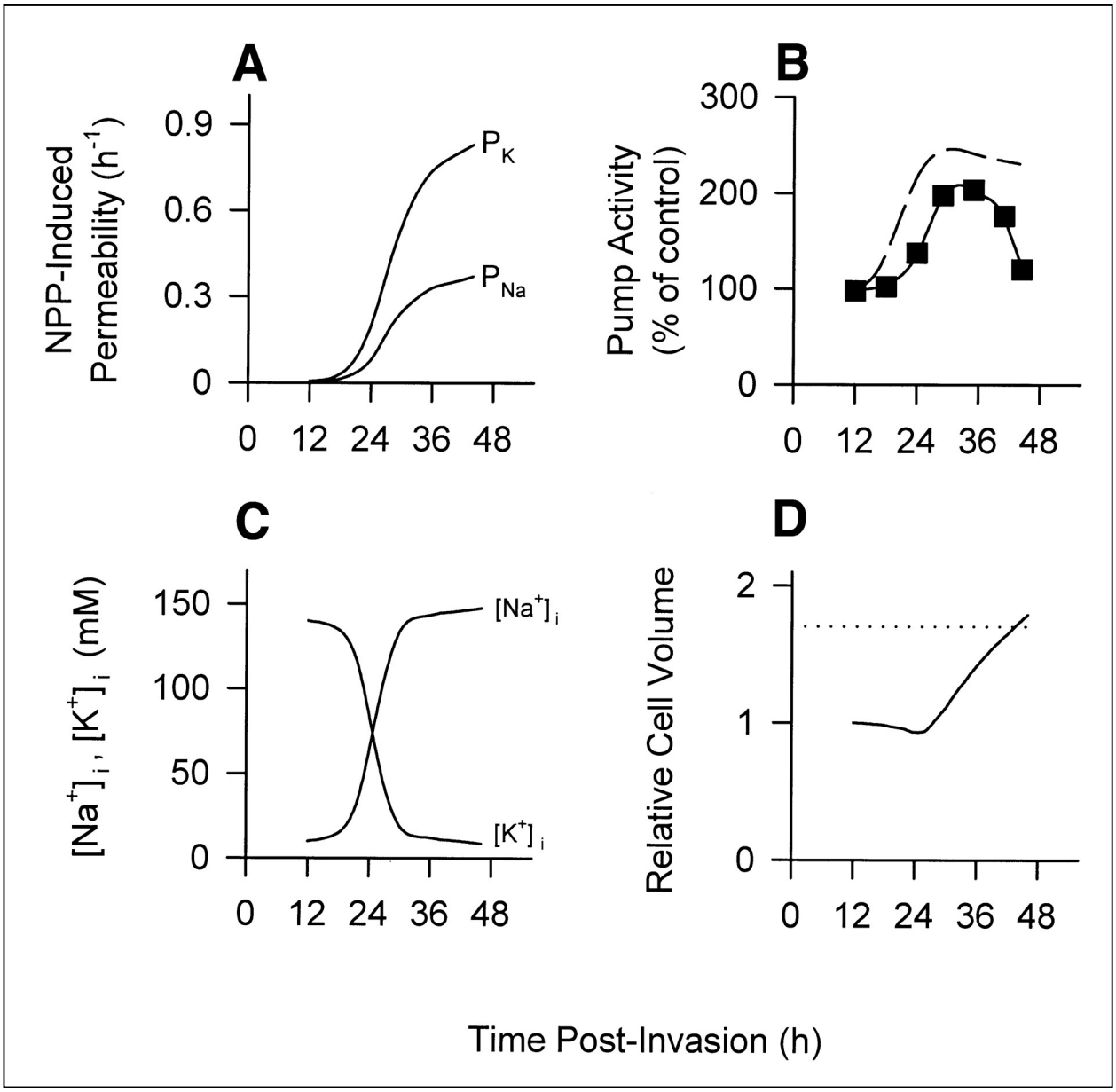


Figure 1.5. Mathematical modeling of the *Plasmodium*-induced changes in Na^+ and K^+ transport and the physiological consequences of these changes for the parasite's 48h intra-erythrocytic development cycle. This modeling was carried out using the integrated Lew-Bookchin erythrocyte model [536]. (**A**) Time-dependent increase in the basal permeabilities of the RBC membrane to Na^+ (P_{Na}) and K^+ (P_K) arising from the furosemide-sensitive *NPP*s. (**B**) Time-dependent variation in the activity of the Na^+/K^+ - pump. The closed squares show 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) [796].

In the first 12 – 15 h p.i., the Na^+/K^+ pump exhibits transport activities comparable to those of non-infected RBCs. In response to the infection-induced increase of cation leakage

through the RBC membrane, an increase of the cytosolic [Na^+] stimulates the RBC Na^+/K^+ pump activity up to twice its normal value between 18 and 36 h p.i. (Fig. 1.5.B). Later on, between 36 h p.i. and at the end of the blood cycle, the Na^+/K^+ pump activity declines progressively whereas cation leakage through the RBC membrane continues. This decline of pump activity results in a dramatic increase of $[Na^+]_i$ and decrease of $[K^+]_i$, respectively within the RBC (Fig 1.5. C).

In particular, during the course of the infection the induced Na^+ and K^+ permeabilities (together with a decreasing host Na^+/K^+ - pump activity) generate Na^+ and K^+ concentrations within the host cytosol that approximate the values of the extracellular space [239,326,528,659,796]. The exact transport rates for the Na^+ / K^+ - pump and the cation leakage have been determined experimentally [796]. According to the output of a mathematical model these values point to a progressive equilibration of the RBC's K^+ and Na^+ concentrations with the blood plasma levels that starts at 12 h p.i. (Fig 1.5.C). The model predicts an almost complete loss of the normal Na^+ and K^+ gradient across the RBC membrane by 36 h p.i [796]. This prediction matches very well the high cytosolic Na^+ and low K^+ concentrations ([Na^+]/[K^+] ~11.6) directly measured in late trophozoite and schizont-infected RBCs using electron probe X-ray microanalysis of cryosections [528]. As a consequence, inwardly and outwardly directed Na^+ and K^+ gradients, respectively, are built up across the plasma membrane of the parasite. In contrast to the infected erythrocyte, the intracellular parasite maintains a low cytosolic [Na^+]/[K^+] ratio throughout the intraerythrocytic cycle, ranging between 0.06 and 0.17 [528,329].

1.9.3 Prevention of premature erythrocyte hemolysis by *P. falciparum*

Breakdown of the host membrane gradients makes the host RBC prone to swelling and eventually colloidosmotic hemolysis due to ~ 7 mM of non-membrane-permeating (impermeant) protein prior to the maturation of the intraerythrocytic parasite (Fig. 1.5.D) [826]. The mathematical model predicts a relative slight cell volume decrease from 12 to 24 h p.i. due to the fact that the efflux of K^+ exceeds the influx of Na^+, followed by an increase from 24 h p.i. onwards due to continuous Na^+ gain until it reaches the critical hemolytic cell volume (~ 150 fl) at 46 h [796]. Premature hemolysis is prevented by a concerted action of the parasite and the host (Fig. 1.6.).

The parasite degrades much more host Hb than needed for its metabolism and the host exports the excess Hb-derived amino acids via the *NPP*s into the serum [543,546]. By this means, the host-parasite interplay lowers the colloidosmotic (oncotic) pressure within the host cytosol

and delays host cell expansion (Fig. 1.6.B). The parasite ingests and digests about 70 - 75% of the host cell hemoglobin (Hb) (Fig. 1.6.A), but uses only up to 16% of the released amino acids for its own protein synthesis [546]. Thus, the *NPP*s accomplish a regulatory volume decrease (RVD) of the volume-challenged host RBC in a similar manner as organic osmolyte and anion channels do in mammalian nucleated cells [804].

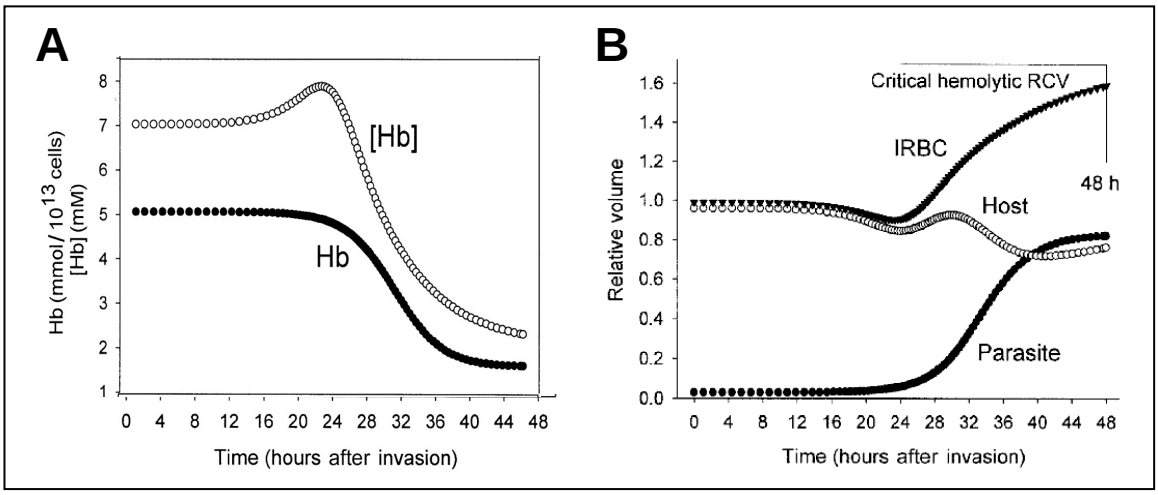


Figure 1.6. (A) Hemoglobin (Hb) digestion by *Plasmodium falciparum* following invasion. The diagram shows the decrease in Hb content (Hb) and the change in Hb concentration ([Hb]). **(B) Predicted changes in relative cell volume of infected RBCs (▼, IRBC), host cell (○, Host) and parasite (●, Parasite) following invasion.** [546]

1.10 Erythrocyte polymorphisms and protection against malaria tropica

1.10.1 Hemoglobinopathies

Hemoglobinopathies is the collective term for the inherited disorders of hemoglobin (Hb). They broadly fall into two main groups. The thalassemiases are classified according to the particular globin chains that are ineffectively synthesized into the α, β, δβ, and εδβ thalassemiases. The structural hemoglobin variants mostly result from single amino-acid substitutions in the α or β chains (HbC, HbE, HbS, or the hereditary persistence of HbF).

In sickle cell anemia /disease (homozygous, HbS/S) and sickle cell trait (heterozygous, HbA/S), there is a mutation in the Hb gene, which codes for the Hbβ chain. The normal allele is HbA ($Hb\beta^A$), but for the sickle cell allele, HbS ($Hb\beta^S$), in the 6th codon after the start codon GAG is substituted by GTG, so that glutamic acid is replaced by valine at amino acid 6 (β6

Glu→Val). The side chain of glutamate is highly polar, whereas that of valine is distinctly nonpolar. In this way a hydrophobic residue comes to lie on the outside of HbS. This change encourages a sickling process in homozygous HbS/S RBCs that takes place at oxygen tensions between 15 and 40 mm Hg, commonly observed in the tissues of the human body. In contrast, sickling in heterozygotes HbA/S RBCs takes places only below 15 mm Hg oxygen partical pressure [31,377]. In the body the oxygen partial pressure drops down to 15 mmHg only under conditions of severe vasoconstriction or anoxemia, *e.g.* high altitude flights in unpressurized aircraft [781]. Sickling is induced only in the presence of deoxyhemoglobin S through binding between Hb molecules by hydrophobic bonds, comparable to the process of formation of amyloid fibrils [617,281,55,551]. In oxyhemoglobin S the complementary site (to the hydrophobic residue) at the EF corner of the β chain is masked. If the complementary site on one deoxyhemoglobin can bind to the hydrophobic residue outside of another deoxyhemoglobin S molecule, long fibers are formed, rigid tactoids that distort the RBC's cell shape [679,589,623]. Thus, polymerization of HbS, deforming RBCs into a sickle shape, occurs when there is a high concentration of the deoxygenated form of HbS. This is usually not the case for sickle cell trait, because not more than half of the hemoglobin is HbS [806]. Irreversibly sickled cells (ISC) have irreversible membrane damage and high mean corpuscular hemoglobin concentration values as they are dehydrated because of water and potassium loss through the activation of the deoxygenation-induced cation pathway P_{sickle} [539,538] and subsequently Gardos channels. ISCs are removed from the circulation by sequestration mechanisms, hemolysis and uptake by macrophages [714]. Heterozygous sickle cell trait, however, provides protection against malaria mortality and severe malaria symptoms [8,30,29,28,38,150,391,414,415,555,896,895].

Studies of globin gene haplotypes based on the patterns of restriction fragment-length polymorphisms in the β globin gene clusters [47,657,389,340] have provided important information on their evolution [292]. The studies suggest that the mutation in HbS originated approximately 2000 years ago with at least four sites of origin in Africa [340] and once in either the Middle East or India. Accordingly, the majority of the chromosomes with the β^S gene have one of the five common haplotypes, designated as Benin, Bantu or Central African Republic, Cameroon, Senegal, and Arab-Indian haplotypes. However, 5 - 10% of the chromosomes have less common haplotypes, usually referred to as atypical haplotypes [924]. The present distribution of the HbS gene and haplotype diversity reflects redistribution on different backgrounds by gene conversion and recombination and has resulted from population migration, founder effects, and other forms of genetic drift [291,292,630].

1.10.2 Genetic traits with a presumable protective role against *falciparum* malaria

The clinical outcome of infectious disease is determined by a complex interaction between microorganism, host genetic factors and environment [413]. Recent epidemiological studies show that the genetic background among the populations exposed to infectious agents may confer partial or complete protection to disease [87,109,178]. As a part of host-pathogen interaction, an effective immune response to infection has contributed to the development of host genetic diversity through selective pressure [754,895]. Among the study of host factors and their genetic contribution to infectious diseases, one of the best known examples of genetically determined factors for the course of disease is found in malaria [265,605,894]. The relationship between malaria and evolution of the human genome has attracted remarkable interest from physical anthropologists and human geneticists since the 1950s [366,555,571,621]. Interest in this topic has continued up to the present [226,350,365,390,392,556,712,873,894]. Experimental, clinical and field research has led to the paradigm that malaria is the key selective factor in attainment and maintenance of relatively high frequencies of several genetic traits [227].

α-thalassemia [26,609,653,865,897,898,921];

β-thalassemia [168,891,892,893];

HbS (β6 Glu→Val) [8,30,29,28,38,150,391,414,415,555,896,895];

HbE (β26 Glu→Lys) [152,174,289,341,414,415,646,736], common in Southeast Asia;

HbC (β6 Glu→Lys) [7,68,152,275,274,302,608,611,827], common in Western Africa;

G6PD-deficiency [34,66,152,555,610,614,726,834];

(Melanesia) ovalocytosis as result of deletions in the band 3 (AE1) gene [27,123,194,195,320];

the human MHC class I HLA-Bw52 (alpha-3 domain) antigen; and

the MHC class II HLA DRB1*1302-DBQ*0501 haplotype [391]

have reached their current gene frequency by heterozygote selection against malaria [873], because they confer refractoriness to the two most common forms of severe malaria - severe malarial anemia and cerebral malaria [227]. For α-thalassemia [921], HbC, and HbE the selective advantage is greatest in homozygotes. These polymorphisms are a major determinant of child survival in malaria endemic countries. How they confer resistance towards *P. falciparum* infection has been recently reviewed [894]. Other polymorphisms are under investigation [493,712]: erythrocyte surface molecules [54,604,122,576,629,671,670]; mutant membrane proteins such as glycophorins [668]; adhesion molecules of endothelial cells like ICAM; receptors such as CD36,

CR1 (complement receptor 1, CD35) [180,628,803,822,931]; surface molecules of platelets like PECAM1; mechanisms of acquired immunity via IL-4, CD40 ligand; low-affinity IIa receptor for Fc fragment of IgG; and mediators of innate immunity. The best-studied influence of the malaria parasite upon the human genome, however, is the sickle cell trait [82,400,669].

1.10.3 Balanced polymorphism in the case of HbS

The geographical coincidence between *falciparum* malaria endemicity and the distribution of HbS, thalassemia and G6PD deficiency has provided the stimulus for the formulation of the "malaria hypothesis" since the 1950s. The "malaria hypothesis" postulates that individuals with with various thalassemia traits [364], sickle cell trait (HbA/S) [30,29,28,31,33,34,37,35,36] and female heterozygotes of G6PDH deficiency whose blood contains a mixture of deficient and non-deficient red cells due to mosaicism [102,726] are more resistant than healthy people towards infection with *P. falciparum* [621,779]. Sickle cell trait is the most clearcut example of a "balanced polymorphism". The considerable selective advantages conferred to heterozygotes under *P. falciparum* pressure are balanced by the disadvantages suffered by the homozygotes such as gene frequency remains very high in malaria endemic regions. Therefore, HbS has been selected in present or recent malaria endemic areas, such as in the Congo [388], on the coast of Libya [612], Greece [75], Arabia [530], and India [770]. In African tribes, sickle cell trait frequency approaches 40 %. About 4% of new-born children are sickle cell homozygotes. However, their viability is less than one fifth of the viability of other genotypes [32]. The death of each homozygote removes two sickle cell genes from the population. In conclusion, for one generation, the removal is more than 2* 4% * 4/5 = 6.4 % of the genes for the ß-chain of Hb. Thus, about 15 % of the sickle cell genes are eliminated in each generation [33].

Heterozygote HbA/S individuals are not protected from parasitemia [391,580,712,896] as they are as easily infected with *P. falciparum*, and show parasites just as frequently as subjects without the trait do [33]. However, with regard to severe malaria, it has been confirmed that heterozygotes suffer less frequently and less severely than do normal individuals. This might be particularly the case when the adaptive immune system is still developing and the innate immune system has a dominant role, as in young children under 5 years of age [8,171,170,290,382,391,648]. Two large cohort studies involving a total of 3000 children in Kenya have demonstrated that HbA/S is almost 90% protective against severe or complicated malaria, 75% against admission to hospital, 50% against mild clinical malaria, but have no effect on

the prevalence of parasitemia, and no significant effect on a wide range of other childhood diseases. HbA/S individuals have parasite densities 4-fold lower than those with HbA/A. The protective effect of HbA/S is equally strong against the two most common forms of severe malaria - severe malarial anemia and cerebral malaria [227], resulting in reduced morbidity and mortality in this group of children [391,580,896].

1.10.4 Mechanisms of protection from severe malaria for HbA/S carriers

The protection mechanisms remain controversial. The very early theories, which until recently were still considered valid by some authors, held the presence of HbS *per se* responsible for the limited multiplication of the trophozoites, resulting in lower parasite counts in HbA/S carriers [33]. Some studies suggested that invasion by the parasite induces sickling, either impairing parasite growth [298,300,302,669,667], or improving clearance of infected RBCs by the reticulo-endothelial system [572]. Other studies suggested that sickling modulates cytoadherence and/or binding of infected HbA/S RBCs to peripheral blood mononuclear cells (PBMCs), this in turn influencing TNF and IFNγ levels and the immune response as a whole [1,5].

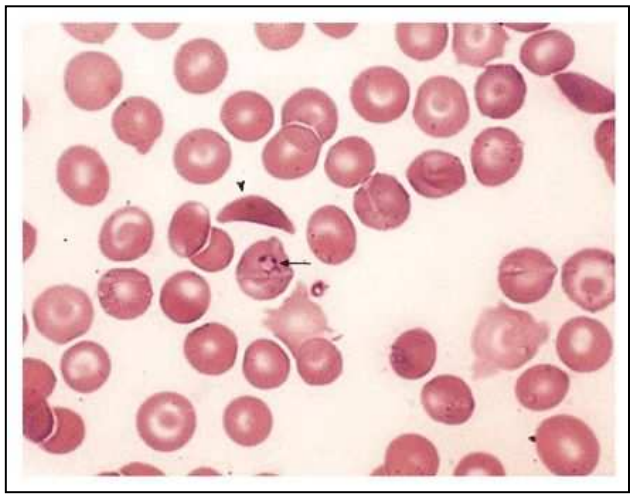


Figure 1.7. Blood smear of HbS/S patient infected with *Plasmodium falciparum*.
The blood smear revealed a few sickle cells (arrowhead) and rare red cells with *P. falciparum* (arrow) [116]

The notion that sickling *per se* impairs parasite growth in HbA/S RBCs was first questioned in the 1970s and repeatedly thereafter [17,523]. Further, that sickling might occur after invasion of HbA/S RBCs by the parasite was disproved by Boctor and Uehlinger in 2002 [116], who examined Giemsa stained blood smears from a *P. falciparum* infected 23-year-old man with sickle cell disease, *i.e.* HbS/S RBCs as an extreme of HbA/S RBCs (Fig 1.7.). They

found no *P. falciparum* in sickled RBCs, suggesting that the altered physical and biochemical properties of hemoglobin S cannot account for protection against *P. falciparum*. Moreover, increased free FP IX released from hemoglobin digestion is high in infected HbS/S RBCs, but low in infected HbA/S and HbA/A RBCs [656]. Thus, other mechanisms involving the immune system must be considered responsible for the protection of sickle trait individuals from malaria tropica [918].

1.10.4.1 Accelerated acquisition of adaptive immunity

Several studies have shown a more rapid acquisition of adaptive immunity in HbA/S individuals [3,5,4,82,84,150,385,417,581,639,638] compared to HbA/A individuals in malaria - endemic regions [129,743]. In fact, a large cohort study with more than 1000 children has shown that the degree of HbA/S protection against clinical malaria changes with age. It is only 20% in the first 2 years of life, peaks at 56% around the age of 10 years, and returns thereafter to 30% in children elder than 10 years [895]. The changing degree of protection may reflect combined effects of innate and adaptive immune responses. Recent advances in molecular pathology and studies of host immunity show that the immune mechanisms that control parasitemia are different from those that control pathology in malaria [743]. Antibody-dependent cellular toxicity is necessary to reduce parasitemia [129]. Because the Fc fragment is responsible for cytophilic binding of antibodies to macrophages [95] or lymphoid cells [848], these antibodies must be of the cytophilic subclasses IgG1 and IgG3 [234].

1.10.4.2 Accelerated clearance by the innate immune system

The instability of HbS leads to increased formation of hemichrome [381,227,226,228]. Hemichromes are low spin derivatives of the high spin form, ferrihemoglobin, brought about through discrete reversible and irreversible changes of protein conformation so that atoms endogenous to the protein are now bound as the sixth ligand of the heme iron that usually is used for oxygen binding [696]. Hemichromes oxidize and cluster RBC membrane protein band 3 (AE1). This results in an increase of autologous immunoglobulin G (IgG) deposition and binding of complement C3b fragments, which accelerates phagocytosis of infected HbA/S RBCs already at ring stage [66].

1.10.4.3 Impaired parasite growth

Another oxidant stress model has been developed to explain the protection from malaria afforded by sickle cell trait [42]. This model proposes that increased formation of oxygen radicals such as superoxide anions (O_2^-), or hydrogen peroxide (H_2O_2) in *P. falciparum* infected RBCs containing HbS results in membrane injury, so that parasite growth is inhibited.

Inhibition of parasite growth in HbA/S RBCs [631] can take on various forms: (1) impaired merozoite liberation and altered RBC rupture, *e.g.* delayed release of merozoites from HbA/S RBCs due to increased buffering capacity of the altered hemoglobin for fatty acids [524]; (2) reduced invasion by merozoites at low partial pressure of oxygen [298,300,302,299,303,669,667]; (3) impaired parasite survival [298,300,302,299,303,572,629,669,667,724]; and / or (4) retardation of intracellular development within the erythrocyte [298,300,302,299,303,669,667].

An imbalance in the MSP-1 allelic distribution of three investigated MSP1 parasite genotype families was attributed to reduced fitness of some parasite strains in individuals with sickle cell trait [640].

1.10.4.4 Impaired rosette formation

Modulation of cytoadherence is a rather unlikely protective mechanism [725], whereas impaired rosette formation was observed to a significant degree in sickle cell trait carriers [155]. In *P. falciparum* infected HbA/A RBCs knob size and knob density change inversely from trophozoite to schizont stage. Knobs of trophozoites (24 - 36 h p.i.) have diameters from 160 - 110 nm and densities ranging from 10 - 35 knobs / μm^2. In schizonts (40 - 44 h p.i.) the knob size is decreased (100 - 70 nm), whereas density is increased (45 - 70 knobs / μm^2) [357]. Heterozygous *P. falciparum* infected HbA/S RBCs showed reduced rosetting, *i.e.* binding of uninfected RBCs to infected RBCs, forming smaller and weaker erythrocyte rosettes than HbA/A erythrocytes *in vitro* [154,155]; this might reduce the risk of cerebral malaria.

1.11 Objective of the study

The objective of this study was to elucidate the mechanisms of programmed cell death in erythrocytes, also referred to as eryptosis, that are induced in RBCs after infection with *Plasmodium falciparum*. In the host cell *Plasmodium* is confronted with a completely different environment in tems of redox state, electrolyte concentrations and signaling molecules as compared to the extracellular fluid, *i.e.* prior to invasion. To survive in the new environment, the parasite thus has to modify the host cell. For instance, it induces new permeability pathways (*NPP*s) in the host erythrocyte membrane, which provide the parasites with essential nutrients, facilitate disposal of metabolic waste products, and modify the electrolyte composition of the host cell [461].

Oxidation of non-infected cells induces membrane permeabilities similar to those obseverd in infected erythrocytes [410,406,407], suggesting that the parasite accomplishes the alterations of host cell function at least in part by inducing oxidative stress. Recent patch-clamp whole-cell recordings have directly demonstrated a nonselective (NSC) conductance (G_{cat}) with low but significant Ca^{2+} permeability in *Plasmodium falciparum*-infected and in oxidized non-infected red blood cells (RBCs) [244,243]. This G_{cat} of infected RBC exhibits an about two-fold higher permeability for K^+ over Na^+ [461]. Accordingly, the infection-induced NSC conductance is responsible for the cation permeability of the *NPP*s. The cation permeable subfraction of the *NPP*s has been characterized previously by tracer flux and isosmotic hemolysis experiments [796]. These experiments suggested that the cation permeable subfraction of the *NPP*s impairs the Na^+/ K^+ pump-leak balance of the host RBC. Moreover, the cation leak might supply the parasite with Ca^{2+}. Furthermore, the parasite might use this cation leak to decrease the colloid osmotic pressure of the erythrocyte to prevent premature hemolysis of the host cell [545]. The functional significance of the infection-induced cation leak for parasite growth is still ill-defined. Therefore the first question to be answered in the present study was:

(1) What is the functional significance of the infection induced increase in Ca^{2+}-permeable NSC conductance (G_{cat}) for intraerythrocytic parasite development?

Elevated cytosolic Ca^{2+} concentrations and oxidative stress have been demonstrated to induce eryptosis in non-infected RBCs [207,243,507]. This leads to exposure of phosphatidylserine (PS) on the erythrocyte surface [507,510]. If and why apoptosis-like mechanisms exist in single cell organisms such as trypanosomes [247,286] or in erythrocytes [499,509] is still a matter of discussion. While viable cells retain PS in the inner leaflet of the membrane, apoptotic cells

are unable to maintain their membrane asymmetry, resulting in exposure of PS on their surface and rapid removal of these cells. Apoptosis with subsequent phagocytosis and degradation of infected cells may result in the elimination of intracellular pathogens and thus serve as host defense mechanism [346,476,739]. Clearance of infected erythrocytes may similarly confer protection against severe malaria. The malaria parasite *P. falciparum* invades host erythrocytes to proliferate, but may also use them to hide from the host's immune responses. Therefore, the induction of suicidal erythrocyte death by *Plasmodium* infection may be of use not only to the parasite but also to the host cell. As erythrocytes lack MHC molecules for presenting foreign antigens to fight an infection, a mechanism such as eryptosis might serve to protect the organism. Therefore, the next question to answer was:

(2) Does *Plasmodium* infection induced eryptosis lead to increased PS exposure?

Two eryptosis signaling pathways are known. One stimulates the Ca^{2+}- permeable NSC conductance (G_{cat}), the other activates a sphingomyelinase that produces ceramide. An enhanced infection-induced NSC conductance (G_{cat}) has already been demonstrated (see above). Therefore the next question to address in this study was:

(3) Is host sphingomyelinase activated during *Plasmodium* infection?

Activation of parasite sphingomyelinase has already been shown [372]. This led on to the next question, namely:

(4) What is the functional significance of ceramide production in infected RBCs?

Elevated cytosolic [Ca^{2+}] and ceramide activate scramblase and inhibit translocase, resulting in PS exposure at the outer leaflet on the membrane of non-infected erythrocytes [507,510,934]. Phospholipid scrambling of the cell membrane with subsequent PS exposure has been shown to be a recognition signal for the innate immune system, *i.e.* for macrophages to rapidly phagocytoze the PS exposing cells, whether they be nucleated cells [271] or erythrocytes [115]. PS exposure contributes to the mechanisms by which aged, *i.e.* senescent, or injured non-infected erythrocytes are removed by macrophages [15,16,455,454,453,516,514,635]. In particular, ring stage infected sickle trait (HbA/S) erythrocytes [66], which show many features of senescent erythrocytes [565], are recognized and cleared more quickly than infected normal (HbA/A) erythrocytes. This may cause the partial resistance of sickle cell trait carriers [66]. Accordingly, the aim of the second part of the present study was to determine whether enhanced eryptosis might be one mechanism that contributes to the partial resistance of sickle cell trait carriers. This threw up the following question:

(6) Does *P. falciparum* infection lead to enhanced PS exposure in sickle trait erythrocytes?

Furthermore, the objective of the study was to investigate the mechanisms of eryptosis

in infected HbA/A and HbA/S RBCs. Homozygous non-infected sickle (HbS/S) RBCs undergo eryptosis more rapidly when subjected to different stress stimuli like oxidative stress or hypertonic conditions [513] compared to HbA/A non-infected RBCs. Therefore the study examined:

(7) Is acid and neutral sphingomyelinase differentially activated in infected HbA/A and HbA/S RBCs under normal culture conditions or under hypertonic conditions?

Recent observations revealed a pivotal role of phospholipase A_2 (PLA_2) and prostaglandin E_2 (PGE_2) in the regulation of PS exposure on human erythrocytes during eryptosis [517]. PLA_2 releases from phospholipids arachidonic acid which is further processed by cyclooxygenase (COX) and prostaglandin E_2 synthase (isomerase) to PGE_2. In erythrocytes, PGE_2 activates Ca^{2+}-permeable cation channels, leading to Ca^{2+} entry [440,518,548] and stimulation of a Ca^{2+}- sensitive scramblase [133,218,517,913]. Moreover, a mild course of malaria infection in *P. falciparum* infected sickle cell trait children correlates with higher levels of PGE_2 [677]. The present study therefore tested whether enhanced PS exposure in *P. falciparum* infected HbA/S RBCs is Ca^{2+}-mediated and PGE_2- induced – with a special emphasis on ring stage parasites:

(8) Is the infection induced Ca^{2+}-permeable NSC channel more active in *P. falciparum* infected HbA/S than in HbA/A RBCs, especially at ring stage?

(9) Do *Plasmodium falciparum* infected HbA/S erythrocytes secrete more PGE_2 than do infected HbA/A RBCs?

2 Materials and Methods

2.1 Materials

If not stated otherwise chemicals were of the highest purity available.

2.1.1 Organisms and cells used

Plasmodium falciparum FCR3 [429], BINH [104] strains were a kind gift from the institute of tropical medicine in Tuebingen, Germany.

Banked erythrocytes and plasma (which could not be used for transfusion) were a kind gift from Marc Weidmann and the institute of transfusion medicine, Tuebingen, Germany.

Acid sphingomyelinase wild-type (ASM +/+) and knock-out (ASM -/-) mice were a kind gift from Dr. Verena Jendrossek. They were originally obtained from Drs. R Kolesnick/E. Gulbins (New York) who maintained ASM -/- mice in a sv129 3 C57BL/6 background, breeding them with genotyped heterozygous mice [402]. These mice were then backcrossed (around 5 times) again on C57BL/6, so that only this background was left. The ASM +/+ and ASM -/- mice used for this study were maintained by using heterozygous breeding pairs and genotyped by the animal husbandry at the Ear-Nose-Throat Clinical Center of Tuebingen University.

This animal model has been created by gene targeting for studying the pathogenesis and treatment of the Types A and B of the Niemann-Pick disease (NPD), which results from the deficient activity of ASM, and for investigations into the role of ASM in signal transduction and apoptosis.

By eight months of age, the disease takes a severe, neurodegenerative course which ends lethally. Autopsy of these animals revealed complete absence of ASM activity in the tissues as well as elevated blood cholesterol and liver and brain sphingomyelin (SM) levels. The atrophy of the cerebellum and marked deficiency of Purkinje cells was evident. Microscopic analysis revealed 'NPD cells' in reticuloendothelial organs and characteristic NPD lesions in the brain [402]. Membrane defects were first observed about three months after birth.

2.1.2 Chemicals

2.1.2.1 Laboratory chemicals

Acetic acid (100%, glacial)	Merck Eurolab GmbH
Adenine	Sigma, Deisenhofen
Agarose	Carl Roth, Karlsruhe
Azure B	Sigma-Aldrich, Taufkirchen
Albumax II	Gibco-Invitrogen, Karlsruhe
Annexin V-FLUOS	Boehringer, Mannheim
Annexin V-568	Roche, Mannheim
ATP disodium salt	Sigma, Munich
t-butylhydroperoxide (8 M)	Sigma, Deisenhofen
Chloroform	Carl Roth, Karlsruhe
$CaCl_2$	Sigma, Deisenhofen
Cardiolipin (from bovine heart)	Sigma, Munich
Citrate-Phosphate-Dextrose-Stabiliser	Baxter S.A. (Fenwal), Maurepas, France
C6 – ceramide	Biomol, Hamburg
C16- ceramide	Alexis, Lausen, Switzerland
DETAPAC ($C_{14}H_{23}N_3O_{10}$)	Sigma (Fluka), Munich
Dextrose monohydrate (D (+) glucose)	Sigma, Deisenhofen
DL-Dithiothreitol (DTT) minimum 99 %	Sigma, Deisenhofen
2,4 –Dichloroisocoumarin	Biomol GmbH, Hamburg
Dipotassium hydrogen phosphate	Merck KGaA, Darmstadt
Dimethylsulfoxid (DMSO)	Sigma, Deisenhofen
Disodium hydrogen phosphate anhydrous	Merck KgaA, Darmstadt
dNTP Mix 100 mM	Roche, Mannheim
EDTA	Sigma, Steinheim
Eosin Y	Merck Eurolab, Darmstadt
EIPA	Sigma, Deisenhofen
Ethanol	Merck Eurolab, Darmstadt
Ethidium bromide (10 mg/ml)	Carl Roth, Karlsruhe
Epoxyquinone	Alexis, Gruenberg

Fluo-3/AM	Calbiochem, Bad Soden
Gas mixture (90% N_2 / 5% O_2 / 5% CO_2)	Hoepfner, Reutlingen; Mast, Tuebingen
Gentamicin sulphate	Gibco InVitrogen, Karlsruhe
Glutamine	Gibco InVitrogen, Karlsruhe
Giemsa staining solution	Sigma, Deisenhofen
Glycerol	Sigma-Aldrich, Taufkirchen
HCL	Merck Eurolab, Darmstadt
HEPES	Sigma, Deisenhofen
HEPES/NaOH 1M solution	Sigma, Deisenhofen
Hypoxanthine	Sigma, Deisenhofen
Ionomycin	Sigma, Munich
Imidazole (1,3- Diazo-2,4-cyclopentadiene)	Sigma-Aldrich, Taufkirchen
Immersion oil Type A	Cargille Laboratories, Cedar Grove, NJ, USA
Inosine	Sigma-Aldrich, Taufkirchen
Instamed RPMI 1640 with Glutamine	Biochrom, Berlin
Instamed RPMI 1640 with Glutamine	Gibco Invitrogen, Karlsruhe
Ionomycin	Sigma, Munich
Isopropanol	Merck Eurolab, Darmstadt
KCl	Sigma, Deisenhofen
Mannitol	Sigma, Deisenhofen
Methanol	Merck KgaA, Darmstadt
Methylene blue	Sigma-Aldrich, Taufkirchen
Multi Twist Top Vials 1.7 ml	Roth, Karlsruhe
Multi Twist Top Vial Caps	Roth, Karlsruhe
NaCl	Sigma, Deisenhofen
Na-gluconate	Sigma, Deisenhofen
NaHCO3	Sigma, Deisenhofen
NaOH	Sigma Diagnostics, St Louis, MO, USA
Na-vanadate	Sigma-Deisenhofen
Nitrogen (Gas)	Mast, Tuebingen
NMDG-Cl	Sigma, Deisenhofen
n-octylglucopyranoside ($C_{14}H_{28}O_6$)	Sigma (Fluka), Munich
PBS	Gibco BRL, Karlsruhe
Potassium dihydrogen phosphate	Merck Eurolab, Darmstadt

Propidiume iodide	Sigma, Munich
PWO polymerase (5 U/µl, 2 x 250 units)	Roche, Mannheim
RPMI medium 1640 (w/o glutamine)	Gibco-Invitrogen, Karlsruhe
SAG-Mannitol solution	Baxter S.A. (Fenwal), Maurepas, France
sn-1,2-diacylglycerol kinase, recombinant, membrane preparation of E. coli, High Purity	Merck, Darmstadt
Raffinose	Sigma, Deisenhofen
Spiroepoxide	Alexis, Gruenberg
Sphingomyelinase (50 U)	Biomol, Hamburg
1-Stearoyl-2-arachidonoyl-*sn*-glycerol	Alexis, Lausen, Switzerland
Syto16 (1mM in DMSO)	Invitrogen (Molecular Probes), Karlsruhe
TaKaRa 10x LA PCR buffer II (Mg^{2+})	BioWhittaker Europe, Verviers, Belgium
TaKaRa dNTP mixture (2.5 mM each)	BioWhittaker Europe, Verviers, Belgium
TaKaRa LA Taq-Polymerase (5U/µl)	BioWhittaker Europe, Verviers, Belgium
Trizma base	Sigma-Aldrich, Steinheim
Sorbitol	Sigma, Deisenhofen
Raffinose	Sigma, Deisenhofen
Urea	Sigma, Deisenhofen

2.1.2.2 Radiochemicals

[^{32}P]γ-ATP, 250 µCi (92.5 KBq), dissolved in 50% Ethanol, 3 mmol/l; 2 mCi/ml	Amersham, Braunschweig
[^{32}P]γ-ATP, 100 µCi (37KBq) dissolved in 50% Ethanol; 3 mmol/l, 2 mCi/ml	Hartmann Analytic GmbH, Braunschweig
$^{45}Ca^{2+}$ as $CaCl_2$ in aqueous solution, specific activity: 0.185 – 1.11 TBq/g Ca	ICN Biomedicals GmbH, Eschwege

2.1.2.3 Antibodies

Monoclonal antibody to ceramide; 200µg/ml; clone MID 15B4; isotype Mouse IgM	Alexis, Gruenberg

Monoclonal antibody to ceramide; 200µg/ml	Sigma, Munich
Isotype matched pure mouse antibody (IgM)	Alexis (Ancell), Lausen, Switzerland
Secondary monoclonal PerCP-Cy 5.5-conjugated rat anti-mouse IgM antibody, *Igh-C*^{a} and *Igh-C*^{b} haplotypes, isotype rat (LOU) IgG2a'κ; 0.1 mg, 0.2 mg/ml	BD Biosciences, Heidelberg

2.1.2.4 Primers

Primers were procured from Invitrogen, Karlsruhe.

ASM – PS	5'agccgtgtcctcttcccttccttac3'
ASM - PA1	5'cgagactgttgccagacatc3'
ASM - PA2	5'ggctacccgtgatattgctg3'
Glo5new	5'aactgtgttcactagcaacctc3'
BPG2	5'atagaccaataggcagag3'
Myco-P1	5'-gtgccagcagccgcggtaatac-3'
Myco-P4	5'-taccttgttacgacttcacccca-3'

2.1.2.5 Kits

Correlate-EIA^{TM} Prostaglandin E_2 Enzyme Immunoassay Kit	Assay Designs, Inc., Ann Arbor, MI, USA
DNA easy tissue kit	Qiagen GmbH, Hilden
Mycoplasma Plus^{TM} PCR Primer Set	Stratagene, Heidelberg
QIAamp DNA (Blood) Mini Kit	Qiagen, Hilden
QIAamp DNA Mini Kit	Qiagen, Hilden

2.1.3 Stock materials

6-, 12-, 24- & 96-well plates	Greiner Bio-One, Frickenhausen
Bottle-top filters for 0.125 l	Millipore, Schwalbach
Bottle-top filters for 0.25 and 0.5 l	Carl Roth, Karlsruhe
CryoTube Vials 2.0 ml	Greiner Bio-One, Frickenhausen

EDTA tubes	Monovette, Sarstedt
FACS tubes	Greiner Bio-One, Frickenhausen
Falcon tubes	Greiner Bio-One, Frickenhausen
Kodak^{TM}X-OmatAR-5 films (V-1651496)	Amersham, Braunschweig
Patch pipettes (8 to 12 MΩ) made of borosilicate glass (150 TF-10)	Clark Medical Instruments, UK
Microcentrifuge Filters Ultrafree-MC, NMWL 5.000 Dalton, PLCC cellulosic membrane	Sigma, Taufkirchen
MµlTI Twist Top Vials (1.7 ml; 10 x 45 mm polypropylene)	Roth, Karlsruhe
MµlTI Twist Top Vial Caps	Roth, Karlsruhe
Paper filter	Macherey- Nagel, Düren
Silica Gel 60 thin-layer (HPTLC), 200µm thick, glass plates (without F), 10cm x 10cm	CAMAG, Berlin
Syringe sterile filter (0.22 µm)	Millipore, Schwalbach
Scintillation vials	PerkinElmer (LAS), Rodgau-Juegesheim
Tissue culture flasks	Greiner Bio-One, Frickenhausen
Tissue culture flasks	Nunc, Wiesbaden
Transfer pipettes, polyethylene, extended fine tip, large bulb, Bulb: 3 ml, sterile	Sigma, Deisenhofen
Tissue culture flasks	Greiner Bio-One, Frickenhausen
Tissue culture flasks	Nunc, Wiesbaden

2.1.4 Devices

Adapter for gas dosing unit EC for 24 Pasteur pipettes (V826.612.000)	VLM, Leopoldshöhe
Autoclave (with outlet air bacterial filter)	Systec Labor Systemtechnik, Wettenberg
8-channel-multi-pipette Research pro	Eppendorf, Hamburg
Bath sonicator (Transsonic 310)	Elma, Singen
Biofuge pico Heraeus	Kendro laboratory products, Hanau
β-scintillation counter Wallac 1409	PerkinElmer (LAS), Rodgau-Juegesheim
Concentrator 5301	Eppendorf, Hamburg

Dissolved Oxygen Meter (DO-5509)	Lutron, Copersburg, PA, USA
Horizontal DMZ puller	Zeitz, Augsburg
Evaporator EVA-EC1-24-S (V832.000.002)	VLM, Leopoldshöhe
EPC 9 patch-clamp amplifier	HEKA, Lambrecht
FACS Calibur	Becton Dickinson, Heidelberg
Hamilton microliter syringe(100 µl, 250 µl)	Carl Roth, Karlsruhe
Hera cell incubator 37 °C	Kendro Laboratory Products, Langenselbold
Heraeus Sepatech Centrifuge	Kendro laboratory products, Hanau
Hair drier	Tuebingen, Germany
Heating block HB-EC1-24-1,5-EP (V804.080.000)	VLM, Leopoldshöhe
Hypercassette neutral RPN11648	Amersham, Braunschweig
ITC 16 interface	Instrutech, USA
Jouan Centrifuge MR 1812	Fernwald, Germany
Light microscope Leica CM E	Leica, Solms
Liquid β-scintillation counter Wallac 1409	Wallac, Freiburg
PCR machine- Peltier Thermal Cycler-200	MJ Research, Waltham, MA, USA
Rotina35 centrifuge	Hettich GmbH & Co KG, Tuttlingen
Safety cabinet class II (Hera Safe)	Kendro Laboratory Products, Langenselbold
Spectronic GENESYS* 6 UV-Vis Scanning Spectrophotometer	Thermo Electron, Bremen
Thermomixer 5436	Eppendorf, Hamburg
VLM-metal block- thermostat digital type EC-1V-130-K1R (V649.061.620)	VLM, Leopoldshöhe

2.1.5 Media, buffers and solutions

2.1.5.1 Solutions for preparing human erythrocytes for storage

Citrate-Phosphate-Dextrose-Stabilizer	(g/l aqua ad iniectabilia)
Citrate acide – monohydrate	3.27
Sodium citrate – monohydrate	26.30
Sodium dihydrogen phosphate – dehydrate	2.50
Dextrose monohydrate	25.50

SAG-Mannitol solution	(g/l)
NaCl	8.77
Dextrose monohydrate (D (+) – glucose)	9.00
Adenine	0.169
Mannitol	5.25

Citrate-Phosphate-Dextrose-Stabilizer (70 ml) and SAG-Mannitol (SAGM) solution (110 ml) were obtained sterile in pockets connected by thin tubes, separated by a leukocyte filter, ready for a blood donation of 500 ml.

Sterile saline solution	(in mM):
NaCl	150
KCl	5
$CaCl_2$	1.4
$MgCl_2$	1
HEPES/NaOH pH 7.4	10
Glucose	10

The saline solution was prepared according to Egee *et al.* [253]. The pH was adjusted to 7.4 and the osmolarity to 320 ± 5 mOsm $(kg\ H_2O)^{-1}$ before sterile filtering the saline solution through a filter unit of 0.22 µm pore size.

2.1.5.2 Media and solutions for the maintenance of *P. falciparum in vitro* culture

Supplemented RPMI 1640 medium	
HEPES/NaOH	25 mM
Gentamicin sulphate	20 µg /ml
Glutamine	2 mM
Hypoxanthine	200 µM
Albumax II™	0.5 %
(Human Serum	2 %)

1 M sterile filtered $CaCl_2$ (0.22 µm pore size) was used for preparing human serum from plasma.

Albumax stock solution 10 x	(in g/l):
Instamed RPMI 1640 with Glutamine	
NaHCO3	10.43
HEPES	5.96
D (+) glucose	2
Hypoxanthine	0.2
Albumax II^{TM}	50
Gentamicin sulphate	0.01

The pH was adjusted to 7.1 - 7.4. The sterile filtered Albumax stock solution was stored in 50 ml aliquots at -20 or -80°C.

Freezing solution	(in %)
Glycerol	28
Sorbitol	3
NaCl	0.65

The freezing solution was sterile filtered (through 0.22 µm pore size) and stored at 4°C.

Defreezing solution	(in %)
NaCl	3.5

The sterile filtered defreezing solution (through 0.22 µm pore size) was stored at room temperature (RT). The solution was warmed up to 37°C before use.

2.1.5.3 Solutions to analyze blood smears of *P. falciparum* infected RBCs

Dipotassium hydrogenphosphate	1 M
Potassium dihydrogenphosphate	1 M

Potassium phosphate buffer of pH 7.2 0.1 M was prepared according to Sambrook *et al.* [733]. Giemsa staining solution was freshly prepared from the purchased Giemsa staining solution by dilution 1:10 with 0.1 M potassium phosphate buffer pH 7.2, filtered over a paper filter.

FIELD'S A is a buffered solution of azure dye	(g/l)
Methylene blue	1.6
Azure	11
Disodium hydrogenphosphate anhydrous	2.6
Potassium dihydrogenphosphate	2.6

All substances were put in a flask in a warm water bath for 30 min, left for 24 h at RT, and then filtered over a paper filter.

FIELD'S B is a buffered solution of eosin	(g/l)
Eosin Y	2
Disodium hydrogen phosphate anhydrous	2.6
Potassium dihydrogen phosphate	2.6

The solution was filtered after dissolving in water.

Field's B was diluted 1 in 4 with distilled water just before use.

2.1.5.4 Solutions for *P. falciparum in vitro* growth assays

All solutions were sterile filtered (through 0.22 µm pore size) and added to the supplemented RPMI1640 media. Changes in media concentration resulting from the addition of solutions were tolerated up to a concentration loss of 10%. In cases where the addition of the stock solution to the RPMI medium would have diluted the supplemented RPMI medium by more than 10%, the relevant chemical was added directly to the supplemented medium, which was then sterile-filtered again.

For the test solutions the different stock solutions or chemicals were diluted with sterile distilled water to obtain the final concentrations shown below.

RPMI 1640 medium	**(mg / l)**
INORGANIC SALTS	
$Ca(N0_3)_2 * 4\ H_20$	100
KCl	400
$MgSO_4 * 7\ H_20$	100
NaCl	6000
$NaHCO_3$	2000
Na_2HPO_4 (anhydrous)	800

OTHER COMPONENTS	
D- Glucose	2000
Gluthathion (reduced)	1
Phenol red	5
AMINO ACIDS	
L-Arginin * HCl	240
L-Asparagin (free base)	50
L-Aspartate	20
L-Cystin	50
L-Glutamate	20
Glycin	10
L-Histidin (free base)	15
L-Hydroxyprolin	20
L-Isoleucin	50
L-Leucin	50
L-Lysin * HCl	40
L-Methionin	15
L-Phenylalanin	15
L-Prolin	20
L-Serin	30
L-Threonin	20
L-Tryptophan	5
L-Tyrosin	20
L-Valin	20
VITAMINS	
D-Biotin	0.2
D-Ca-Panthothenate	0.25
Cholinchloride	3
Folic acid	1
i-Inositol	35
Nicotinamid	1
Para-Aminobenzoic acid	1
Pyridoxal * HCl	1
Riboflavin	0.2

Thiamin* HCl	1
Vitamin B_{12}	0.005

Stock solutions (1 M):

$CaCl_2$

KCl

NaCl

Na-gluconate

NMDG-Cl titrated with HCl to pH 7.4

D (+) glucose

HEPES (titrated with TRIS (Trizma base) to pH 7.4 or titrated with NaOH to pH 7.4)

Raffinose

Urea

C6-ceramide (D-erythro-N-hexanoylsphingosine) was dissolved in dimethylsulfoxid (DMSO) to give a 50 mM stock solution that was stored at -20°C.

Synchronization solution	(in mM)
Sorbitol isosmotic (5%)	290
HEPES / NaOH pH 7.4	10
Glucose	5

When erythrocytes were to be used further, *e.g.* for western blots (membrane proteins) or for measuring $^{45}Ca^{2+}$ uptake, the 5% sorbitol solution was supplemented with HEPES and glucose. This was done to prevent infected RBCs from being stressed or oxidized through glucose depletion.

Ca- Ringer	(in mM)
NaCl	140
KCl	5
HEPES	10
MgCl2	1
CaCl2	1
Glucose	5

For **Hyperosmolar Ca-Ringer** 650 mM sucrose was added.

NaCl test solution	(in mM)
NaCl	120
HEPES / TRIS (titrated with TRIS to pH 7.4)	30
KCl	5
Glucose	5
$CaCl_2$	1.7

NMDG test solution	(in mM)
NMDG (titrated with HCl to pH 7.4)	140
HEPES / TRIS (titrated with TRIS to pH 7.4)	30
KCl	5
Glucose	5
$CaCl_2$	1.7

KCl test solution	(in mM)
KCl	120
HEPES / TRIS (titrated with TRIS to pH 7.4)	30
Glucose	5
$CaCl_2$	1.7

Na-gluconate test solution	(in mM)
Na-gluconate	120
HEPES / TRIS (titrated with TRIS to pH 7.4)	30
KCl	5
Glucose	5
$CaCl_2$	1.7

Raffinose test solution	(in mM)
Raffinose	280
HEPES / TRIS (titrated with TRIS to pH 7.4)	30
KCl	5
Glucose	5
$CaCl_2$	1.7

2.1.5.5 Solutions for fluorescence assisted cell sorting (FACS) analysis

Annexin binding buffer	(in mM)
NaCl	140
HEPES / NaOH pH 7.4	10
CaCl2	5

PBS	(in mM)
NaCl	140
PO_4 Buffer pH 7.4	10
KCl	3

Each tablet of 5 g PBS had to be dissolved in 500 ml distilled water, giving a final pH 7.45.

Syto16 was diluted before use in a dark environment in PBS or annexin binding buffer to a final concentration of 10 nM - 1 µM.

***t*-butylhydroperoxide** (~ 8 M) was diluted in distilled water to a final concentration of 1 mM just before use.

Modified NaCl test solution	(in mM)
NaCl	125
KCl	5
D (+) glucose	5
$CaCl_2$	1
$MgSO_4$	1
HEPES / NaOH, pH 7.4	32

Sphingomyelinase (50 U) was diluted with 140 µl sterile distilled water for use. 0.2 Units of sphingomyelinase were applied for the positive control to detect ceramide in the outer RBC membrane leaflet by a monoclonal antibody to ceramide.

2.1.5.6 Solution for PCR and agarose gel electrophoresis

dNTP Mix	(in µl)
100 mM stock solution	20
Distilled H_2O	720

TAE (TRIS-acetate-EDTA) buffer was prepared according to Sambrook *et al.* [733].

TAE (TRIS-acetate) buffer 50 x stock solution (for 1 L, pH 8.5)

TRIS (Trizma) base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

A concentrated (50 x) stock solution of TAE was made by weighing out 242 g TRIS base (MW: 121.14 g/mol) and dissolving it in approximately 750 mL distilled water. Carefully 57.1 ml glacial acid acid and 100 mL of 0.5 M EDTA (pH 8.0) was added and the solution was adjusted to a final volume of 1 L. This stock solution was stored at RT. The pH of this buffer was not adjusted and was about 8.5.

TAE buffer working solution	(in mM)
TRIS-acetate	40
EDTA	1

2.1.5.7 Solutions for patch-clamp experiments

Standard bath NaCl solution	(in mM)
NaCl	115
$MgCl_2$	10
$CaCl_2$	5
HEPES / NaOH (pH 7.4)	20

Na- gluconate bath solution	(in mM)
Na-gluconate	140
HEPES / NaOH, pH 7.4	10
$CaCl_2$	1
$MgCl_2$ (pH 7.4)	1

Standard pipette solution	(in mM)
K-gluconate	140
NaCl	10
EGTA	1
Mg-ATP	1
HEPES / NaOH (pH 7.4)	5

Ca-(gluconate)$_2$ pipette solution	(in mM)
Ca-(gluconate)$_2$	95
MgATP	1
HEPES / NaOH (pH 7.4)	10

EIPA Stock solution (in DMSO)	100 mM
Final concentration for use:	1 µM

2.1.5.8 Solutions for DAG Biotrak enzyme assay

2.1.5.8.1 Standard preparation

Preparation of stock solutions, standards as well as the assay itself were performed in Multi Twist Top Vials 1.7 ml, 10 x 45 mm polypropylene microtubes with screw caps with a rubber seal to prevent evaporation.

1-Stearoyl-2-arachidonoyl-*sn*-glycerol (DAG), a clear oil with the (total molecular) formula $C_{41}H_{72}O_5$ (MW: 645 g/mol), was dissolved in ethanol to give a 1 mM stock solution. This mixture was again diluted 1:10.

DAG stock solution (1 µmol/ml = 1 mM)	
DAG (15.5 µmol)	10 mg
Ethanol 100 %	1.55 ml

C16 – ceramide (D-erythro-N-palmitoylsphingosine), a white amorphous to crystalline solid, $C_{34}H_{67}NO_3$ (MW: 519,751 g/mol) was dissolved in 37°C warm ethanol to give a 1 mM stock solution. This mixture was again diluted 1:10.

C16 - ceramide stock solution (1 µmol/ml = 1 mM)

C16 - ceramide (9.62 µmol)	5 mg
Ethanol 100 %	962 µl

The stock solutions were stored in Multi Twist Top Vials 1.7 ml at -20°C for maximally two months and brought to RT just before use.

Standards (pmol)	**Dilution**
0	
123	1:810
370	1:270
1111	1:90
3333	1:30
10000	1:10

Chloroform was pipetted with Hamilton syringes into Multi Twist Top Vials 1.7 ml. For the 10000 pmol standard, 100 µl from a 1:10 dilution of the stock solution was transferred to a tube. The rest of the 1:10 dilution was used for a 1:3 dilution series (150 µl chloroform + 75 µl of each dilution) to give the other standards (100 µl of each dilution). 100 µl pure chloroform were taken for the 0 pmol ceramide standard.

2.1.5.8.2 Extraction and separation of lipids

NaCl Ringer	(in mM)
NaCl	125
$MgSO_4$	1.2
HEPES	32.2
Glucose	5
$CaCl_2$	1

The solution was adjusted to pH 7.4 with NaOH.

Methanolic HCl	0.1 N

2.1.5.8.3 Enzymatic reaction

Detergent solution (Final volume: 10 ml, 240 µl aliquots, storage at -20°C)

- cardiolipin (biphosphatidylglycerol), dissolved in ethanol at 4.8 mg/ml, MW = 1467 g/mol, (C9 +(4*C18)+(13*O)+(4xO)+ 2P+(4*[(2H)*16 +3H])+19
- *n*-octylglucopyranoside, MW = 292,38 g/mol, ($C_{14}H_{28}O_6$)
- DETAPAC (Diethylene triamine pentaacetic acid), MW = 393,35 g/mol, ($C_{14}H_{23}N_3O_{10}$), chelating agent

Cardiolipin (15.28 ml)	5 mM
DETAPAC (3.9335 mg)	1 mM
n-octylglucopyranoside	7.5 %

Cardiolipin was transferred to a 15 ml Falcon Tube and dried by a nitrogen gas stream. The other components were added. The volume was adjusted to 10 ml. Cardiolipin was dissolved by ultrasound.

***sn*-1,2-diacylglycerol kinase** (261.8 µl aliquots, storage at -80°C)

sn-1,2-diacylglycerol kinase was reconstituted in 238 µl sterile distilled water to restore the pre-lyophilization solution:

	(in mM)
NaCl	300
Imidazole	250
Sodium phosphate buffer, pH 7.5, containing 1 % n-decyl-ß-D-maltopyranoside.	50

Assay buffer (20 ml sterile filtered, storage at 4°C)

	(in mM)
Imidazole (1,3- Diazo-2,4-cyclopentadiene)	100
NaCl	100
$MgCl_2$	25
EGTA	2

The solution was adjusted with HCl to pH 6.6, then brought to the final volume.

DTT (20 mM, 120 µl aliquots, storage at -20°C)

DL-DTT was dissolved on ice and directly deep-frozen.

Buffer for cold tracer solution	(in mM)
Imidazole	100
DETAPAC	1

The buffer was adjusted to pH 6.6 with HCl and to 4/5 of the final volume.

Cold tracer solution (5 mM ATP, pH 6.6, 10 ml, 110 µl aliquots, storage at -20°C)

ATP disodium salt	5 mM

The buffer for cold tracer solution was added. The pH was checked before adding dH_20 to obtain the final volume.

Chloroform: methanol: 1 N HCl (100:100:1)

50 ml to stop the reaction, 50 ml to rinse in glass flasks

Buffered saline solution (100 ml, sterile filtered, storage at 4°C)

	(in mM)
NaCl	135
$CaCl_2$	1.5
$MgCl_2$	0.5
Glucose	5.6
HEPES	10

The pH was adjusted to 7.2, the volume to 100 ml.

EDTA-solution (100 mM, 10 ml, sterile filtered, storage at 4°C)

Reaction mix (770 µl)	(in µl)
sn-1,2-diacylglycerol kinase	262
Assay buffer	634
DTT 20 mM (final concentration: 2.8 mM)	110

Radioactive tracer solution (final volume 110 µl, freshly prepared before use)

Cold ATP 5 mM (around 100µl)

10 µCi [^{32}P]γ-ATP was added (10 µl at reference date) for the mice experiments,

20 µCi for human RBCs. The volume was adjusted according to the specific activity

of the relevant [^{32}P]γ-ATP solution as determined by the ladders shown in Fig. 2.1. The lower the specific activity, the more volume was added.

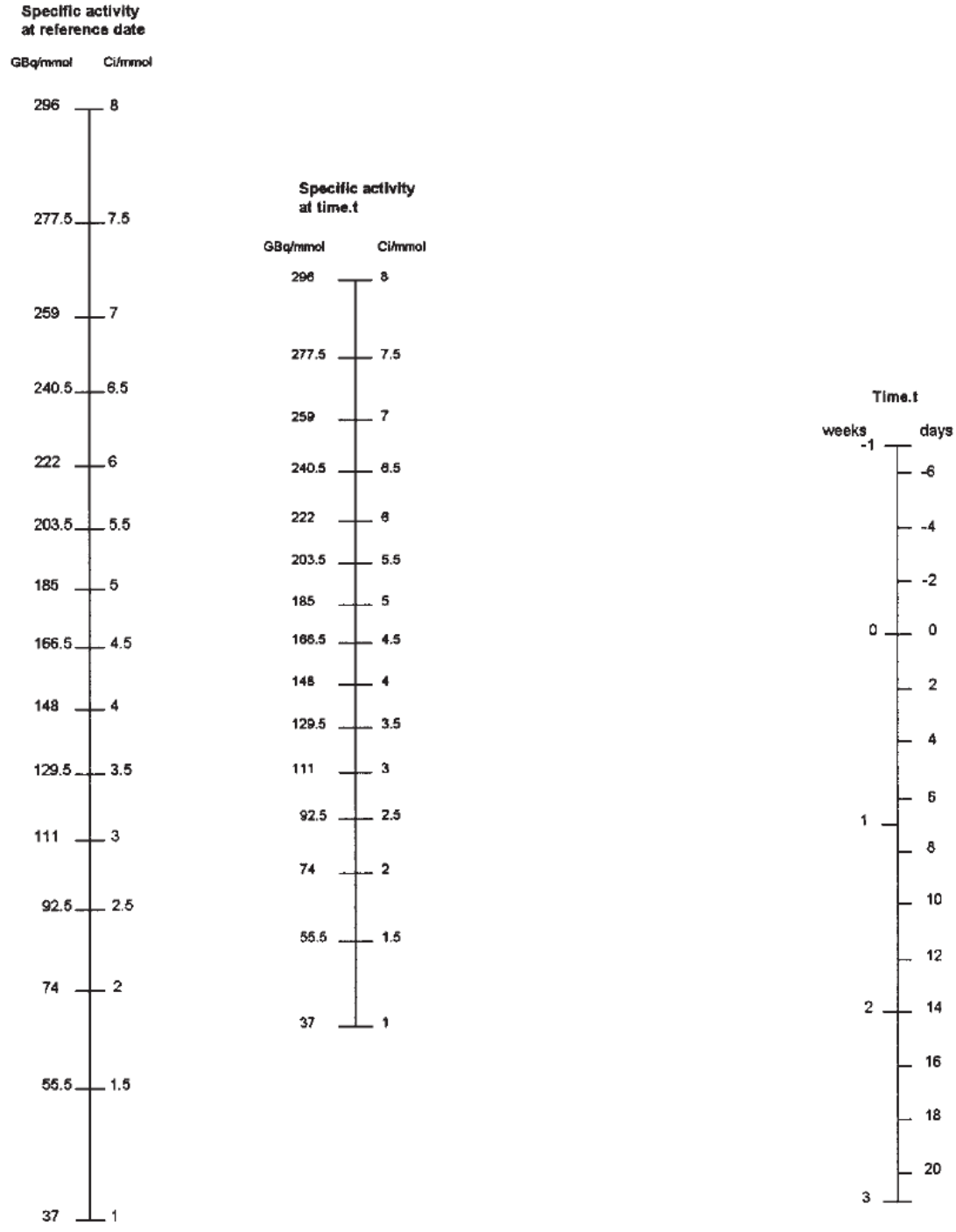


Figure 2.1. Specific activity of [^{32}P] γ-ATP.

A ruler was used to determine the specific activity of the [^{32}P]γ-ATP solution.

Chloroform: methanol (1:1)

50 ml for the assay, 50 ml to rinse in glass flasks.

2.1.5.8.4 Thin-layer chromatography

Running buffer: Chloroform : methanol : acetic acid (65:15:5)

2.1.5.9 Solutions for $^{45}Ca^{2+}$ flux experiments

6% Trichloroacetic acid (TCA)

EGTA-free KCl/NaCl solution (1L)	(in mM)
KCl	80
NaCl	70
Na-HEPES (HEPES titrated with NaOH)	10
Glucose	5
$MgCl_2$	0.2

KCl / NaCl solution (100 ml)	(in mM)
KCl	80
NaCl	70
Na-HEPES	10
Glucose	5
$MgCl_2$	0.2
EGTA	0.1

Supplemented KCl / NaCl solution (500 ml)	(in mM)
KCl	80
NaCl	70
Na-HEPES	10
Glucose	5
$MgCl_2$	0.2
Inosine	10
Na-Vanadate	1

For the KCl / NaCl solution, EGTA was added to 100 ml of EGTA-free KCl/NaCl solution. For the supplemented KCl / NaCl solution 10 mM inosine, 1mM Na-vanadate was added to 500 ml EGTA-free KCl / NaCl solution.

2.1.6 Software

CellQuest Version 9 and 10	BectonDickinson, Heidelberg
InStat statistic program	GraphPad Software, San Diego, California, USA
Pulse software	HEKA, Lambrecht

2.2 Methods

2.2.1 Preparation of human erythrocytes

Banked erythrocyte concentrate from the institute of transfusion medicine (in a 280 ± 28 ml pocket) was prepared as follows: 500 ml blood was taken from volunteers, and directly transferred to a pocket containing 70 ml citrate-phosphate-dextrose-stabilizer. The blood in the stabilizer was left for 3 h at RT, centrifuged for 10 min at RT at 4950 g with a special centrifuge, thus transferring the different components of the blood to different pockets. Plasma was obtained in one pocket and erythrocyte concentrate in another. The erythrocyte concentrate was conserved in 110 ml SAGM. The buffy coat was nearly completely retained by a filter. As storage life increases with leukodepletion [134], the erythrocyte concentrate stored at 4°C could be used for 42 days (expiry date) and another 14 days for parasite culture [428].

Blood was obtained from volunteers with their informed consent, collected in EDTA – tubes and left at RT for 3 h post collection to avoid contamination, as white cells will actively remove infectious material during the first 3 hours of incubation. Then the plasma was removed. The blood cells were washed three times in a sterile saline solution [253] (hematocrit 25%). After each centrifugation step (450 g, 5 - 8 min at RT), the buffy coat and upper 10-20% of the red blood cells (RBCs) were removed with a transfer pipette. The washed, leukocyte-depleted RBC concentrates were stored in SAGM solution (ratio: 5.5 ml SAGM / 10 ml erythrocyte concentrate) for a maximum of 35 days at 4 °C according to official guidelines [564]. One day before use, RBCs were washed once in original RPMI 1640-medium, to which was added sterile HEPES solution (final concentration: 20 mM) as necessary, depending on the pH. Blood from sickle cell trait patients and their controls was further genotyped (as described below). All experiments were performed under a class II safety cabinet.

2.2.2 *In vitro* culture of *P. falciparum* infected human erythrocytes

Plasmodium falciparum FCR3 [429] and BINH [104] strains were cultured according to the modified method of Trager and Jensen [841,842,840]. Cultures were maintained by routine passage in fresh or banked human erythrocytes (1-2 % hematocrit), diluting the RBCs to a parasitemia of around 0.5 % and growing them up to a parasitemia of around 10 % in supplemented RPMI 1640 medium at 37°C in an atmosphere of 90% N_2 / 5% O_2 / 5% CO_2 or in an exsiccator according to the candle jar method [428]. Only for some experiments *P. falciparum* was grown separately to higher parasitemias. Although the medium was additionally supplemented with 2% of heat-inactivated human serum during the first part of the experiments, this was found unnecessary for maintaining parasite growth consistent [203]. Therefore, all further experiments were performed with 0.5 % Albumax only. This provided batch-to-batch consistency, eliminated the requirement of blood group (ABO) compatibility between RBCs and the serum,, and reduced exposure to a potentially biohazardous blood product.

The parasitemia was determined by FACS analysis with Syto16, by Giemsa or Field´s rapid staining, respectively. Syto16 is a DNA/RNA binding dye; Giemsa or Field´s rapid stain the parasites in methanol fixed blood smear slides.

The dilution factors (Df) for splitting the infected RBCs suspension in order to maintain the *P. falciparum in vitro* culture were calculated as follows:

$Df = mP * V_{(RBC)} / dP * dV_{(RBC)}$

Fresh blood was added according to the following formula: $dV_{(RBC)} - (V_{(RBC)})/ Df$

mP: measured parasitemia; $V_{(RBC)}$: volume of packed erythrocytes in the culture flask; fP: desired parasitemia; $fV_{(RBC)}$: desired volume of packed erythrocytes in the flask

The volume of the medium was adjusted according to the degree of parasitemia and the time until the next medium change [557,742]. Parasite culture was regularly tested for *Mycoplasma* contamination (as described below).

2.2.2.1 Serum preparation from plasma

The plasma was split in 45 ml aliquots in 50 ml falcon tubes and heat inactivated for 45 min at 56°C. To each 45 ml aliquot 800 µl sterile filtered 1M $CaCl_2$ solution was added. This mixture was incubated at 37°C for at least 30 min and then at 4°C overnight (ON) and subsequently centrifuged for 30 min / 4°C at 600 g. The supernatant (serum) was kept, while the white pellet discarded. If the supernatant was not clear, the liquid was centrifuged again for another 30 min.

2.2.2.2 Determination of parasitemia

2.2.2.2.1 Syto16 staining

RBCs were incubated with Syto16, diluted in PBS or annexin binding buffer (~20 nM to 1 µM) at 37°C. The staining procedure was performed for around 30 – 40 min for infected human RBCs (20 - 30 nM) and for around 1 h (1 µM) for mice RBCs. Syto16 green fluorescent nucleic acid stain bound to DNA has a maximum excitation/absorption wavelength of 488 nm, which corresponds to the argon line of the single-laser of the FACS Calibur used, and a maximum emission wavelength of 518 nm. Bound to RNA the absorption maximum is at 494 nm, the emission maximum at 525 nm. This green emission is detected in the Fluorescence 1 (FL-1) channel with a detector for an emission wavelength of 530 ± 15 nm. FACS analysis proved a more sensitive technique for determining parasitemia than either Giemsa or Field´s rapid staining: 1% parasitemia counted through a light microscope corresponded to approximately 2 % parasitemia in FACS analysis. However, Giemsa and Field´s rapid staining remained useful for distinguishing parasite developmental stages.

2.2.2.2.2 Giemsa stain for thin films

A drop of the parasite culture was transferred to an object slide with a transfer pipette. With the help of a second object slide a thin blood smear was obtained. This smear was immediately dried by the air-flow of a hair-drier to avoid echinocytes (crenated RBCs) developing or parasites leaving RBCs before being dried. The blood smear was fixed in methanol for 1 minute and air-dried. The object slide with the blood smears were flooded with freshly prepared Giemsa staining solution for 25 – 30 minutes, rinsed under tap water to float off the stain and to prevent deposition of the precipitate on the film. The slides were vertically air-dried, and then examined using the 100 x objective of a Leica CME microscope with immersion oil.

2.2.2.2.3 Field´s rapid stain

Field’s rapid stain, a modification of the original Field’s stain for thick blood films, permits rapid staining of fixed thin films, making it a good choice for quick analyses. This method is suitable for examining malaria parasites, *Babesia spp*., *Borrelia spp*. and *Leishmania spp*. The air-dried, methanol-fixed films were flooded with 1 ml of 1: 4 diluted

Field's stain solution B. Then an equal volume of undiluted Field's stain solution A was added immediately. After 1 min the slide was well rinsed under tap water, air-dried, and examined with a light microscope (100 x objective, oil immersion, and 1000 x magnification). This method is useful for rapid presumptive species identification, showing adequate staining of all stages of malarial parasites including stippling (mainly Maurer's clefts).

Nevertheless, staining with Giemsa solution remains the method of choice for definitive species differentiation. As a means of distinguishing parasite stages Giemsa staining provided better visibility of parasites inside the erythrocytes than did Field's rapid staining.

2.2.2.3 Freezing parasites

From time to time samples with high, *i.e.* ≥ 20 % parasitemia, predominantly containing ring stages were deep frozen in liquid nitrogen for stock purposes. The cell pellet obtained after centrifugation (450g / 8 min at RT) was mixed with an equal volume of freezing solution, sterile transferred to a 2.0 ml CryoTube Vial and directly deep frozen in liquid nitrogen.

2.2.2.4 Defreezing parasites

Four cryotubes containing infected RBCs (> 20 % parasitemia) were thawed quickly at 37°C in a water bath. The contents were transferred to a 15 ml Falcon Tube and centrifuged (450g / 4 min at RT). The supernatant was discarded. A volume of sterile filtered 3.5 % NaCl solution equal to $V_{(RBC)}$ was added at a rate of 1 - 2 drops per second while gently shaking the tube. Finally the liquid was mixed with a pipette. After centrifugation at 450g for 4 min the supernatant was discarded. The RBC pellet was carefully suspended in original RPMI 1640 medium ($2*V_{(RBC)}$). Washing was repeated until the supernatant became clear. The remaining cell pellet was resuspended in complete medium and transferred to a culture flask. Fresh RBCs were added (amount depending on $V_{(RBC)}$). The culture flask was filled with 90% N_2 / 5% O_2 / 5% CO_2.

2.2.2.5 Mycoplasma detection in *P. falciparum* infected erythrocytes by PCR

DNA was prepared from 5-10 million cells (~ 200 µl blood sample) using the QIA Amp DNA Mini Kit. DNA was eluted in 200 µl buffer AE. 1 µl from the elution solution was

used for the PCR, carried out in a final volume of 50 µl. Primers P1 and P4 were designed according to Spaepen *et al.* [787].

PCR mixture	(µl)
DNA	1
10x PCR buffer	5
dNTP mix	8
Primer Myco-P1 (~ 300 ng/µl)	1
Primer Myco-P4 (~ 300 ng/µl)	1
Taq Pol 1 U	0.2
dH_2O	33

PCR conditions for mycoplasma detection

24 cycles	min	°C
Denaturing:	1	95
Annealing:	1	60
Extension:	1.5	72

Controls

Each DNA with GAPDH-Primer

Positive DNA control

Negative DNA control

Master Mix without DNA (only dH_2O)

15 µl of the PCR was run on 1 % agarose gel. A DNA fragment of around 900 bp was considered positive. Alternatively, the Mycoplasma Plus™ PCR Primer Set was used following the manufacturer's instructions. In brief, an aliquot of the medium was boiled and cleaned using StrataClean™ resin. The extract was tested in a PCR.

2.2.3 Patch-clamp recording in human erythrocytes

The patch clamp technique was developed by Sakman and Neher almost 30 years ago [633,370,728]. It allows to measure transmembrane currents and voltages in voltage-clamp and current-clamp mode, respectively, by the use of a single glass electrode connected to an

extracellular reference electrode. Fig. 2.2 provides a brief introduction to the technique of whole-cell voltage-clamp recording in human RBCs, which was were performed in the present study as has been described by Huber *et al.* and Duranton *et al.* [243,410].

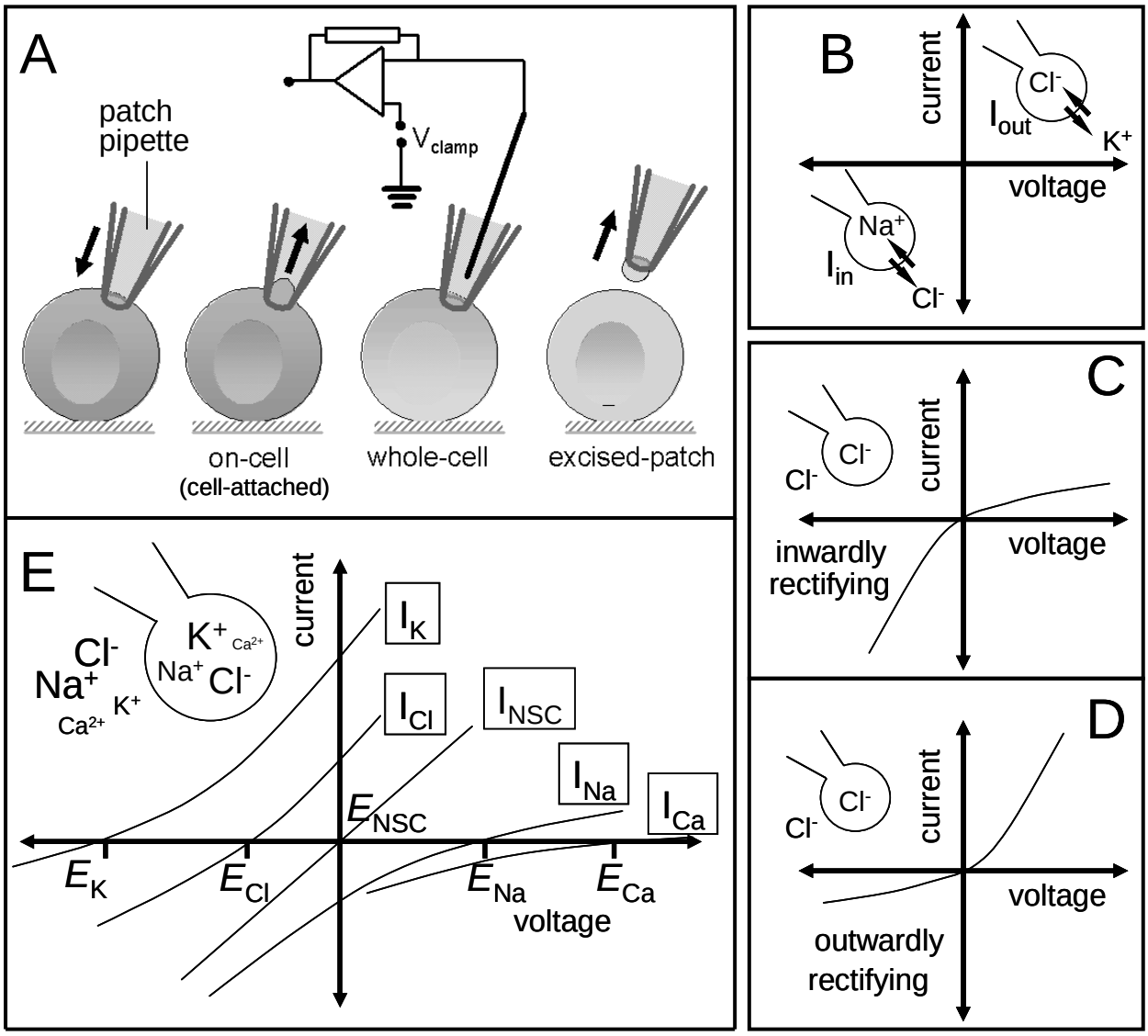


Figure 2.2. Whole-cell voltage-clamp recording in human RBCs.

(**A**) Recording modes. To obtain a gigaΩ seal resistance between the pipette and the bath solution, the tip of the recording pipette is impressed on the RBC surface (outer left). Application of negative pressure to the pipette lumen aspirates the RBC membrane into the pipette tip. Under these conditions a a gigaΩ seal occasionally develops. This on-cell (cell-attached) mode records in intact cells the activity of individual channels residing in the aspirated patch (middle left). Further negative pressure or current injection disrupts the aspirated membrane, thus restoring the whole RBC membrane to its initial electrical conductance. Simultaneously, the RBC cytosol is dialyzed with the pipette solution. In whole-cell mode, recordings are made of macroscopic ion channel activity, *i.e.* macrocopic currents, over the whole RBC membrane (and at high input resistances of the activity of individual channels). Here the intracellular and extracellular ion concentrations are defined by the pipette and bath solutions, respectively (middle right). Withdrawal of the pipette excises a membrane patch from the cell surface. Inside-out patches are obtained when the excision starts from on-cell mode, and outside-out patches

when starting from whole-cell mode (outer right).

(**B**) Convention: The applied voltages refer to the electric potential between the cytoplasmic face of the membrane and the extracellular space. The inward currents (I_{in}), defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces. Consequently, flow of Cl^- from the bath solution into the cell generates an outward current (I_{out}).

(**C**, **D**) Rectification: Some channel types do not show a linear current-voltage relationship with varying voltage when recorded at equal concentrations of the membrane-permeating ion species on either side. This might be due to various voltage-dependent phenomena: a blockade by physiological ions, a change in unitary conductance or a variation in open probability. As a result, the current-voltage relationship is either inwardly (**C**) or outwardly rectifying (**D**).

(**E**) Selectivity. In the whole-cell or excised patch modes, the compositions of the pipette and bath solutions together determine the Nernst equilibrium potential for each ion species. In the present example, the equilibrium potentials for K^+, Cl^-, nonselective cations (NSC^+), Na^+ and Ca^{2+} are set to different voltages (intracellular and extracellular ion concentrations are indicated by the letter size). In the current-voltage diagram (I) an ion-selective current can be identified by the fact that its value is zero at the ion-specific reversal potential for the used solutions [407].

Briefly, patch pipettes (8 to 12 MΩ) made of borosilicate glass were pulled using a horizontal DMZ puller. Pipettes were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier. Data acquisition and data analysis were performed using a computer equipped with an ITC 16 interface with Pulse software. For current measurements, cells were held at a holding potential (V_h) of –30 mV and 400 ms pulses from –100 to +80 mV were applied in increments of +20 mV. Whole-cell currents were recorded at RT with morphologically intact late trophozoite-stage-infected erythrocytes selected optically as described previously [245,410]. 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 in increments of +20 mV. In the original current traces the individual current sweeps are superimposed and are depicted without filtering (acquisition frequency of 5 kHz). Currents were analyzed by averaging the values between 350 and 375 ms of each square pulse. The conductance was estimated by linear regression for outward currents from +40 to +80 mV.

Voltages refer to the cytosolic membrane side in respect to the earthed extracellular side. Outward currents, 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. Inward currents are defined as flow of positive charge from the extracellular to the cytoplasmic membrane face. The liquid junction potentials between bath and pipette solutions

and between bath solution and salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch [76]. Data were corrected for liquid junction potentials.

The standard bath NaCl solution and the standard pipette solution were used for control whole-cell recording. Some experiments were performed with a Ca-$(gluconate)_2$ pipette solution. After attaining whole-cell mode, cells were superfused (21°C) with Na-gluconate bath solution.

2.2.4 *In vitro P. falciparum* growth assays

Synchronization to ring-stage was achieved by incubation of the infected RBC in isosmotic sorbitol solution (5 % w/v, *i.e.* 290 mM) for 15 min at RT [495,401]. The RBCs were then washed twice with original RPMI 1640 medium, and the supernatant was discarded. Ring stage-synchronized infected RBCs were aliquoted in 96-well plates (200 µl aliquots, 1 % hematocrit, 1% parasitemia) and grown for 48 h with the candle-jar technique [428] in the absence (control) or presence of different chemicals in concentrations as described in the results. Chemicals in complete culture medium were prepared double-concentrated, and then mixed with the same volume of synchronized parasite culture of 1 % parasitemia and 2 % hematocrit to give a final hematocrit of 1% and 1% parasitemia.

Partial O_2 pressure was determined with a dissolved Oxygen Meter calibrated at 20.9% partial O_2 pressure (air). This corresponds to 4.7 mg/l oxygen dissolved in distilled water. At flame extinction partial O_2 pressure was measured as 5.4 % (1.2 mg/l dissolved oxygen in distilled water), which corresponds to a partial oxygen pressure of around 35 mm Hg, found in venous blood. These conditions provided a culture environment that will not induce sickling in heterozygotes HbA/S RBCs, as this occurs only below 15 mm Hg [31,377] and under nutrient deprivation. Accordingly sickling could not be observed (data not shown).

In all *P. falciparum in vitro* re-invasion experiments (with normal or sickle trait RBCs), if not stated otherwise, parasites were grown for 48 h in culture medium under culturing conditions. Parasitemia was assessed by Syto16 staining at time 0 h, *i.e.* just as the experiment had been started, and after 48h of growth at 37°C. After 48 h a 6 µl aliquot (1 % hematocrit) of the well-mixed cell suspension was stained in 44 µl of Syto16 solution for 30 min at 37°C, giving a final concentration of Syto16 of 30 nM in PBS. Prior to FACS analysis the samples were diluted 1: 5 with PBS. Parasitemia was defined by the percentage of RBCs stained with Syto16. Additionally, the percentages of annexin-binding cells were determined by double-staining (see below) in several growth assays.

The *in vitro* reinvasion by *P. falciparum* of RBCs kept in culture medium, test solutions with different electrolyte compositions, in supplemented RPMI medium containing the cation channel inhibitor EIPA, or $CaCl_2$ ± the chelator EGTA was studied. For this purpose washed ring-stage synchronized infected RBCs were grown for 48 h to a parasitemia of about 5 - 10% (starting with a parasitemia of around 1 %). Only growth assays in which parasites multiplied at least 5 fold were considered. EIPA in concentrations varying from 0 to 100 µM, or EGTA in a concentration of 1 or 2.5 mM was added to the culture medium at time 0 h. $CaCl_2$ (1 or 2.5 mM, respectively) was added together with EGTA (1 or 2.5 mM, respectively) to control for direct EGTA effects. The concentrations of free Ca^{2+} were calculated according to Fabiato *et al.* [266].

Other *P. falciparum in vitro growth* experiments were performed in the presence of C6-ceramide (D-erythro-N-hexanoylsphingosine) (0 – 20 µM), in the presence of 2,4 - dichloroisocoumarin (DC) ± NaCl (50 mM) and varied concentrations of NaCl (0 - 100 mM), raffinose (0 - 200 mM) and urea (0 - 200 mM). Additional parasite growth experiments tested the dependence of growth on extracellular Na^+, K^+ and Cl^- concentrations. After 24 h of synchronized culture, when most of the parasites were in the trophozoite stage, cells were spun down and the culture medium was washed twice and replaced by isosmotic NaCl, NMDG-Cl, Na-gluconate or raffinose test solution or mixtures of these solutions. The test contained undiluted 120 mM NaCl, 140 NMDG titrated with HCl to pH 7.4, 120 Na-gluconate or 240 raffinose and in addition [in mM]: 30 Hepes, 5 glucose, and 1.7 $CaCl_2$ (5 KCl in the case of NMDG test solution). After 4 h, 8 h, or 16 h of incubation in these solutions, cells were spun down, freed from the test solution, washed with culture medium and further incubated for 20 h, 16 h, and 8 h, respectively, under normal culture conditions (the total incubation time was 48 h). Parasitemia was assessed as described above. Where not indicated otherwise, experiments were performed with 8h medium replacement by the test solution.

2.2.4.1 Determination of free concentration of EIPA in parasite growth medium

To determine the free EIPA concentration in parasite growth medium, EIPA was prepared at 0, 1, 10 and 100 µM concentration in pure distilled water or in distilled water with the same concentrations of ALBUMAX II (0.5%) and serum (2%) as used for the parasite culture medium. The latter solutions were ultracentrifuged at 1942 g at 4°C for 40 min using Microcentrifuge Filters Ultrafree-MC, which bind proteins, to determine the free

concentrations of EIPA. The absorption spectrum of EIPA was determined with 100 µM EIPA, dissolved in distilled water, over a range from 200 nm to 700 nm using a UV-VIS Scanning Spectrophotometer. Maximum absorption wavelength was at 370 nm. To obtain a standard curve the absorption at of different EIPA concentrations in distilled water was determined. Then the absorption of the used EIPA concentrations (1, 10, 100 µM) in the ALBUMAX/serum/water mixture were measured at 370 nm. Finally, the free EIPA concentration was calculated from the standard curve.

2.2.4.2 Annexin binding experiments

Suspensions with non-infected RBCs were stained with annexin V-FLUOS. Suspensions with *P. falciparum* infected RBCs were stained with annexin V-568 and/or with the DNA dye Syto16 to assess PS exposure in the outer leaflet of the RBC membrane and the percentage of infected RBCs, respectively.

For annexin binding, RBCs were washed, resuspended in annexin-binding buffer, stained with annexin V-568 (dilution 1:50) or annexin V-FLUOS (dilution 1:100), and incubated for 20 min at RT in Ca^{2+}-free annexin binding buffer. AnnexinV binding to negatively charged phospholipids (with a high specificity for PS) is Ca^{2+}-dependent [44,92,151,321,753,876]. Therefore an annexin binding buffer containing Ca^{2+} was used. Syto16 (final concentration of 30 nM) was co-incubated in the annexin binding buffer for 20 min at RT for double-staining purposes. Samples were diluted 1:5 with annexin binding buffer just before FACS analysis. Cells were analyzed by flow cytometry. Fluorescence 1, FL1 (detector for an emission wavelength of 530 ± 15 nm) was either an indicator of annexin V-FLUOS fluorescence intensity (excitation: 450 - 500 nm, emission: 515 - 565 nm) (simple staining for annexin); or of Syto16 fluorescence (maximum excitation wavelength: 488 nm for DNA, 494 nm for RNA, maximum emission wavelength: 518 nm for DNA, 525 nm for RNA). Syto16 fluorescence was taken to reflect the degree of parasitemia. Fluorescence 2, FL2 (detector for an emission wavelength of 585 ± 21 nm) or FL1 was used to determine the annexin fluorescence of Annexin V-568 or Annexin Fluos, respectively, *i.e.* the percentage of PS exposing cells. Annexin V-568 has an absorption spectrum from 450-650 nm, a maximum at 578 nm, an emission spectrum from 560 nm up to > 700 nm with a maximum at 603 nm. Double staining necessitated compensation for overlapping emission spectra by electronic subtraction of any unwanted spectral “spillover” from the signal of interest. Simple stained infected erythrocytes (with Syto16 or annexin V-568) containing dye in the relevant

concentrations were used as compensation controls for this multicolor study.

To induce annexin binding according to Lang *et al.* [507], non-infected RBCs were incubated for 1 h at 37°C with the Ca^{2+} ionophore ionomycin (0 and 1 µM, respectively) or oxidized by *t*-butylhydroperoxide (*t*-BHP, 1 mM for 15 min followed by 24 h of post-incubation) in a modified NaCl test solution consisting of (in mM): 125 NaCl, 5 KCl, 5 D (+) glucose, 1 $CaCl_2$, 1 $MgSO_4$, 32 HEPES titrated to pH 7.4 with NaOH.

2.2.4.3 Detection of ceramide in the RBC outer membrane leaflet by an monoclonal antibody to ceramide

Ceramide is detected in brief as follows: A suspension with infected RBC ($1{,}1*10^6$ cells; 1 µl of packed RBCs contain ~$11*10^6$ erythrocytes) was incubated with a first monoclonal antibody to ceramide. After washing twice, the cells were incubated with a secondary rat anti-mouse IgM antibody, labelled with the fluorescent PerCP-Cy5.5. In a third step, after washing twice, the cells were stained with Syto16 (final concentration 30 nM).

PerCP-Cy5.5 is a tandem fluorochrome, composed of peridinin chlorophyll a protein (PerCP), a naturally occurring algal pigment that is excited by the 488-nm line of the FACS Calibur Argon ion laser (absorption maximum at 490 nm). This permits the use of conjugated PerCP as a third color on bench-top single-laser instruments. PerCP serves as energy donor in this procedure. It is coupled to the cyanine dye Cy 5.5™, which acts as energy acceptor and emits fluorescence at 695 nm (within the Fluorescence 3 (FL-3) channel of BD FACS Calibur (detector for an emission wavelength of 650-700 nm). The key advantages of using PerCP-Cy5.5-conjugated reagents are that they are useful for analysis on stream-in-air flow cytometers, do not photobleach like PerCP, and cause less Fc receptor-mediated non-specific staining than PE-Cy5 tandem fluorochromes. This secondary antibody was chosen to avoid spectral overlap with Syto16 fluorescence dye.

To test for the quality of the anti-ceramide antibody and to determine working concentrations, dilutions of 1:5 (Sigma) or 1:10 (Alexis) of the first antibody together with different concentrations of the secondary PerCP-Cy 5.5-linked rat anti-mouse IgM antibody were used (0.2, 0.4, 0.6, 0.8 µg/ml). A good result of anti-ceramide staining (procedure see below) was obtained by using the Sigma monoclonal antibody to ceramide and a concentration of 0.8 µg/ml of the secondary antibody.

In addition, appropriate compensation controls had to be prepared for this multicolor study. For positive control, RBCs with high parasitemia (around 15 %) of *P. falciparum* were incubated overnight (8 - 15 h) in a hyperosmolar Ca-Ringer-Solution (300 mOsm Ringer and

650 mOsm Sucrose). In addition, 200 µl infected and non-infected RBCs were treated with 0.2 U Sphingomyelinase for 2 - 5 min and washed thoroughly thereafter. For negative control, one sample of each NaCl concentration (0, 10, 25, 50 mM) was left untreated by the first antibody (instead 10 µl PBS was used). Then, the secondary antibody was added to test for unspecific binding. For isotype control, isotype matched pure mouse antibody (IgM) (1 µg/ml) was used as first antibody. To test for background Syto16 fluorescence and spectral overlap with PerCP-Cy 5.5 emission, each control was incubated with and without Syto16 (± Syto16) (30 nM final concentration).

Parasite growth assays were performed as described above with 0, 10, 25, 50 mM NaCl (n = 6), which was added to the culture medium. 10 µl *P. falciparum* infected (n = 3) RBCs ($V_{(RBC):}$ 0.1 µl, *i.e.* $1.1*10^6$ cells) were cooled down on ice. The samples were incubated for 45 - 60 min on ice with 30 µl ice-cold PBS containing 2 % human serum to block unspecific binding and 10 µl monoclonal antibody to ceramide (1 µg/ml from Sigma). Hereafter, cells were washed twice with ice cold 400 µl PBS, which contained 1 % human serum, and centrifuged at 225g at 4°C for 5 min. The supernatant was discarded. RBCs were incubated on ice with the secondary rat anti-mouse IgM PerCP-Cy 5.5-conjugated antibody (0.8 µg/ml, final volume: 50 µl) for 30-45 min. The washing steps were repeated twice. Syto16-staining was performed (30 nM at 37°C) in a final volume of 50 µl. 200 µl PBS was added prior to FACS analysis. The settings were adjusted according to negative and positive controls to permit differentiation between 4 populations within the 4 quadrants of a dot plot (± Syto16 (FL1) ± ceramide (FL3)).

2.2.5 In vivo proliferation of *Plasmodium berghei* ANKA

Male and female C57BL/6 mice (2-3 months old) with ASM knock-out (-/-) (10 female, 7 male) or ASM wild-type (+/+) (15 female, 5 male) genotype were infected by intraperitoneal injection of 10^6 *P. berghei* ANKA-infected mouse erythrocytes, which had been deep frozen in liquid nitrogen. From the seventh day post infection, parasitemia was estimated daily by Syto16 DNA-RNA staining as described above, neglecting the presence of white blood cells (1 µM final concentration, incubation at 37°C for 30 - 60 min).

2.2.5.1 Analysis of genomic mice DNA

DNA preparation from ear tissue or mouse tail was done using the DNA easy tissue kit. Briefly, DNA was eluted in 200 µl buffer. 5 µl of the elution was taken for the PCR, performed in a final volume 50 µl.

PCR mixture	(in µl)
Genomic DNA	5
10x PCR buffer (+ $MgSO_4$)	5
dNTP mix	8
Primer ASM - PS (100 ng/ µl)	1
Primer ASM - PA1 (100 ng/ µl)	1
Primer ASM - PA2 (100 ng/ µl)	1
Distilled H_2O	28
PWO polymerase (5 U/µl)	0.2

.

PCR conditions for mice genotyping		
30 cycles	min	°C
Denaturing:	1	95
Annealing:	1	58
Extension:	1	72

PCRs were carried out analogously with DNA of a wild-type, a heterozygote, a knockout mice and a negative control without any DNA. 10-15 µl of each PCR mixture was used for agarose (1.5 %) gel (5 µg/ml ethidium bromide) electrophoresis according to Sambrook *et al.* [733]. The size of the ASM wild-type DNA fragment was 269 bp, while that of the ASM knockout DNA was 523 bp; in the case of heterozygous mice both fragments appeared.

2.2.6 Genotyping of human blood samples

Genomic DNA was isolated using the QIAamp DNA (Blood) Mini Kit, following the supplier's instructions. Briefly, total DNA was purified from 200 µl of whole blood using

QIA Spin Columns fitted in 2 ml Eppendorf tubes. DNA bound to the QIAamp membrane was washed twice, and then eluted with elution buffer, following the supplier's instructions. DNA yield was determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. A_{260}/A_{280} ratio was 1.7 to 1.9, corresponding to pure DNA.

The HbS mutation occurs in one allele for HbA/S blood, in the 6th codon (GAG → GTG) after the start codon, leading to a substitution of glutamic acid by valine in the 6th position in the β-globin amino acid sequence (β-6). To confirm the absence or presence of the mutation, DNA extracted from blood of Hb A/A (7 individuals) and Hb A/S donors (5 individuals) were PCR amplified and sequenced. Total PCR volume was 25µl.

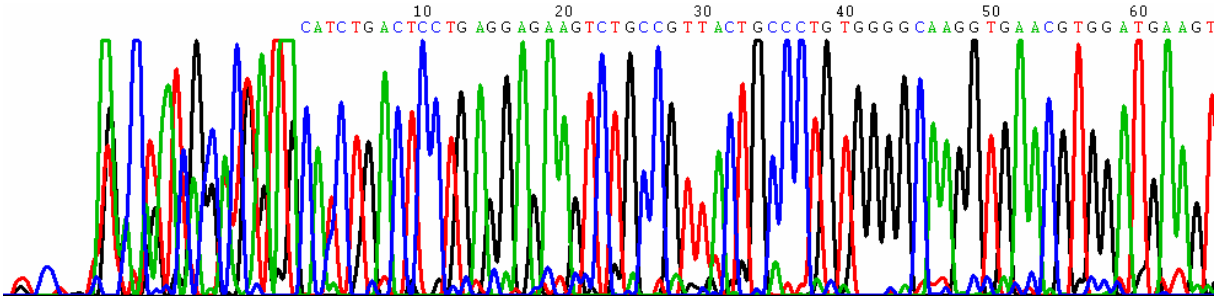
PCR mixture	(in µl)
Genomic DNA	2.5
TaKaRa 10x LA PCR buffer II (Mg^{2+} plus)	2.5
TaKaRa dNTP mixture 2.5 mM each	2
Primer Glo5new (20 µM)	1
Primer BPG2 (20 µM)	1
Primer ASM - PA2 (100 ng/ µl)	1
Distilled H_2O	28
TaKaRa LA Taq polymerase (5 U/µl)	0.5

PCR conditions for human blood genotyping

	min	°C
Start:	4	94
35 cycles		
Denaturing:	1	94
Annealing:	1	55
Extension:	1	72
Last extension, in addition:	2	72
	∞	4

The PCR mixture was stored at –20°C until sequencing was performed by 4baselab, Reutlingen, Germany. Sequencing results of one healthy (HbA/A) and one sickle cell trait (HbA/S) individual are shown in Fig. 2.3. For sequencing results of all donors, see appendix.

A HbA/A, position 14 only one signal A



B HbA/S, position 14 double signal A/T

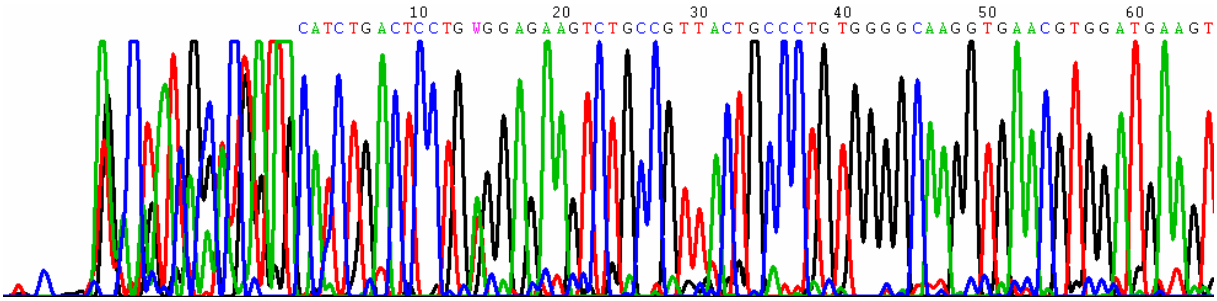


Figure 2.3. Confirmation of the genotype of the blood samples used. Sequencing results of an amplified DNA fragment of a healthy and a sickle cell trait individual containing the codon 6 of the β-hemoglobin chain.

A-B Sequences of an amplified DNA fragment containing the codon 6 of the β-hemoglobin chain. Genomic DNA, extracted from whole blood of a healthy (HbA/A) and a sickle cell trait (HbA/S) individual were PCR amplified and sequenced. Normal hemoglobin (HbA) has the code GAG (position 13-15) for glutamine (**A**), while sickle cell hemoglobin (HbS) has the code GTG for valine. The DNA from sickle trait (HbA/S) therefore shows a double signal A/T at position 14 (**B**).

2.2.7 DAG Biotrak enzyme assay for the determination of ceramide levels

In principle, this radio-enzymatic assay employs abundant *E. coli* diacylglycerol (DAG) kinase and defined mixed micelle conditions. These conditions effectively solubilize the crude extract of ceramide and allow its quantitative conversion to [^{32}P] phosphatic acid in the presence of [^{32}P]γ-ATP and DAG-kinase, which also acts as a ceramide-kinase in this case. The radio-enzymatic reaction of assay is shown in Figure2.4. Following its enzyme-catalyzed phosphorylation, the [^{32}P] phosphatic acid reaction product is extracted and separated from [^{32}P]γ-ATP, which is added in excess, by thin-layer chromatography. The more lipophilic product moves with the running buffer, whereas the more hydrophilic [^{32}P]γ-ATP remaines at the baseline. The radioactivity attributable to the [^{32}P] phosphatic acid product is scratched off the plate and determined by liquid scintillation counting. The amount

of ceramide present in the sample is calculated from the amount of [^{32}P] phosphatic acid produced and the specific activity of the [^{32}P], corresponding to the standard curve.

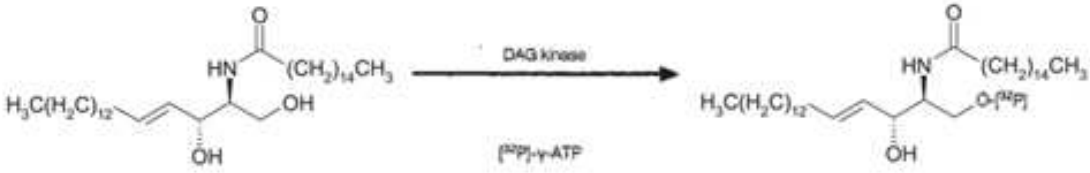


Figure 2.4. Radio-enzymatic reaction used in the DAG Biotrak Enzyme assay.

The substrate used for the standard curve is C16-ceramide (D-erythro-N-palmitoylsphingosine). It is quantitatively converted into [^{32}P] phosphatic acid in the presence of [^{32}P]γ-ATP and DAG kinase.

2.2.7.1 Standard preparation

The prepared standards (including the zero value) were air-dried by the Eppendorf concentrator at 30°C for about 15 min.

2.2.7.2 Extraction and separation of lipids

The whole assay was performed in MµlTI Twist Top Vials with screw caps with a rubber seal to prevent evaporation and spill. 20 µl of erythrocyte pellet were washed twice in NaCl Ringer and centrifuged at 225 g for 2 min at RT. 10µl of the washed RBCs were suspended quantitatively in 90 µl NaCl Ringer. This was followed by a modified lipid extraction method [316,111] briefly described in the following: 250 µl methanol, 125 µl chloroform were added to the 100 µl RBC suspension using a Hamilton syringe. The samples were vortexed for 2 min and centrifuged at 9460g for 2 min at RT. Phase separation was accomplished by addition of 125 µl chloroform with a Hamilton syringe and 125 µl distilled water and subsequent repetition of the suspension (vortexing for 2 min) and centrifugation steps. The lower phase was collected with a 250 µl Hamilton microliter syringe, air-dried in a concentrator maintaining a temperature of 30°C for around 25 - 30 min. If no degradation of DAG was performed, samples were dried just before undergoing the enzymatic reaction.

DAG was degraded by alkaline hydrolysis in 100 µl of 0.1 N methanolic HCl for 1 h at 37°C. The extraction protocol described above was modified here to take account of the 99 µl methanol already present, as follows: 90 µl NaCl Ringer, 151 µl methanol and 125 µl chloroform were added as a first step before suspension and centrifugation.

After complete extraction, the lower phase was collected, dried, and stored at –20°C. Samples and standards were analysed by thin layer chromatography within 72 h.

2.2.7.3 Enzymatic reaction

To provide defined mixed micelle conditions 20 µl of detergent solution were added to the dried samples and standards, which were sonicated in an ice-cold ultrasound bath for 10-30 min until the dried extract was dissolved.

70 µl of reaction mix and 10 µl of freshly prepared radioactive tracer solution were added to each solubilized sample and standard, which were immediately incubated for 30 min at RT, *i.e.* 25°C, in a shaking thermomixer. The enzymatic reaction was stopped by adding 1 ml of chloroform: methanol: 1 N HCl (100:100:1). After vortexing 170 µl of buffered saline solution and then 30 µl of 100 mM EDTA-solution were added. The samples were again vortexed for 15 s, centrifuged at RT at 6300g for 5 min. 200 µl of the lower phase was collected with an appropriate Hamilton microliter syringe, eventually stored at -20°C overnight. The organic phase was air-dried at RT overnight in an extractor hood or alternatively at 30°C in a heating block using a nitrogen flow for evaporation in either (the latter) case .

2.2.7.4 Thin-layer chromatography

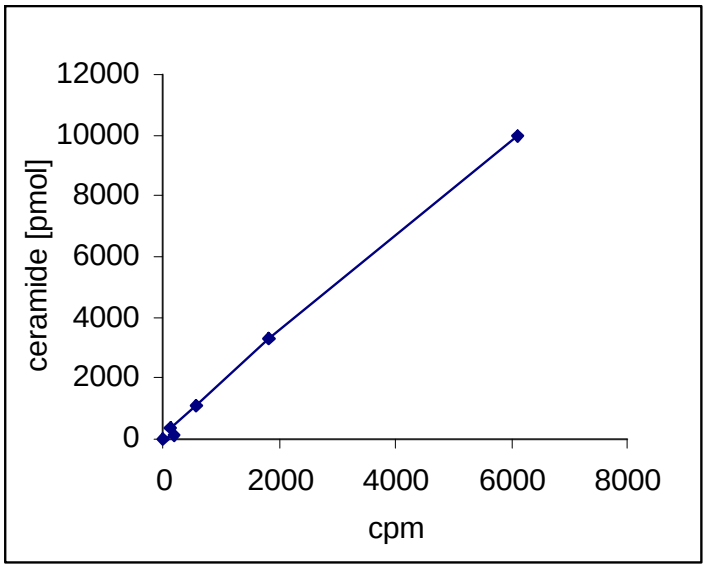


Figure 2.5. Standard curve to determine the ceramide content in pmol from scintillation counter measurements. Standards with a content of 0, 123, 370, 1111, 3333, and 10000 pmol ceramide were prepared alongside the samples to determine a standard curve.

9-10 dried (but not overdried!) samples were each dissolved in 20 µl 1:1 chloroform-methanol solution and separated by thin-layer chromatography. The silica gel plate was air-dried. An autoradiography was developed overnight. The spots of phosphatic acid of ceramide remaining as reaction product were detected by autoradiography, scraped off the plate and transferred to scintillation vials. Scraped silica gel samples of each spot were dissolved in 8 ml of scintillation liquid. [^{32}P] radiation was quantitatively measured in a liquid β-scintillation counter. The ceramide level of the RBCs was determined in terms of pmol of the radio-enzymatically labeled ceramide using a standard curve (Fig. 2.5.) and extrapolated for 100 % parasitemia according to the following formula:

$\text{Ceramide}_{\text{measured}} = [\text{ceramide}]_{\text{infected}} * (\text{parasitemia})\ \% + [\text{ceramide}]_{\text{non-infected measured}} * (100 - \text{parasitemia})\ \% \leftrightarrow$

$\text{Ceramide}_{\text{infected}} = ([\text{ceramide}]_{\text{measured}} - [\text{ceramide}]_{\text{non-infected measured}} * (100 - \text{parasitemia})\ \%) / (\text{parasitemia})\ \%$

2.2.8 Determination of intracellular free Ca^{2+} concentration

To determine intracellular free Ca^{2+} concentration, infected human HbA/A and HbA/S erythrocytes (24 h after ring-stage synchronization) were stained in NaCl Ringer solution with ethidium bromide (1 µM, 30 min / 21°C), washed, post-stained with Fluo-3/AM (2 µM, 30 min / 21°C) and washed again. Ca^{2+}-dependent Fluo-3- and DNA-dependent ethidium bromide fluorescence intensity was measured by flow cytometry.

2.2.9 Determination of $^{45}Ca^{2+}$ flux

For determination of $^{45}Ca^{2+}$ uptake into *P. falciparum* ring stage-synchronized human erythrocytes, infected RBCs were synchronized with 5% sorbitol /HEPES/glucose solution, washed and immediately suspended in KCl/NaCl solution to remove extracellular Ca^{2+}. Around 120 µl of synchronized infected RBCs (10 µl RBCs in triplicate for 4 times points) were washed twice for 10 - 15 min in EGTA-free KCl/NaCl solution to remove EGTA and centrifuged at 225g for 5 min at RT. After this, RBCs were preincubated for 30 min (37°C) in supplemented EGTA-free KCl/NaCl solution and then incubated in the same solution (final hematocrit: ~7.4%) supplemented with radioactive tracer from a 100 mM $CaCl_2$ stock solution with a specific activity of around 10^7 c.p.m. / mmol to reach a final free calcium concentration of 100 µM (~1 µCi / ml $^{45}Ca^{2+}$). 100 µl aliquots were successively delivered into 1 ml ice-cold EGTA-free KCl/NaCl solution at predefined points in time (0, 10, 20 and 30 min). The cells were washed twice using 1 ml of the same solution, and the supernatant

was discarded. The cells were lysed and precipitated by addition of 600 µl 6% TCA. After another spin (2800g for 10 min, RT), 0.5 ml of the clear supernatant (diluted 1:5 with scintillation liquid) was used for measuring $^{45}Ca^{2+}$ radioactivity using a liquid β-scintillation counter.

The total calcium content of the cells $[Ca_T]_i$ was calculated by dividing the activity of the samples by the specific activity of $^{45}Ca^{2+}$ and by the number of cells.

2.2.10 PGE_2 measurements

For PGE_2 determination [517], infected HbA/A and HbA/S erythrocytes were grown to high parasitemia, synchronized and washed. Then they were adjusted to a hematocrit of 0.1 %, a parasitemia of ~10%, and cultured in a 12-well plate (1 ml cell suspension / well) in duplicate for each time point. The supernatant was collected after 1, 3, 6, 12, 24 and 48 h. After incubation for 0 - 48 h at 37°C, cells were spun down at 4 °C, 225 g for 5 min and the supernatant was stored at -80°C. PGE_2 concentrations in the supernatant were determined using the Correlate-EIA^{TM} Prostaglandin E_2 (PGE_2) Enzyme Immunoassay (EIA) Kit according to the manufacturer's instructions. Briefly, the samples were diluted 1: 2.5 with assay buffer. Then, 100 µl sample, 50 µl alkaline phosphatase PGE_2 conjugate and 50 µl monoclonal anti-PGE_2 EIA antibody were applied to goat anti-mouse IgG microtiter plates and incubated at RT for 2 h. After washing, 200 µl of p-nitrophenyl phosphate substrate solution was added, and samples were incubated at RT for 45 min. Finally, the optical density at 405 nm was measured in a spectrophotometer microplate reader. PGE_2 concentrations in the samples were calculated from a PGE_2 standard curve (39.1 – 5000 pg/ml), which was determined alongside the experimental work. PGE_2 levels in the supernatant and lysate of control treated erythrocytes were 98 ± 26 pg/10^9 infected HbA/A RBCs, 127 ± 32 pg /10^9 infected HbA/S RBCs, and 26 ± 2 pg/10^9 non-infected HbA/A RBCs at time point 0 h, and were defined as 100 %.

2.2.11 Data analysis and statistics

Differences between means were estimated by one-way ANOVA, Bonferroni multiple comparison method or two-tailed Student's *t*-test where appropriate using the InStat statistic program.

3. Results

3.1 Dependence of *P. falciparum in vitro* growth on the cation permeability and the induction of eryptosis of the human host erythrocyte

3.1.1 Whole-cell currents of erythrocytes induced by *P. falciparum* infection

Patch-clamp measurements of whole-cell cation currents were performed in late-trophozoite-infected human red blood cells (RBCs) as a means of characterizing the cation-permeable subfraction of the New Permeability Pathways (*NPP*s). Infected RBCs exhibit whole-cell currents which are 2-3 orders of magnitude higher than those of non-infected RBCs. The principal current fraction of infected RBCs is anion-selective, as indicated by the whole-cell reversal potential which approximates the Nernst equilibrium potential for Cl^- (Fig. 3.1.A. 1st trace and corresponding I-V-curve in Fig. 3.1.B.). Replacement of Cl^- in the bath by gluconate, *i.e.* removal of extracellular Cl^-, changes the Cl^- equilibrium potential. After this type of bath solution exchange, Cl^- influx-generated outward currents were no longer detectable, whereas the current amplitude increased progressively. Some kind of cation conductance must have been activated, as the observed current increase was paralleled by a change of the current reversal potential from Cl^- equilibrium potential towards 0 mV. The equilibrium potential for nonselective cation condutances (NSCs) is 0 mV (Fig. 3.1.A 2nd trace and corresponding I-V- curve in Fig. 3.1.B.). In conclusion, *Plasmodium falciparum* infected RBCs exhibit in addition to the predominant anion conductance a small cation conductance. Hence, these data indicate voltage-independent NSC currents in infected RBCs that are similar to those in non-infected RBCs stimulated by removal of extracellular Cl^-. The current amplitudes in infected RBCs after extracellular Cl^- removal demonstrate a 7 to 8-fold stimulation of NSC currents as compared to non-infected erythrocytes [243].

During patch-clamp measurements in Na-gluconate bath solution combined with KCl/K-gluconate in the pipette, the whole cell current reversed at -25 mV, suggesting an approximately two-fold higher permeability for K^+ than for Na^+. As has been demonstrated recently [243], Ca^{2+} permeability is also due the the infection-induced cation conductance. In the present study, $CaCl_2$/Ca $(gluconate)_2$ pipette solutions generated outward currents in infected RBCs in the absence of Cl^- in the bath solution (Fig. 3.1.C., first two traces), indicating that

these outward currents were carried by Ca^{2+} efflux and not by Cl^- influx into the cell. Moreover, EIPA (1µM) which has been demonstrated to inhibit the infection-induced cation but not the anion conductance [243], inhibited the outward current (Fig. 3.1.C., 2nd and 3rd traces and Fig. 3.1.D.). Taken together, these data confirm previous results [243] showing the induction of a small Ca^{2+}-permeable NSC conductance by infection of human RBCs with *Plasmodium falciparum*.

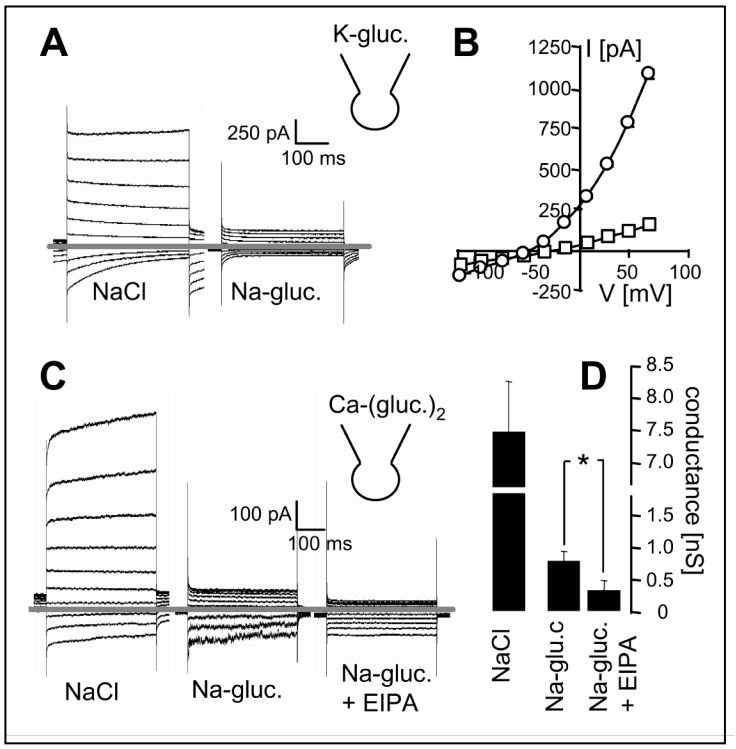


Figure 3.1. Cl^- dependent cation conductance in *Plasmodium falciparum* infected human RBCs.

(**A**) Whole-cell current traces from a *Plasmodium falciparum*-infected RBC recorded first with NaCl (left) and then with Na-gluconate bath solution (right). Currents were obtained in fast whole-cell voltage-clamp mode with a pipette solution containing K-gluconate. The membrane potential was held at -10 mV and currents were elicited by 400 ms square pulses to test potentials between -100 and +100 mV. Currents of the individual voltage sweeps are superimposed; a thick line indicates zero current.

(**B**) Mean current-voltage (I-V) relations (number of measurements (n) = 6, ± standard error (SE)) of *Plasmodium falciparum*-infected cells recorded as in (A) with NaCl (circles) and Na-gluconate bath solution (squares).

(**C**) Whole-cell current traces from a *Plasmodium falciparum*-infected RBC recorded first with Ca-$(gluconate)_2$ pipette solution. Records were performed with NaCl (left) and Na-gluconate bath solution prior to (middle) and upon bath application of EIPA (1 µM, right). EIPA inhibited a Ca^{2+}-carried outward current.

(**D**) Mean outward conductance (n = 4, ± SE) of infected RBCs recorded as in (C) with Ca-$(gluconate)_2$ in the pipette and bath solutions containing NaCl, Na-gluconate or Na-gluconate and EIPA (1 µM).

* $p \leq 0.05$; paired two-tailed student's t-test.

3.1.2 Sensitivity of *P. falciparum in vitro* growth to EIPA

The infection-induced NSC conductance of RBCs was inhibited by EIPA with an IC_{50} of 0.75 µM. EIPA inhibited parasite growth with an IC_{50} of 8.5 µM when applied throughout 48 h of culture (Fig.3.2.A.-D.). Inhibitors of the infection-induced *NPP*s are much less effective in parasite growth assays than they are in tracer flux measurement or hemolysis experiments. This might be explained by the adsorption of inhibitors to serum proteins in the culture medium [464], a phenomenon which could also account for the low effect of EIPA on parasite growth in the present study. Therefore the free EIPA concentration (10 µM = 3.7 µM free EIPA) was determined and is shown in Fig. 3.2.E. The IC_{50} for free EIPA was 3 µM.

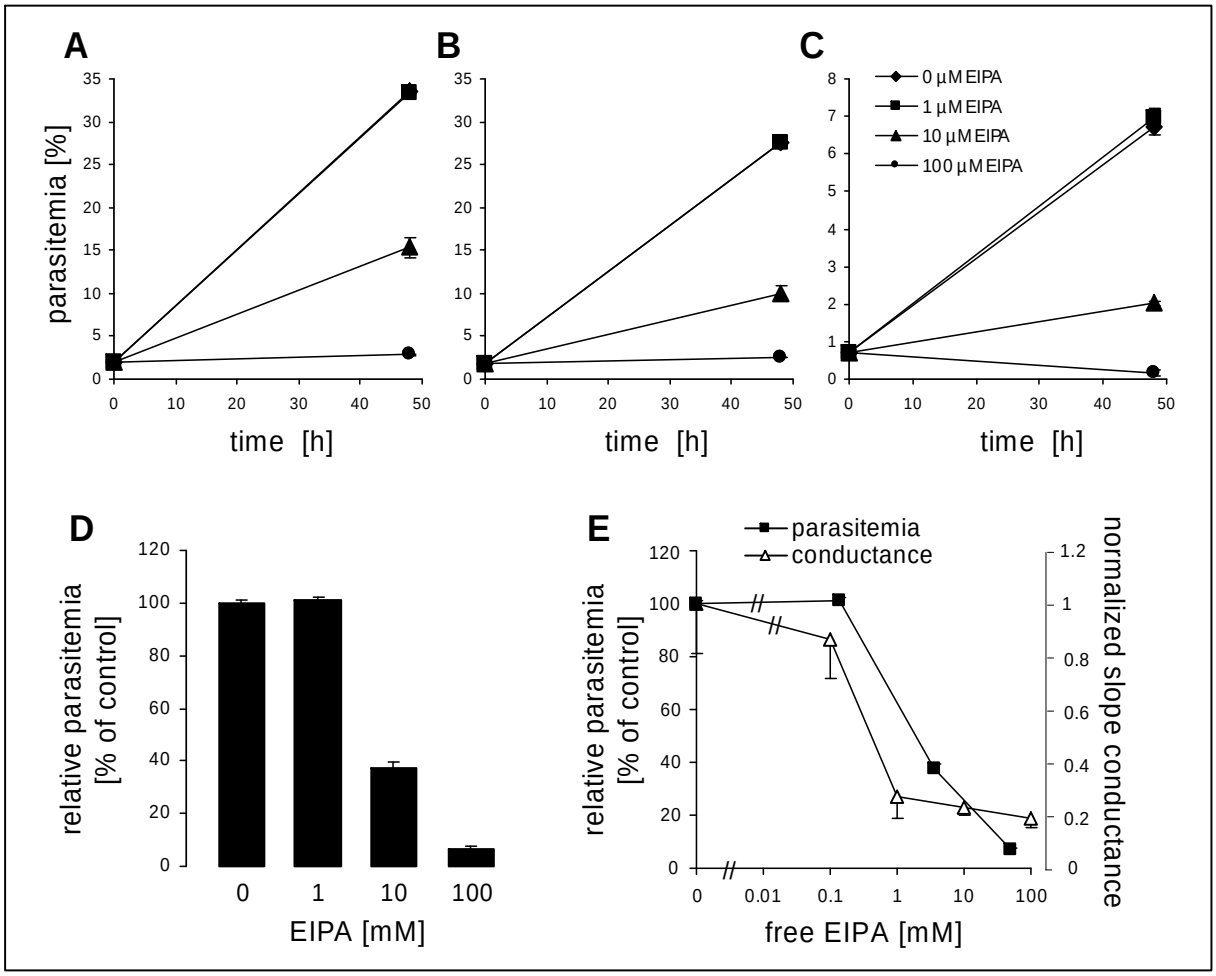


Figure 3.2. Inhibition of conductance and intraerythrocytic *in vitro* growth of *Plasmodium falciparum* by 5-(N-ethyl-N-isopropyl) amiloride (EIPA).

(**A** – **C**) Parasitemia in % at 0 h and after 48 h of synchronized *P. falciparum in vitro* culture. Each curve represents the average of a six-fold determination (n = 6, ± SE). The applied concentrations of EIPA were 0, 1, 10, and 100 µM. (**D**) Initial parasitemia has been subtracted to show relative parasitemia [% of control] observed in the three independent parasite growth assays (A-C, n = 18, ± SE). (**E**) Relative parasitemia (n = 18, ± SE)

after 48 h of growth and normalized slope conductance of infected RBCs as measured by patch clamping, respectively, containing increasing concentrations of free EIPA (as indicated).

3.1.3 Dependence of *P. falciparum in vitro* growth on external Ca^{2+}

As a means of defining the functional significance of the infection-induced Ca^{2+} permeable NSC conductance the dependence of intraerythrocytic *in vitro* growth of *Plasmodium falciparum* on the Ca^{2+} concentration in culture medium was studied. Parasites were grown for 48h with EGTA (0, 1 or 2.5 mM, respectively) continuously present in the medium. EGTA severely inhibited the intraerythrocytic growth of *Plasmodium falciparum*, confirming previous results [867] (Fig. 3.3.A., B.). This effect was fully reversed upon re-increase of extracellular Ca^{2+} activity by addition of 1 or 2.5 mM $CaCl_2$, respectively, corresponding to the concentrations of EGTA used (Fig. 3.3.A. and B.). Thus, the observed inhibition of growth was evidently not due to a toxic effect of EGTA.

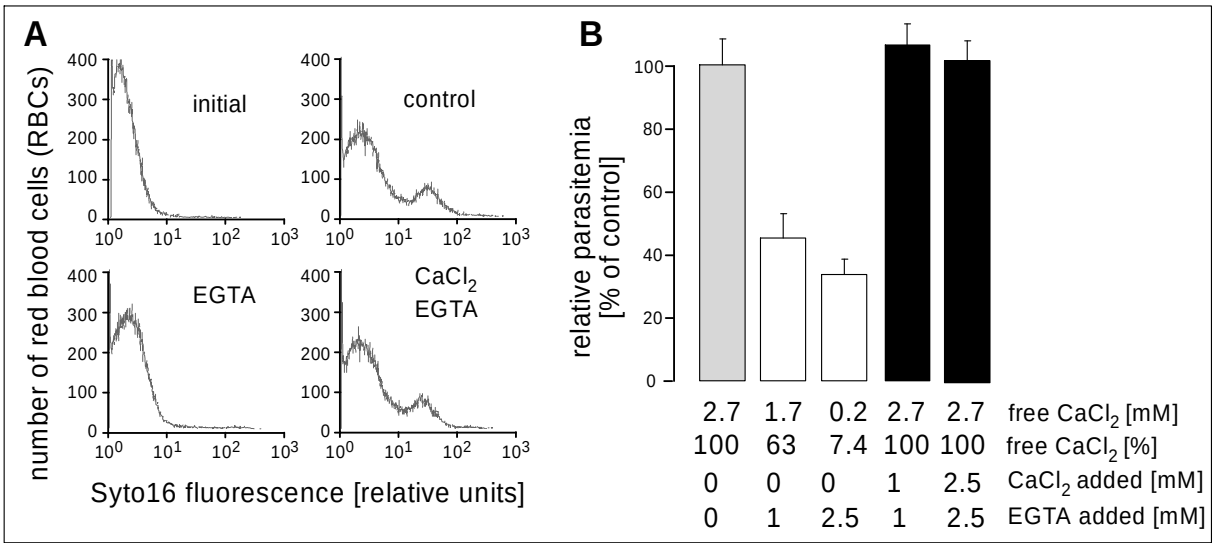


Figure 3.3. Dependence of intraeythrocytic *in vitro* growth of *Plasmodium falciparum* on the free Ca^{2+} concentration in the culture medium.

(**A**) Histograms showing the fluorescence of the DNA dye Syto16 in *Plasmodium falciparum* infected RBCs as recorded by flow cytometry. Histograms are given for the RBCs at the start of the experiments (initial), after 48h of growth in normal culture medium (control), in medium supplemented with EGTA (2.5 mM), and in medium supplemented with EGTA and $CaCl_2$ (2.5 mM each), respectively. Prior to the experiments infected RBCs were synchronized to the ring stage of infection and adjusted to 1% of parasitemia and a 5 % hematocrit. (**B**) Relative parasitemia (n = 11, ± SE) during 48 h of synchronized culture in normal medium (grey bar) or in medium supplemented with various concentrations (as indicated) of EGTA or EGTA and $CaCl_2$. Two independent growth assays were carried out in five or six-fold determination, respectively. Free calcium concentration was calculated according to the WEBMAXC standard webpage [672].

3.1.4 Dependence of *P. falciparum in vitro* growth on external Na^+

As a means of determining the dependence of parasite growth on Na^+ concentration, the culture medium was replaced by isosmotic NaCl or NMDG-Cl test solution for 4 h, 8 h and 16 h beginning after 24 h of synchronized culture (*i.e.* at the trophozoite stage of infection). The data presented in Fig. 3.4.I.A. (closed bars) suggest that replacement of the culture medium by isotonic NaCl test solution, which contained [in mM]: 120 NaCl, 30 HEPES, 5 glucose, 1.7 $CaCl_2$, 5 KCl, was tolerated by the parasites for up to 8 h. The dependence of growth on the electrolyte composition was then studied within an 8 h window (between 24 h and 32 h after synchronization) during which the medium was isosmotically replaced by test solutions. The only protein source for the parasites during this time interval was hemoglobin. Parasite growth upon replacement of the medium by NaCl test solution for 8 h was taken to represent control conditions for the following growth assays. Replacement of the medium by NMDG-Cl test solution, in which 120 mM NaCl was replaced by 140 mM NMDG titrated with HCl to pH 7.4, dramatically inhibited growth when applied for 8h or longer (Fig. 3.4.I.A; open bars).

To exclude a direct toxic effect of NMDG-Cl as a potential cause of the observed growth inhibition (Fig. 3.4.I.A), the medium was replaced for 8 h by NaCl isotonic test solution or by a 1:1 mixture of NaCl and NMDG-Cl, NaCl and raffinose, or NMDG-Cl and raffinose isotonic test solution, respectively. Total replacement of Na^+ (NMDG-Cl/raffinose) inhibited 48 h parasite growth by about 80 % as compared to the growth in normal culture medium (Fig. 3.4.I.B.). Decrease of the extracellular Na^+ concentration to about 50 % by substituting the medium with NaCl / raffinose or NaCl / NMDG-Cl had only little effect on parasite growth when compared to that obtained upon medium replacement by NaCl (Fig. 3.4.I.B.). Most importantly, no difference between parasite growth in NaCl / raffinose and NaCl / NMDG-Cl was apparent (Fig. 3.4.I.B.), indicating that the effect of Na^+-replacement was indeed due to external Na^+ depletion rather than to a toxic effect of $NMDG^+$.

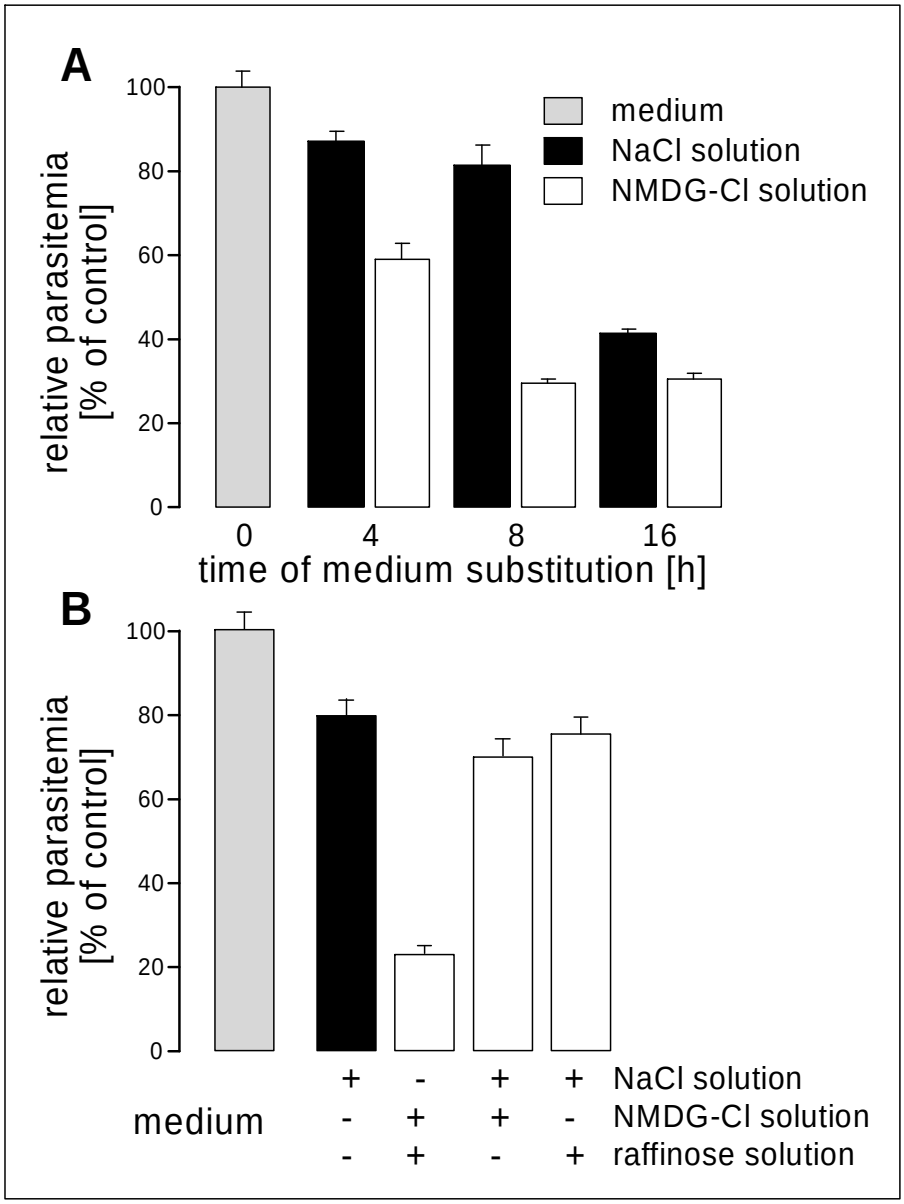


Figure 3.4. Effect of Na^+ removal from the culture medium on parasite growth.

I. Time dependence and toxicity of N-methyl-D-glucamine (NMDG).

(**A**) Relative parasitemia (n = 6, ± SE) of synchronized infected RBCs grown for 48 h in normal culture medium (0 h) or in culture medium replaced for 4 h, 8 h, and 16 h, respectively, by NaCl or (NMDG)-Cl test solution The test solutions contained [in mM]: 120 NaCl or 140 NMDG, titrated to pH 7.4 with HCl, and in addition: 30 HEPES, 5 glucose, 1.7 $CaCl_2$, and 5 KCl. Medium replacement started after 24 h of culture in the trophozoite stage of infection.

(**B**) Relative parasitemia (n = 6, ± SE) of synchronized RBCs grown for 48 h in normal culture medium (gray bar) or in culture medium replaced for 8 h (between 24 and 32 h of culture) by NaCl test solution (closed bar) (with 120 mM NaCl and salts as indicated above, and by 1:1 mixtures of isotonic NMDG-Cl (72.5 mM) and raffinose (140 mM) test solution, NaCl (60 mM) and NMDG-Cl (72.5 mM) test solution, or NaCl (60 mM) and raffinose (140 mM) test solution, respectively (as indicated; open bars). All replacement solutions contained in addition [in mM]: 30 HEPES, 5 glucose, 1.7 $CaCl_2$, and 5 KCl. The only protein source during the replacement interval was hemoglobin.

In order to better define how parasite growth depends on the external Na^+ concentration, isotonic NaCl test solution (containing in [mM]: 120 NaCl, 30 Hepes, 5 glucose, 1.7 $CaCl_2$, 5 KCl) was progressively replaced by isosmotic NMDG-Cl or KCl test solutions (Fig. 3.4. II.). Instead of 120 mM NaCl the NMDG-Cl test solution contained 140 mM NMDG titrated with HCl to pH 7.4 and the KCl test solution 120 mM KCl. As indicated in Fig. 3.4.I.B. the medium was replaced for 8 h after 24 h of culture. Under both experimental conditions, parasite growth decreased when external Na^+ concentration was lowered. The data in Fig. 3.4.II. show that when Na^+ was replaced by $NMDG^+$ or K^+ significantly different concentrations of external Na^+ were necessary in order for parasites to multiply half-maximally as compared to control conditions. If NMDG replaced NaCl, the IC_{50} was at 24 mM NaCl (and 112 mM NMDG). If KCl replaced NaCl, the IC_{50} was already at 58 mM NaCl and 65 mM KCl.

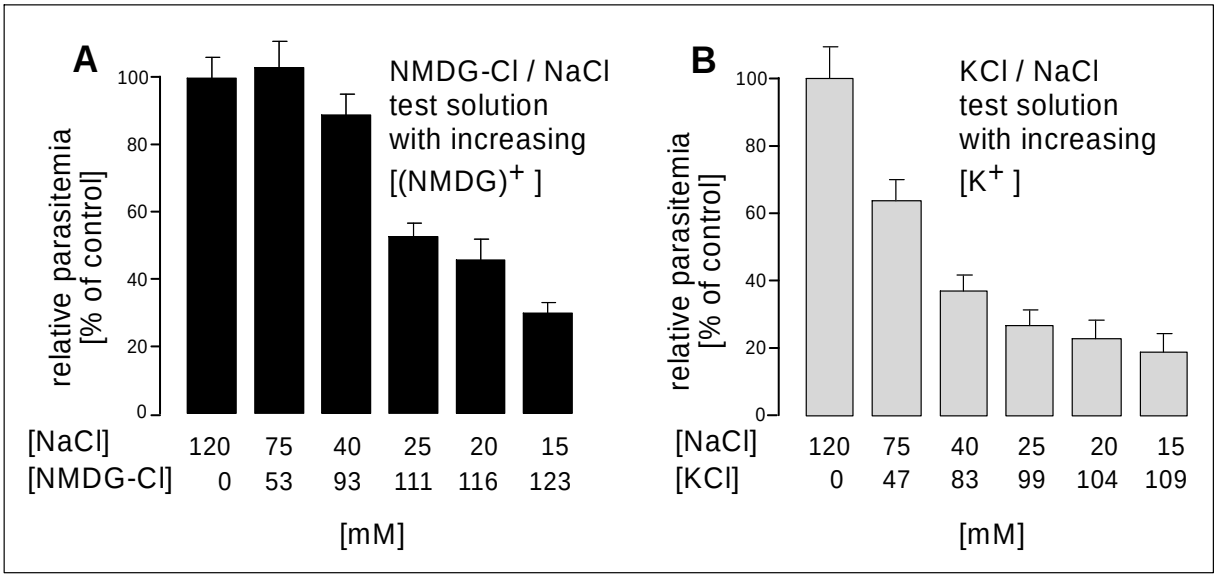


Figure 3.4. Effect of Na^+ removal from the culture medium on parasite growth.

II. Concentration dependence.

Parasite growth in synchronized infected RBCs incubated for 48 h in normal culture medium (gray bar) or in culture medium replaced for 8 h (between 24 and 32 h of culture) by different mixtures of isotonic NaCl and NMDG-Cl test solution (**A**) or isotonic NaCl and KCl test solution (**B**). The remaining NaCl, (NMDG)-Cl or KCl concentration are depicted, respectively. The test solutions contained (in mM): 120 NaCl, 140 (NMDG)-Cl (black bars) or 120 KCl (gray bars), in addition: 30 Hepes, 5 glucose, 1.7 $CaCl_2$, and 5 KCl. The bars represent relative parasitemia (n = 11 - 24, ± SE) as a function of the NaCl concentration of the test solutions. Two - four independent growth assays were carried out in six-fold determination.

3.1.5 Dependence of *P. falciparum in vitro* growth on external Cl^-

In additional 8h replacement experiments the culture medium was substituted with Na-gluconate test solution (containing in [mM]: 120 Na-gluconate, 30 Hepes, 5 glucose, 1.7 $CaCl_2$, 5 KCl). This was done in order to test whether the parasite requires external Cl^-. Upon medium substitution with NMDG-Cl test solution parasite growth inhibition differed from that upon medium substitution with NaCl test solution. By contrast, parasite growth inhibition effected by substitution with Na-gluconate test solution (Fig. 3.5.) did not differ from that exerted by NaCl test solution. This suggests that the parasites' development was not impaired by low extracellular Cl^- concentration.

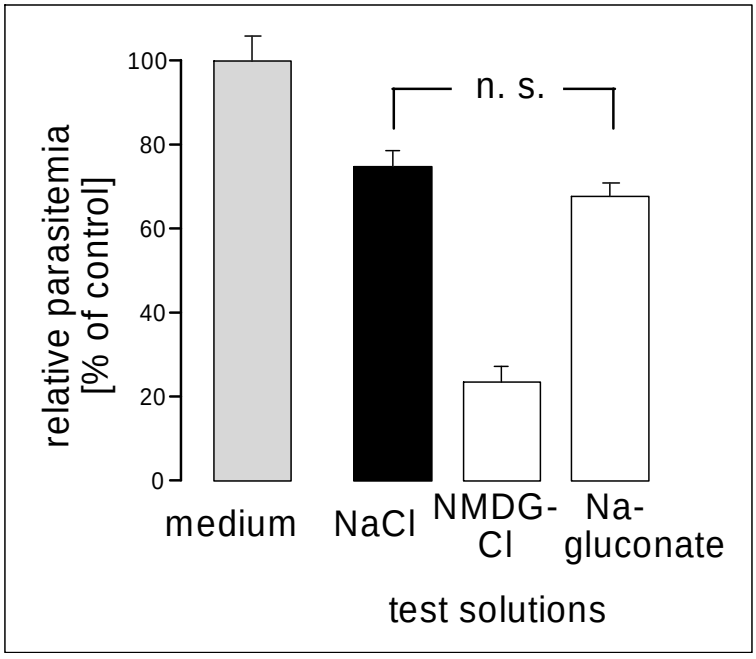


Figure 3.5. Effect of Cl^- removal from the culture medium on parasite growth.

Relative parasitemia (n = 12 – 18, ± SE) of 2 - 3 independent assays carried out in six-fold determination with synchronized infected RBCs grown for 48 h under normal culture conditions (gray bar) or in culture medium replaced for 8 h (between 24 and 32 h after synchronization) by NaCl test solution (closed bar), by NMDG-Cl test solution, or by Na-gluconate test solution, respectively (open bars, n. s. = not significantly different, $p>0.5$). Test solutions contained 120 mM NaCl, 140 mM NMDG (titrated with HCl to pH 7.4, or 120 mM Na-gluconate and in addition [in mM]: 30 Hepes, 5 glucose, 1.7 $CaCl_2$, and 5 KCl.

3.1.6 Breakdown of erythrocyte phosphatidylserine asymmetry

Elevated Ca^{2+} permeable NSC conductance induces breakdown of the PS asymmetry of the RBC membrane in non-infected RBCs [244,513,512,507], which is an important feature of erythrocyte programmed cell death [499]. In order to test whether the infection-induced Ca^{2+} permeable NSC conductance also induces a loss of phospholipid, PS exposure at the outer leaflet of the membrane of *Plasmodium falciparum*-infected RBCs was measured by annexin binding using a flow cytometer. For these experiments, the parasite culture was grown to high parasitemia, monitored by Syto16 fluorescence staining. Fig. 3.6.I. (C-F) shows individual dot plots of RBCs from such high parasitemia *in vitro* cultures.

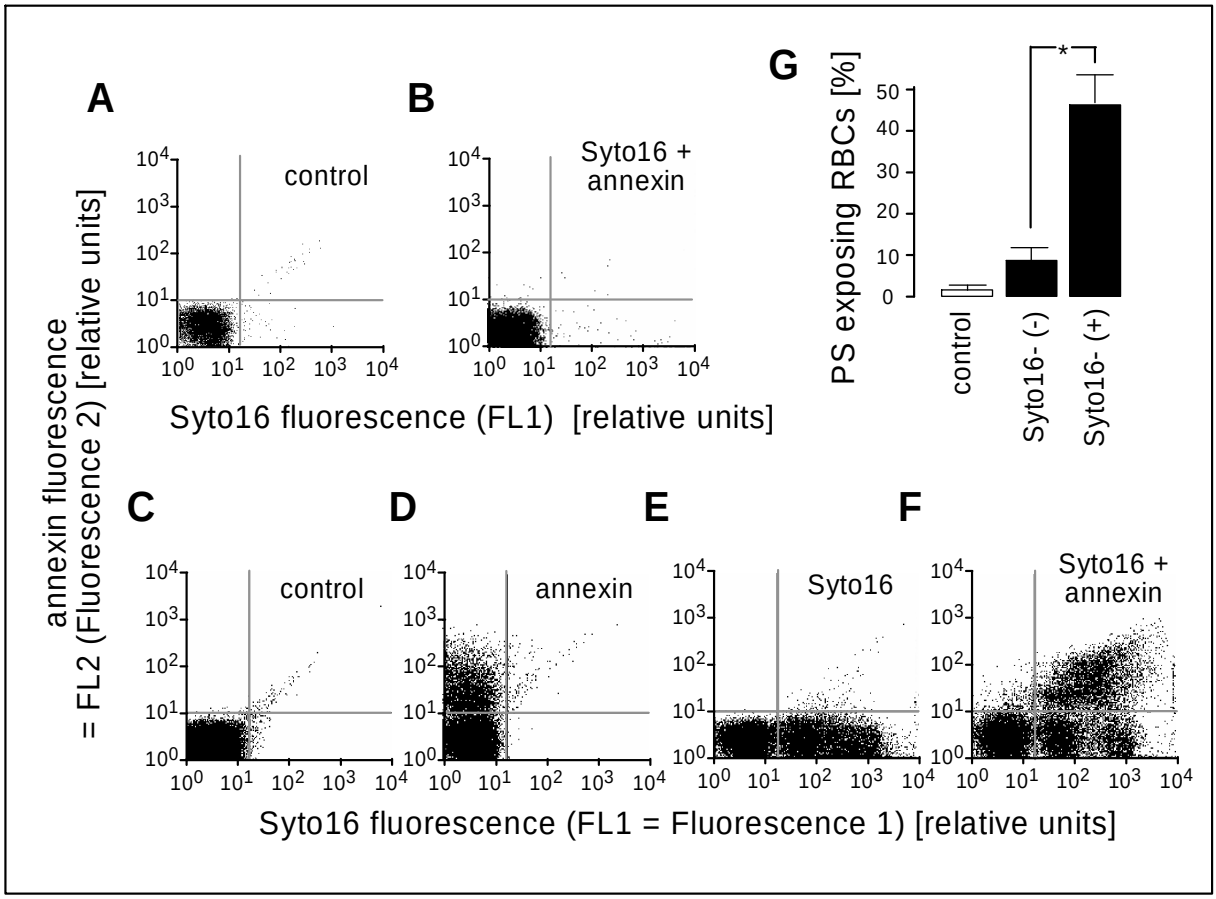


Figure 3.6.I. Breakdown of the phospholipid asymmetry in the RBC membrane induced by infection with *Plasmodium falciparum*.

(**A-F)** Dot plots recordings by flow cytometry of non-infected RBCs, co-culture (**A**-**B**), and of *Plasmodium falciparum*-infected RBCs (**C**-**F**). The blots were obtained from separately cultured unstained non-infected RBCs (**A**), from non-infected RBCs stained with annexin V568 fluorescence dye (annexin) alone or (**B**) together with Syto16 fluorescence dye (Syto16), from unstained infected RBCs (**C**), from infected RBCs stained with annexin V568 fluorescence dye (annexin) (**D**), with Syto16 fluorescence dye (Syto16) (**E**), or with both fluorescence dyes (Syto16 + annexin) (**E**). Annexin binds specifically to phosphatidylserine in the outer membrane leaflet and

and Syto16 to RNA/DNA, thus providing a measure of breakdown of phospholipid asymmetry and parasite infection, respectively. The dot blots show individual samples obtained by counting 100,000 RBCs. **F.** Mean percentage of annexin binding cells (n = 3 – 5, ± SE) in populations of non-infected co-cultured RBC (open bar, co-culture, *i.e.* non-infected, separately incubated cells) and of non-infected cohorts in *Plasmodium falciparum* - infected RBC cultures (closed bars). Syto16-negative refers to the non-infected bystander cells; Syto16-positive refers to the infected RBCs in the *Plasmodium falciparum* infected culture, respectively.

Negative controls were unstained non-infected erythrocytes that had been maintained in a co-culture under the same conditions as the *P. falciparum in vitro* culture or unstained *P. falciparum* infected RBCs, respectively. Positive controls were single stained *P. falciparum* infected RBCs with Syto 16 or with annexin V568, respectively. The fluorescence intensities recorded at two wavelengths (each one corresponding to the emission spectra of one dye) are given for unstained co-cultured non-infected (Fig. 3.6.I.A.) and double stained co-cultured non-infected RBCs (Fig. 3.6.I.B.), unstained infected (Fig. 3.6.I.C.), Syto16-stained infected (FL1), (Fig. 3.6.I.D.), annexin-stained infected (FL2) (Fig. 3.6.I.E.), and Syto16 and annexin-stained infected (Fig. 3.6.I.E.) samples of the *P. falciparum in vitro* culture, clearly indicating at least four distinct RBC populations.

Analysis of these populations demonstrated that non-infected co-cultured RBCs did not expose PS (Fig. 3.6.I.B. and G.; open bar), as the annexin-binding was close to zero; this amount of fluorescence is equal to background levels. Syto16-negative (*i.e.* non-infected cohorts) RBCs from the parasite culture showed a slightly elevated PS exposure (Fig. 3.6.I.F., upper left quadrant; Fig 3.6.I.G., closed left bar), while Syto16-positive (*i.e.* infected) RBCs exhibited a high percentage of annexin binding, *i.e.* PS-exposing RBCs (Fig. 3.6.I.F., upper right quadrant; Fig 3.6.I.G., closed right bar).

This observation points to breakdown of the PS asymmetry of the cell membrane in infected RBC, which increased with the percentage of parasitemia in the *in vitro* culture (Fig. 3.6.II.).

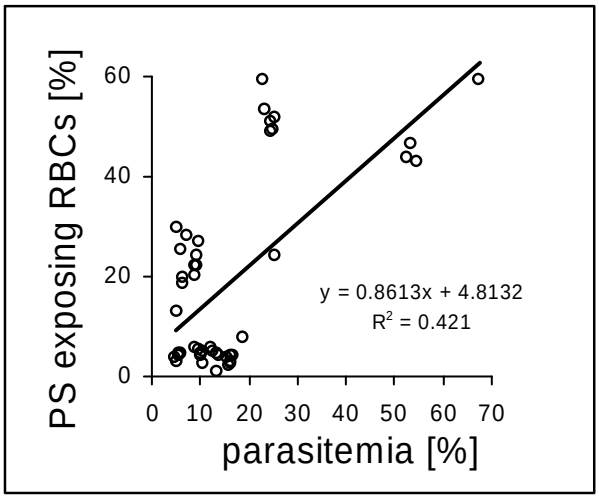


Figure 3.6.II. Dependence of phosphatidylserine-exposure by infected RBCs on parasitemia in the *in vitro* culture.

Increase of phosphatidylserine (PS) exposing cells in % in relation to the increase in parasitemia in % (n = 45). Parasitemia and PS exposure are correlated with a correlation coefficient of (r) = 0.6489; this is within the 95% confidence interval, *i.e.* $p \leq 0.05$ %. The coefficient of determination (r squared, R^2) is 0.421, *i.e.* 42.1 % of the values obey a linear relationship that is shown by the trend line.

A similar increase of annexin binding was observed following treatment of non-infected RBCs with the oxidant *t*-BHP (1 mM for 15 min; Fig. 3.6.III.A., B.), which was shown to activate the Ca^{2+}-permeable NSC conductance of non-infected RBCs [244].

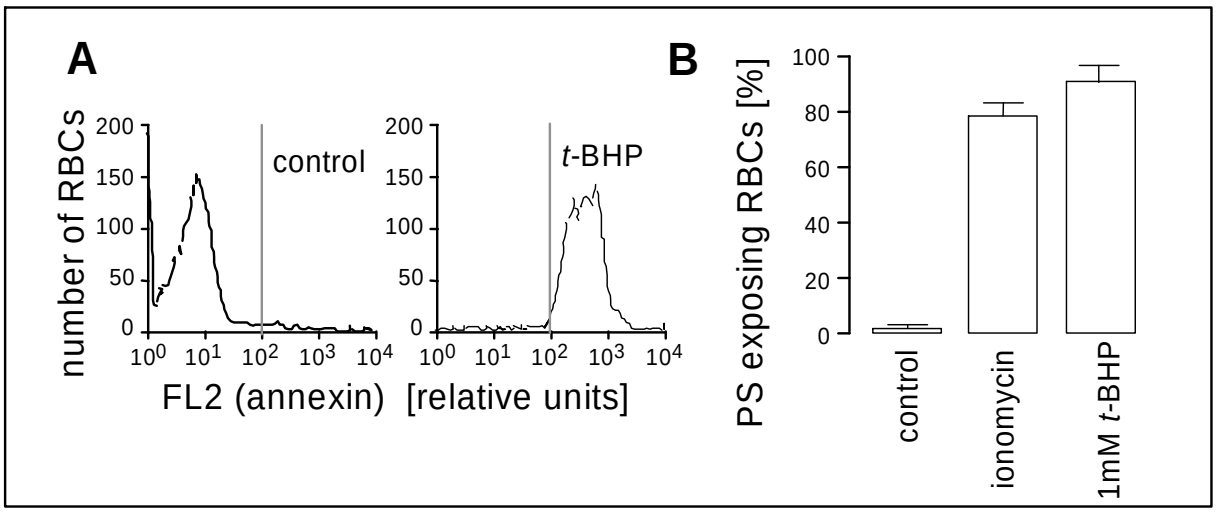


Figure 3.6.III. Breakdown of the phospholipid asymmetry in the RBC membrane induced by Ca^{2+} permeabilization or by oxidative stress.

(**A**) Histograms showing the annexin binding by non-infected control RBCs (left) and by RBCs oxidized with *tert*-butylhydroperoxide (*t*-BHP; right). Cells were oxidized for 15 min (1 mM *t*-BHP) followed by further 24 h of incubation in a modified NaCl test solution consisting of (in mM): 125 NaCl, 5 KCl, 5 D-glucose, 1 $CaCl_2$, and 1 $MgSO_4$. (**B**) Average percentage of annexin binding cells (n = 5 – 6, ± SE) in non-infected RBCs

populations (control) and in RBC populations permeabilized with the Ca^{2+} ionophore ionomycin (1µM in modified NaCl test solution for 1h) or oxidized with *t*-BHP (same protocol as in A).

Incubation of non-infected RBCs with the Ca^{2+} ionophore ionomycin (1 µM for 1 h) mimicked the stimulatory effect of infection or oxidation on annexin binding (Fig. 3.6.III.B.). This suggests that infection-induced activation of the Ca^{2+}-permeable NSC conductance, which results in increased $[Ca^{2+}]_i$, leads to PS exposure [913,218], thus contributing to the observed annexin binding of infected cells. Interestingly, EIPA (10 µM) reduced and Na-gluconate (Cl^--removal) increased annexin-binding in *P. falciparum* positive RBCs (data not shown, n = 6), providing further evidence of the involvement of the NSC cation channel in PS exposure.

The possibility that annexin-binding is due to disruption of the erythrocyte membrane was ruled out by counterstaining *P. falciparum* infected RBCs with propidium-iodide, which was negative (data not shown), and moreover by the fact that hemolysis could not be observed.

Taken together, infection stimulates a NSC conductance that plays an important role in the signaling pathways that converge to trigger eryptosis [499].

3.1.7 *Plasmodium berghei* ANKA infection increases ceramide in blood with significant involvement of host cell acid sphingomyelinase

Activation of erythrocyte sphingomyelinase has been demonstrated to belong to the second signaling pathway of eryptosis [255,498,499,511,510,509,519,635]. *P. falciparum* express neutral sphingomyelinase (NSM) activity [526,371]. During TNF- and CD95 ligand-mediated cell-death of nucleated cells an endosomal acid sphingomyelinase (ASM) is activated, resulting in enhanced intracellular ceramide levels that contribute to apoptosis [213,344,360,533]. To investigate whether the increase in PS exposure of *Plasmodium* infected RBCs resulted from host sphingomyelinase activity, ASM wild-type (+/+) and knock-out (-/-) mice were infected with *P. berghei* ANKA.

P. berghei ANKA infection significantly increased ceramide production in infected blood. Infected wild-type (ASM +/+) and knock-out (ASM -/-) mouse blood had significant higher ceramide levels as compared to non-infected blood.

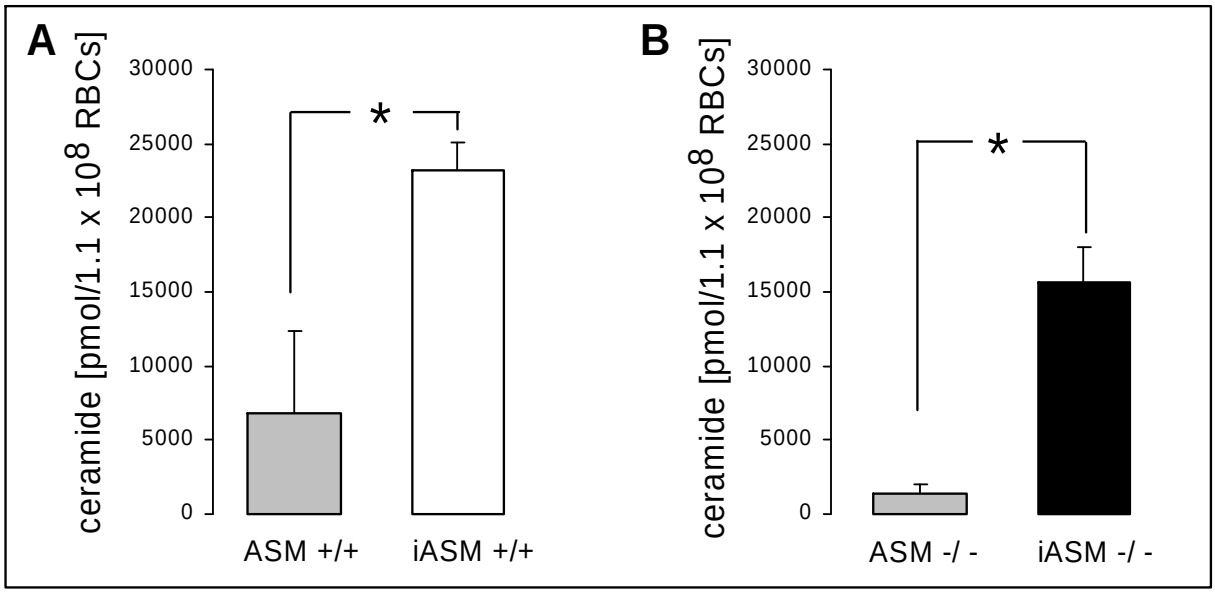


Figure 3.7. *P. berghei* ANKA infection significantly increases ceramide levels in infected RBCs of ASM knock-out mice and their wild-type litter mates.

RBCs of *P. berghei* ANKA infected ASM wild-type (ASM +/+) and knock-out mice (ASM -/-) were collected retro-orbitally and their ceramide levels were determined radioenzymatically (n = 4 – 5, ±SE). For details, see Materials and Methods. (**A**) Ceramide level in pmol in the ASM +/+ blood of control uninfected (gray bar, n = 4, 6844 ± 5555 pmol ceramide/ 1.1 x 10^8 erythrocytes) and *P. berghei* ANKA infected ASM +/+ mice (iASM+/+, open bar, n = 5, 23157 ± 1977 pmol ceramide/ 1.1 x10^8 erythrocytes) (**B**) Ceramide level in pmol in the ASM -/- blood of control uninfected (gray bar, n = 4, 1379 ± 597 pmol ceramide / 1.1 x 10^8 erythrocytes) and *P. berghei* ANKA infected ASM -/- mice (iASM-/-, closed bar, n = 5, 15676 ± 2359 pmol ceramide / 1.1 x10^8 erythrocytes). *: $P \leq 0.05$; unpaired two-tailed student's t-test.

Absolute ceramide values varied considerably between the individual assays. Therefore, the results were normalized by defining the average ceramide level in infected ASM +/+ mouse blood in each assay as 100 %. Normalized ceramide levels in infected ASM -/- were significantly lower than those of ASM +/+ mice (Fig. 3.7.A.). This suggests that the sphingomyelinase of the host erythrocyte contributed significantly to the ceramide formation needed for parasite growth, as this decrease in ceramide production was accompanied by a significantly lower increase in parasitemia (Fig 3.7.B.). The increase in parasitemia differed significantly between ASM -/- mice and their wild-type litter mates between the 10^{th} and 21^{st} day post infection (Fig 3.7.C.). The survival rate of ASM wild-type was lower than that of ASM knock-out mice were similar, with 29 and 35 days maximal survival, respectively (Fig.3.7.D.). However, the difference was not significantly different.

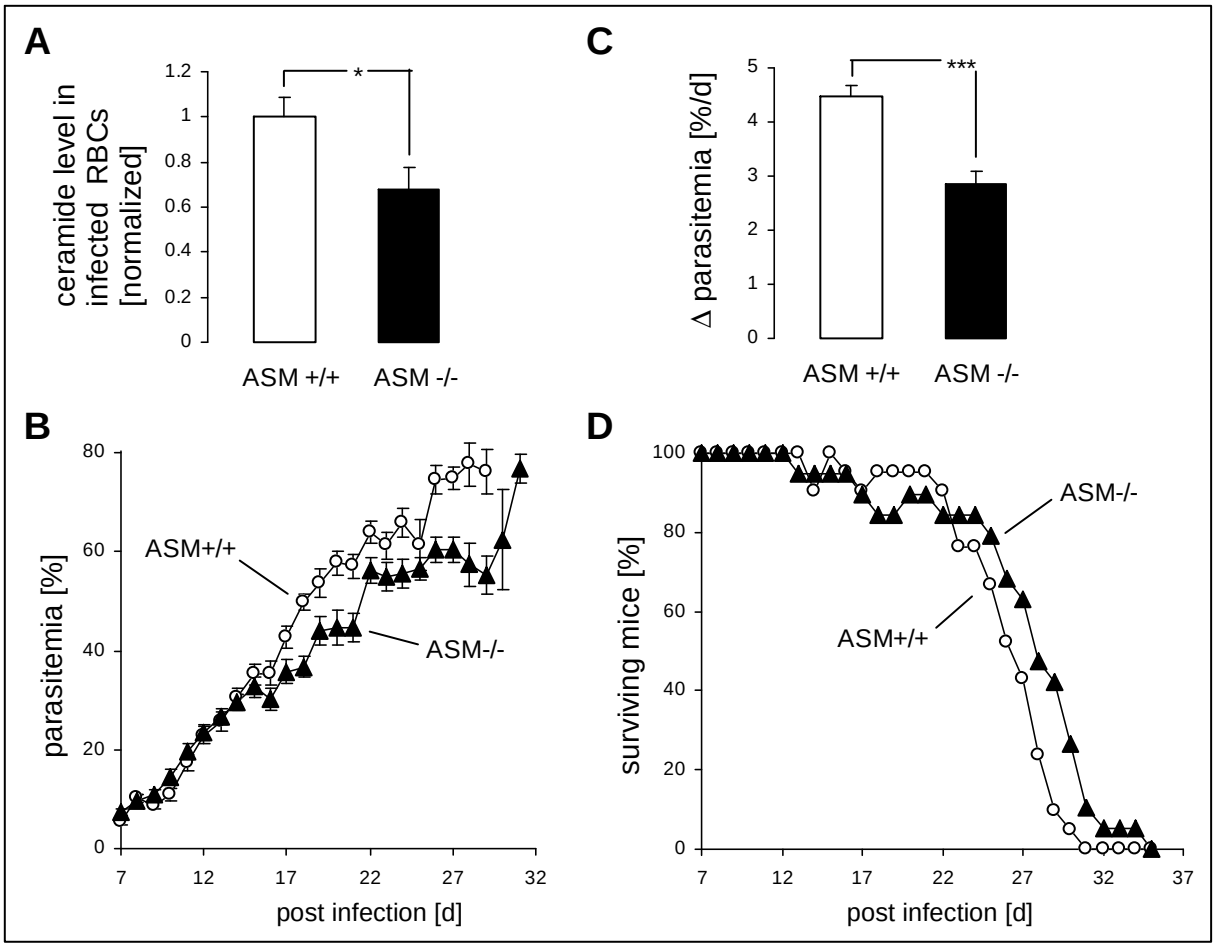


Figure 3.8. Functional significance of host acid sphingomyelinase ceramide production in *P. berghei* ANKA infected mice.

(**A**) Normalized ceramide level in infected ASM +/+ (open bar, n = 5, ±SE) and ASM -/- mouse blood (closed bar; n=5, 68 ± 10 % of infected ASM +/+), *: $P \leq 0.05$, significant, unpaired two-tailed student's t-test. *P. berghei* ANKA infection increased the ceramide level of infected RBC significantly less in ASM knockout mouse blood compared to the ceramide level in ASM wild-type mouse blood. (**B**) Time course of parasitemia in *P. berghei* ANKA-infected ASM -/- mice (closed triangles, n = 19, ±SE) and their wild-type littermates (open circles, n = 21, ±SE) (**C**) Increase in parasitemia as calculated from (**B**) by linear regression between day 10 and 21 post infection in infected wild-type (open bar, n = 20, Δ parasitemia [% / day] = 4.47 ± 0.22 %) and ASM -/- mouse blood (closed bar; n= 17; Δ parasitemia [%/ day] = 2.84 ± 0.25 %), *** : $p \leq 0.001$, two-tailed student's test. (**D**) Survival of *P. berghei* ANKA-infected ASM -/- (closed triangles, n = 19, ± SE) and wild-type mice (open circles, n = 21, ± SE); same experiments as in (**B**)).

3.2 Enhanced entry of *P. falciparum* infected sickle trait (HbA/S) RBCs into eryptosis as compared to normal (HbA/A) host erythrocytes

Sickle cell trait (HbA/S) carriers are partially protected against a severe course of malaria infection [1,8,17,25,29,82,84,154,185,193,226,290,296,318,387,524,555,581,602,639,677,896,895,894]. This partial resistance results, among other potential mechanisms, from early clearance of HbA/S RBCs [66,193,226] mediated by the phagocytic action of monocytes and macrophages. This mechanism acts as a first-line defense of the innate immune system against malaria infections [782]. Pronounced band 3 (AE1) aggregation of ring stage-infected HbA/S erythrocytes leads to binding of autologous IgG antibodies against band 3 and complement. These recognition signals (or "eat-me"-signals) facilitate phagocytosis and thus the clearance of malaria-infected HbA/S erythrocytes by macrophages already at ring-stage.

As a means of testing whether enhanced suicidal erythrocyte death (eryptosis) of the host erythrocyte [509,499] might also contribute to the partial malaria resistance of HbA/S carriers, *P. falciparum* infected sickle trait and normal RBCs were tested for differences in parasite growth and PS exposure. Furthermore, ceramide level, NSC channel activity, *i.e.* Ca^{2+} permeability, cytosolic Ca^{2+} activity and PGE_2 secretion were determined.

3.2.1 *P. falciparum* infected HbA/S and HbA/A RBCs differ in phosphatidylserine exposure but not in growth

Impaired *P. falciparum* growth and reinvasion into HbA/S RBCs has been considered as one mechanism of partial resistance of HbA/S carriers towards severe forms of *P. falciparum* malaria [298,300,669,667,193,226,381,724]. However, parasite prevalence and densities are usually similar in asymptomatic normal (HbA/A) and HbA/S carriers [581]. The results obtained in *in vitro* growth assays [298,300,669,667] could be of artefactual origin due to the hypoxanthine deficiency of the culture [298,300], or due to unphysiological conditions that do not mimic the situation *in vivo* [17,523]. *P. falciparum* infected HbA/A and HbA/S RBCs were synchronized to ring stage and grown for 48 h under normal culture conditions as a means of testing whether impaired *in vitro* growth and reinvasion occur under optimized culturing conditions. In the present study, *P. falciparum* developed similarly in HbA/A and HbA/S RBCs, confirming the results of previous authors [66].

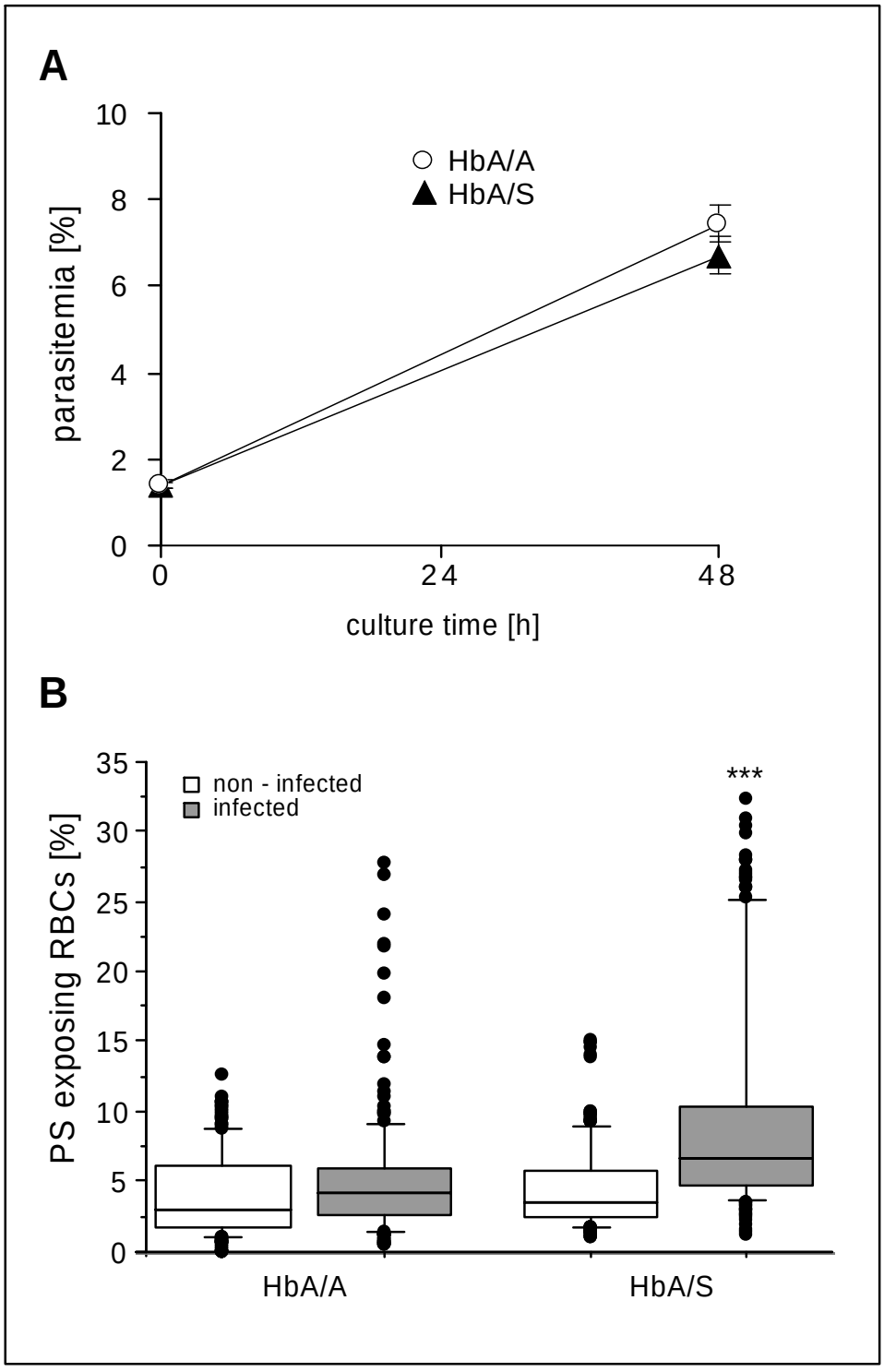


Figure 3.9. Normal and sickle cell trait RBCs infected with *P. falciparum* grown *in vitro* shows differences in phosphatidylserine exposure, but not in parasitemia.

(**A**) Parasitemia of synchronized *P. falciparum* infected normal (HbA/A) (open boxes, n = 187, 7.4 ± 0.4 %) and sickle cell trait (HbA/S) (gray boxes, n = 184, 6.7 ± 0.4 %) RBCs grown for 48 h under normal culturing conditions, starting with a parasitemia of 1.4 % ± 0.09 %. (**B**) Box-whisker plots of the average percentage of annexinV-binding RBCs, 44 - 54 h in culture after ring-synchronization. Same experiments as in (**A**). Box-whisker plots give medians, 25th and 75th percentiles, whiskers denote the 10th and 90th percentiles. All values are shown. Non-infected co-cultured (cohorts) HbA/A RBCs (open plot, left side, n = 187) had a median of 2.87 %

of PS exposing cells. Infected HbA/A RBCs (gray plot, left side, n = 187) had a median of 4.24 % PS exposing cells, whereas non-infected co-cultured HbA/S RBCs (open plot, right side, n = 184) had a median of 3.54 % PS exposing cells. These groups were not significantly different from each other. Infected HbA/S RBCs (grey plot, right side, n=184) demonstrated a significantly higher median of 6.56 % PS exposing cells. The highest annexin-binding value was 32.3 %. ***: $p \leq 0.001$, the average percentage of phosphatidylserine exposing cells among infected HbA/S erythrocytes was very significantly higher than in any of the other groups; nonparametric ANOVA (Kruskal-Wallis-test). 30 independent growth assays were carried out in six-fold determination. HbA/A and HbA/S RBCs were matched for age and storage conditions. Two different parasite strains FCR3 and BINH were used.

Annexin binding was determined by FACS analysis in order to investigate whether this human Hb gene mutation facilitates PS exposure on the outer leaflet of *P. falciparum* infected erythrocytes. *In vitro* infection of human erythrocytes with *Plasmodium falciparum* led to a significant increase in annexin-binding, *i.e.* to a higher PS exposure in infected HbA/S RBCs than in infected HbA/A RBCs and non-infected RBCs of either kind. Experiments were performed with nine different HbA/A and five different HbA/S blood samples (see appendix).

3.2.2 The ceramide level of *P. falciparum* infected HbA/S and HbA/A RBCs is similar

Ceramide production by sphingomyelinase stimulates either a scramblase (thereby enhancing the bilayer migration of PS), and/or inhibits the ATP-dependent aminophospholipid translocase (flipase) which actively transports PS from the outer to the inner membrane [510,499]. Ceramide was determined radioenzymatically in non-infected and infected HbA/A and HbA/S RBCs (Fig. 3.10.) as a means of testing whether the enhanced PS exposure in infected HbA/S RBCs results from a higher sphingomyelinase activity in HbA/S erythrocytes. In addition, in order to test for a functional significance of ceramide for parasite growth, infected HbA/A and HbA/S RBCs were synchronized and grown over 48 h in the presence of increasing concentrations of ceramide and subjected to annexin binding assays (Fig. 3.11.).

Non-infected HbA/S RBCs (open bar right, Fig. 3.10.) produced twice as much ceramide as non-infected HbA/A RBCs (open bar, left, Fig.3.10.). This effect was significant according to a two-tailed student's t-test for normalized values. However, none of the observed differences, including those found in *P. falciparum* infected RBCs, were significant in analysis of variance (ANOVA) due to enormous variations between individual samples, especially among infected RBCs.

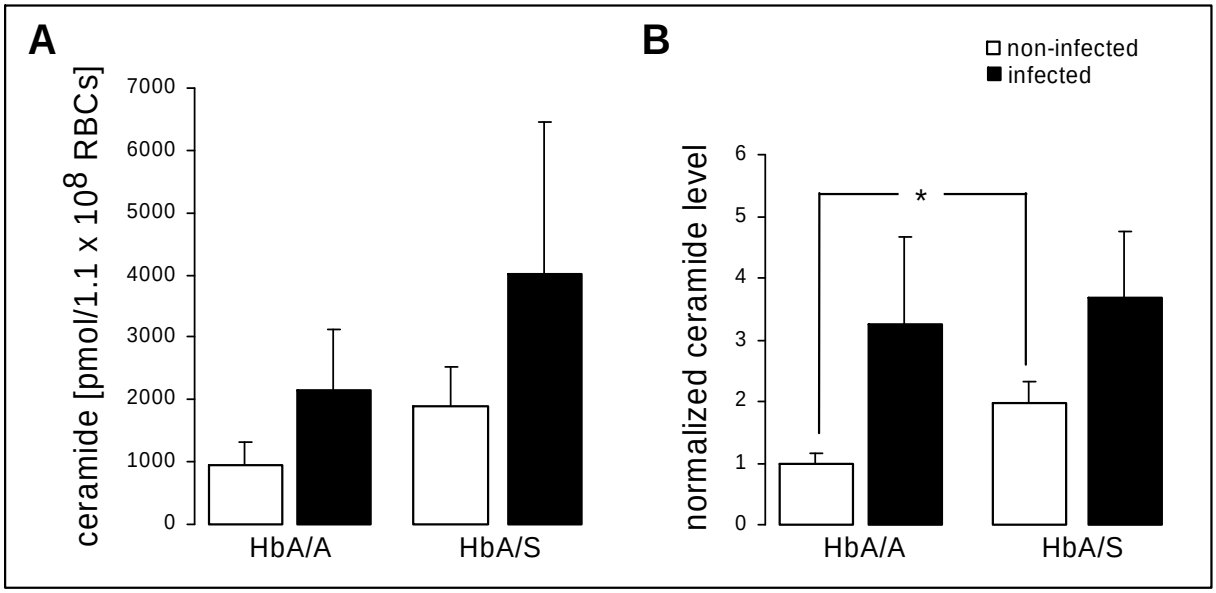


Figure 3.10. Ceramide level in non-infected and infected HbA/A and HbA/S RBCs.

A. The C16-ceramide level of 1.1 x 10^8 of HbA/A and HbA/S RBCs was determined radioenzymatically with the DAG Biotrak enzyme assay (n = 8, ±SE, for non-infected; n = 6 for infected RBCs; 8 or 6 independent experiments, performed as duplicates). Ceramide concentration was determined from the whole population of *P. falciparum* infected RBC cultures, which were all grown to high parasitemias (between 11.5 % and 30 %). Ceramide concentrations were determined as follows, neglecting any influence of infected on non-infected cells: $\text{Ceramide}_{\text{measured}} = [\text{ceramide}]_{\text{infected}} * (\text{parasitemia})\ \% + [\text{ceramide}]_{\text{non-infected measured}} * (100 - \text{parasitemia})\ \% \leftrightarrow$ $\text{Ceramide}_{\text{infected}} = ([\text{ceramide}]_{\text{measured}} - [\text{ceramide}]_{\text{non-infected measured}} * (100 - \text{parasitemia})\ \%) / (\text{parasitemia})\ \%$

B. Non-infected HbA/S RBCs (open bars, right, n = 8, ± SE) show a nearly two-fold concentration of ceramide as compared to non-infected HbA/A RBCs (open bars, left, n = 8, ± SE). * = $p \leq 0.05$; significant in student's t-test when values has been normalized (100 % = 1 for non-infected HbA/A RBCs). Infected RBCs (closed bars, n = 6, ± SE) produced more ceramide than non-infected RBCs, with a slightly higher ceramide concentration for infected HbA/S RBCs, which, however, was not statistically significant (ANOVA) due to enormous variations in the ceramide level among individual samples.

3.2.3 Ceramide impairs growth of *P. falciparum in vitro* more in HbA/S than in HbA/A erythrocytes

Ceramide has been demonstrated to play an important role in regulating signaling pathways such as cell senescence, cell cycle arrest, and programmed cell death in mammalian cells and yeast. Ceramide is able to activate or inhibit protein kinases and phosphatases, ion transporters, and other regulatory pathways [69,371,375,472,488,319,360,790,820]. It is produced either by SM hydrolysis or *de novo* synthesis. Synthesis of SM might play a role in the regulation of ceramide levels. By analogy, ceramide produced by *Plasmodium* or exogenously administered

ceramide and inhibition of ceramide production might modulate the intraerythrocytic development cycle of this parasite or induce eryptosis [371].

Therefore, additional experiments were performed to determine the effect of exogenous addition of ceramide to the culture medium on parasite growth and on PS exposure in infected and co-cultured non-infected HbA/S and HbA/A RBCs. Moreover, the effect of various SMase inhibitors on parasite growth was examined.

While increasing concentrations of short-chain cell permeable C6-ceramide did not induce PS exposure in non-infected RBCs (Fig. 3.11.I.A.), at concentrations greater than 10 µM C6-ceramide stimulated PS exposure to a similar degree in infected HbA/A and infected HbA/S RBCs (Fig. 3.11.I.B.). Also at lower concentrations (below 10 µM) ceramide impaired *P. falciparum in vitro* growth (Fig. 3.11.II.), confirming other recent results [663].

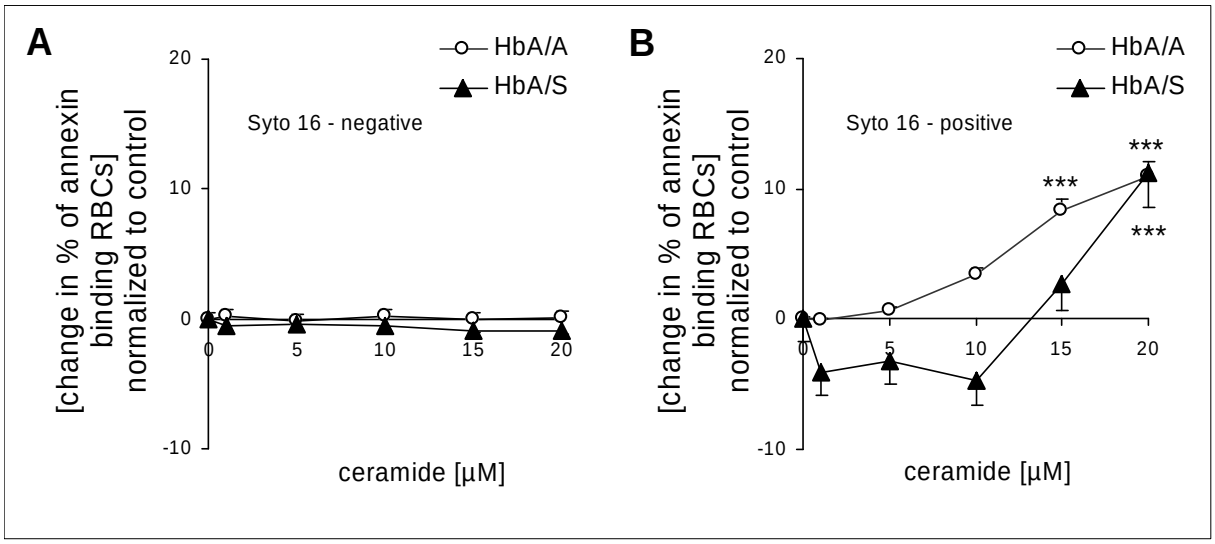


Figure 3.11.I. C6-ceramide stimulated PS exposure in *P. falciparum*-infected HbA/S and HbA/A RBCs to a similar degree.

A.B. Annexin-binding in % of non-infected (cohorts, Syto16-negative) (**A**) and infected (Syto16-positive) (**B**) normal (HbA/A, open circles, n = 29 - 30, ± SE) and infected sickle cell trait (HbA/S, closed triangles, n = 29 - 30, ±SE) RBCs grown for 48 h under normal culture conditions with different concentrations of C6-ceramide. Measured values have been normalized by subtracting annexin-binding as determined under control conditions. Annexin-binding of RBCs under control conditions: non-infected HbA/A = 3.4 ± 0.4, non-infected HbA/S = 4.3 ± 0.2 %, infected HbA/A = 3.3 ± 0.2 %, infected HbA/S = 19.7 ± 1.7 %. ***: $P \leq 0.05$; extremely significantly different from control conditions, ANOVA. Five independent growth assays were carried out in six-fold determination.

Importantly, parasite growth in HbA/S RBCs was more sensitive to exogenously added ceramide than parasite growth in HbA/A RBCs, suggesting that toxic concentrations of ceramide were reached earlier in HbA/S than in HbA/A RBCs (Fig. 3.11.II.). Relative *in vitro*

growth of *P. falciparum* in HbA/S RBCs was already significantly inhibited at an exogenous concentration of 5 µM ceramide, whereas more than 5 µM ceramide had to be applied to significantly inhibit parasite growth in HbA/A RBCs.

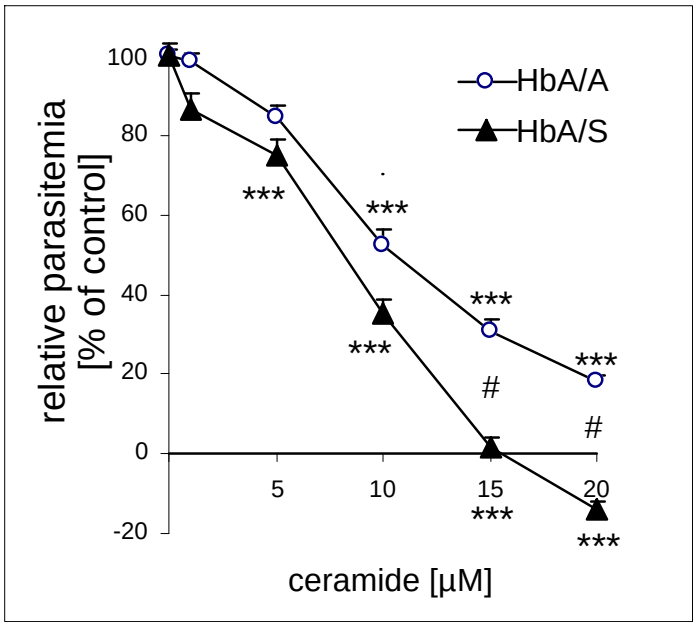


Figure 3.11.II. In the presence of C6-ceramide *in vitro* growth of *P. falciparum* is more inhibited in sickle cell trait than it is in normal infected RBCs.

Relative parasitemia in % of control of synchronized infected RBCs grown as described in Fig. 12. (HbA/A, open circles, n = 24 - 33, ± SE; HbA/S, closed triangles, n = 59 - 60, ± SE). Four to 10 independent growth assays were carried out in six-fold determination. Two different parasite strains FCR3 and BINH were used. Initial parasitemia has been subtracted.

***: $p \leq 0.001$, ANOVA, relative parasitemia highly significantly different from that under control conditions (0 µM ceramide): Relative parasitemia (HbA/A at 10 µM ceramide, open circles, n = 24) = 52.1 ± 4.4 %. Relative parasitemia (HbA/S at 5 µM ceramide, closed triangles, n = 60) = 74.7 ± 4 %.

#: $p \leq 0.01$; ANOVA; very significant difference in relative parasitemia between HbA/A and HbA/S RBCs under identical culturing conditions: Relative parasitemia (HbA/A at 15 µM ceramide, open circles, n = 24) = 30.8 ± 2.9 %. Relative parasitemia (HbA/S at 15 µM ceramide, closed triangle, n = 59) = 1.7 ± 2.4 %.

Taken together, these data suggest that the elevated PS exposure of infected HbA/S cells as compared to infected HbA/A cells did not result directly from elevated ceramide formation. Rather, ceramide in concentrations below 15 µM did not induce PS exposure (Fig. 3.11.I.B.). *P. falciparum* grew normally in untreated HbA/S erythrocytes (Fig. 3.9.), whereas (exogenously added) ceramide inhibited parasite growth (Fig. 3.11.II.). Therefore, there is nothing to suggest that under normal culture conditions ceramide might reach toxic concentrations leading to PS exposure.

3.2.4 Influence of sphingomyelinase inhibitors on *P. falciparum in vitro* growth

Ceramide is released by the activity of at least five forms of sphingomyelinases possessing either an acidic (I and II), neutral (III and IV), or basic (V) pH optimum. Isoforms of sphingomyelinases discovered in mammalian cells and bacteria include:

I. Acid sphingomyelinase (ASM) in lysosomes or endosomal compartments [788,586,746,279];
II. secreted Zn^{2+}-stimulated sphingomyelinase (functions best at acidic pH, but is also active at neutral pH and may be a posttranslationally modified product of ASM) [741];
III. neutral Mg^{2+}-dependent sphingomyelinase, bound to the plasma membrane [789,166,553,606,396,96] and found in the nucleus [634,692];
IV. cytosolic Mg^{2+}-independent neutral sphingomyelinase (NSM) [919,647]; and
V. alkaline sphingomyelinase localized in the gastrointestinal tract [641,236].

Apparently there is no cross-talk between the ASM and NSM ceramide-mediated signaling pathways in mammalian cells, but there is probably significant cross-talk between ceramide signaling and other signal transduction pathways (*e.g.* the PKC and MAP kinase pathways)[69]. However, little is known about sphingomyelinase expression and signaling pathways in infected erythrocytes. Therefore, the effect of exogenous addition of ASM and NSM inhibitors to the culture medium during parasite growth assays was investigated in the following experiments.

3.2.4.1 Ceramide production by acid sphingomyelinase is enhanced in *P. falciparum* infected HbA/S erythrocytes under hyperosmotic conditions

Infected normal and sickle trait RBCs were grown in the presence and absence of 2,4-DC, an effective ASM inhibitor. Parasite growth inhibition was concentration dependent and similar in both cell types, indicating similar ASM activities in infected HbA/A and HbA/S RBCs (Fig. 3.12.). Parasite growth was inhibited by up to one third. These results suggest that ASM only contributes to a minor part to the production of ceramide (unless the ASM inhibitor is not very specific).

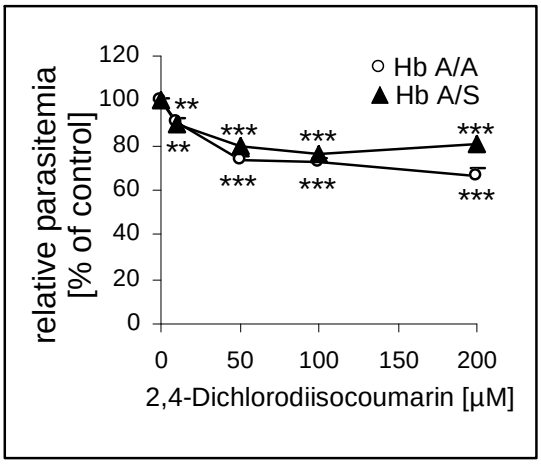


Figure 3.12.I. Effects of the acid sphingomyelinase inhibitor 2,4-dichloroisocoumarin (2,4-DC) on infected HbA/A and HbA/S RBCs.

Influence of 2,4-Dichloroisocoumarin (2,4 DC) on parasite growth (HbA/A: open circles, n = 23 - 24, ± SE; HbA/S, closed triangles, n = 24, ± SE). [2,4-DC] ≥ 10 µM – 200 µM inhibits *P. falciparum in vitro* growth highly significantly (**, P ≤ 0.01), extremely significantly (***, P ≤ 0.001), *i.e.* by up to 33 % (relative parasitemia ≥ 64 %). Four independent growth assays were carried out in six-fold determination. Two different parasite strains FCR3 and BINH were used.

PS exposure and ceramide formation are induced in non-infected RBCs by hyperosmotic shock [510,509,500]. In the present study, hyperosmotic culture conditions significantly inhibited *P. falciparum in vitro* growth ($IC_{50\ (NaCl)}$ =25 mM, raffinose (IC_{50} = 50 mM) and urea (IC_{50} = 140 mM); data not shown). Moreover, 50 mM NaCl very significantly increased PS exposure.

The possibility that hyperosmotic conditions might be able to disrupt the erythrocyte membrane, allowing annexin to bind to PS facing the cytosol, was ruled out. There was no hemolysis of infected RBCs under hyperosmotic conditions. Infected HbA/A RBCs, incubated for 8 h at 37°C under culturing conditions at 950 mOsm, *i.e.* with 650 mM sucrose added to the culture medium, showed 78.2 ± 2.3 % (n = 3) annexin-positive cells, while 17.8 ± 7.3 % (n = 3) were propidium iodide-positive [510]. These results show that propidium iodide-positive cells cannot account for the annexin-binding of infected RBCs.

Infected HbA/S RBCs incubated in 950 mOsm overnight served as positive control for the detection of ceramide in the outer membrane leaflet in infected HbA/S RBCs grown for 48 h hours in different concentrations of NaCl. Binding of monoclonal antibodies to ceramide was significantly increased after culturing parasites in HbA/S RBCs with an additional 50 mM NaCl RBCs, but not in non-infected HbA/S RBCs (data not shown; n = 6; binding of anti-ceramide at 0 mM NaCl for infected RBCs (with 7 ± 0.46 % parasitemia) = 0.72 ± 0.16 %, for non-infected RBCs of the same culture = 0.08 ± 0.02 %, at 50 mM NaCl (1.5 ± 0.07 %

parasitemia) = 5.3 % ± 0.67 %, for non-infected RBCs of the same culture = 0.15 ± 0.07 %).

We therefore examined whether inhibition of parasite growth in hyperosmotic medium is mediated by activation of erythrocyte ASM. Moreover, as infected HbA/S RBCs are microcytic and differ in osmotic resistance from HbA/A RBCs [17], it was investigated whether there might be a difference in sphingomyelinase activation between infected HbA/A and HbA/S RBCs under hyperosmotic conditions.

P. falciparum infected HbA/A RBCs were grown for 48 h in the presence or absence of an additional 50 mM NaCl or / and different concentrations of 2,4 DC (10 -200 µM). Addition of 50 mM NaCl to the culture medium inhibited parasite growth by 93 ± 1 %. Although 200 µM 2,4- DC alone decreased parasite growth, addition of 200 µM 2,4-DC together with additional 50 mM NaCl partially but significantly restored parasite growth (Fig. 3.12.II.). Addition of 100 µM 3,4-DC, another ASM inhibitor, in the absence and presence of 50 mM NaCl for 48 h to the parasite culture with HbA/A RBCs gave consistent results (data not shown).

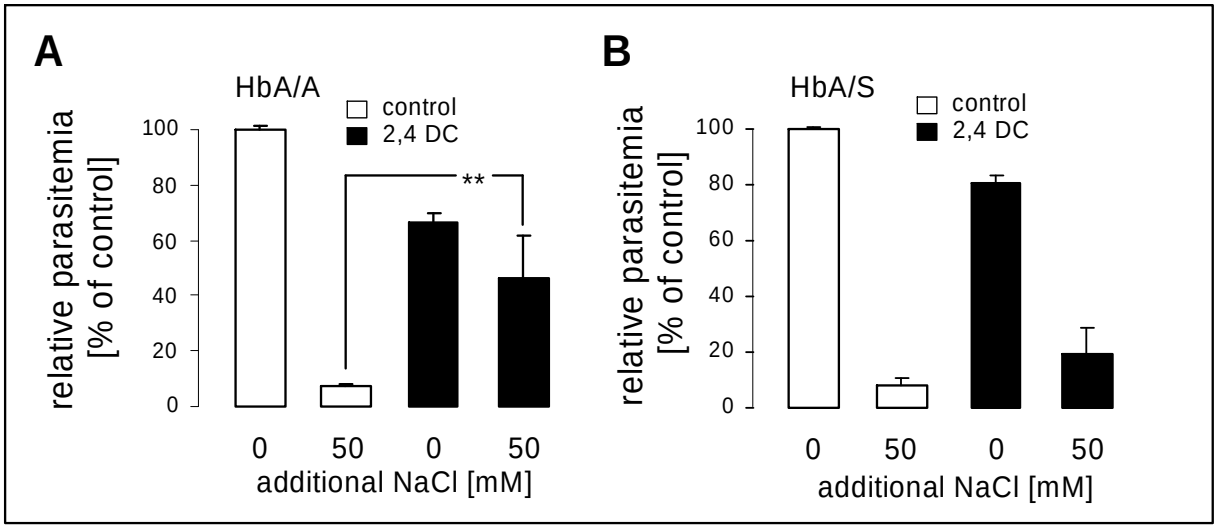


Figure 3.12.II. Acid sphingomyelinase inhibitor 2,4-DC blunts the hyperosmotically induced growth inhibition *in vitro* of *P. falciparum* in HbA/A RBCs, but not in HbA/S RBCs.

Relative parasite growth of synchronized infected HbA/A RBCs grown for 48 h under normal culture conditions (closed left bar) in the presence of 50 mM NaCl ± 2,4 DC (closed right bar without 2,4 DC, 7 ± 1 % relative parasitemia; and open right bar with 2,4 DC, 47 ± 15 % relative parasitemia) or 2,4 DC alone (open left bar, 67 ± 3% relative parasitemia). (**B**) Relative parasite growth of synchronized infected HbA/S RBCs grown for 48 h under normal culture conditions (closed left bar) in the presence of 50 mM NaCl ± 2,4 DC (closed right bar without 2,4 DC; 7.7 ± 2.6 % relative parasitemia, and open right bar with 2,4 DC; 19 ± 9.7 % relative parasitemia) or 2,4 DC alone (open left bar; 80.6 ± 2.4 % relative parasitemia).

Four independent growth assays were carried out in six-fold determination: n =24; ±SE; **: p≤0.01, ANOVA. Two different parasite strains FCR3 and BINH were used.

Similarly as with HbA/A RBCs, parasite growth in ring-synchronized HbA/S RBCs to which 50 mM NaCl had been added was inhibited significantly by 92.3 ± 2.6 %. DC alone decreased relative parasitemia less in HbA/S RBCs than in HbA/A RBCs, whereas DC up to 200 µM was not able to blunt significantly parasite growth inhibition in HbA/S RBCs under identical hyperosmotic conditions, in contrast to the results obtained with HbA/A RBCs.

NSM inhibitors, applied at concentrations facilitating about half-maximal parasite growth (Spiroepoxide, $IC_{50(HbA/A)}$ = 5 µM, $IC_{50(HbA/S)}$ = 3 µM and Epoxyquinone, $IC_{50(HbA/A)}$ =1.5 µM, $IC_{50(HbA/S)}$ = 1.5 µM) did not reverse the inhibitory effect of hyperosmotic shrinkage exerted by 50 mM NaCl on parasite growth in either HbA/A (n = 14 - 18) or HbA/S RBCs (n = 10 - 12; 2- 3 independent growth assays, data not shown).

3.2.4.2 *P. falciparum* infected HbA/A and HbA/S RBCs differ in their sensitivity to neutral sphingomyelinase inhibitors

Infected normal and sickle trait RBCs were grown in the presence and absence of epoxyquinone and spiroepoxide, both effective NSM inhibitors. Parasite growth inhibition was concentration dependent and similar in both cell types, but more effective in HbA/S RBCs.

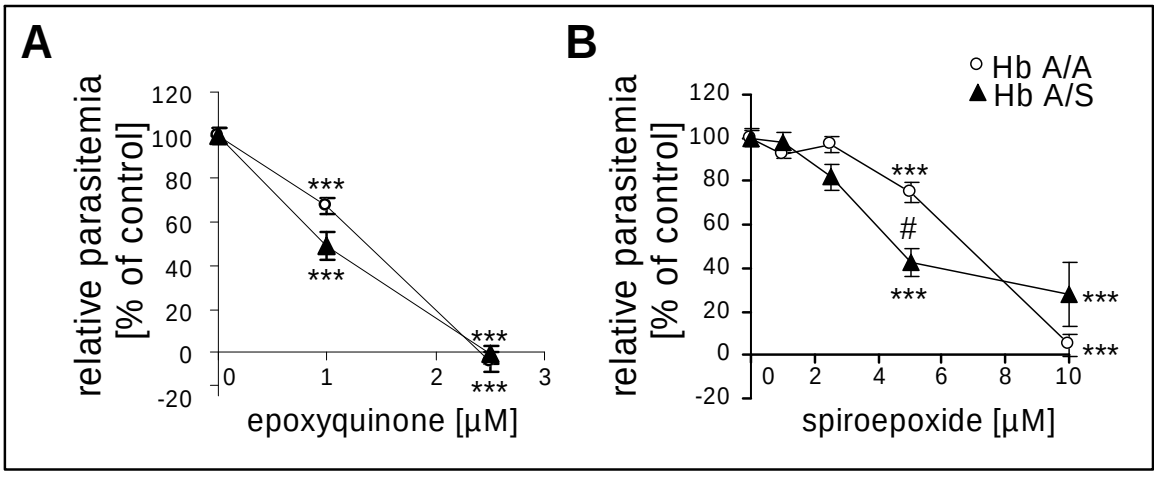


Figure 3.13. The IC_{50} of neutral sphingomyelinase inhibitors for *in vitro* growth of *P. falciparum* is lower in HbA/S than in HbA/A erythrocytes.

(A-B) Relative parasitemia [% of control] of synchronized infected HbA/A RBCs grown for 48 h under normal culture conditions in the presence of the neutral sphingomyelinase inhibitors (**A**) epoxyquinone 0 – 2.5 µM (HbA/A, circles, n = 18 - 53, ± SE; HbA/S, triangles, n = 12 - 36, ± SE); and (**B**) spiroepoxide 0 – 10 µM (HbA/A, circles, n = 16 - 51, ± SE; HbA/S, triangles, n = 18 - 42, ± SE).

The IC_{50} values for the neutral SMase inhibitor epoxyquinone applied during 48 h of parasite growth did not differ significantly for the two cell types: $IC_{50\ (HbA/A)}$ = 1.2 µM, $IC_{50\ (HbA/S)}$ = 1.0 µM (Fig. 3.13.A.). Nonetheless, the IC_{50} value for spiroepoxide was significantly lower for infected HbA/S than for infected HbA/A RBCs: $IC_{50\ (HbA/A)}$ = 5 µM, $IC_{50\ (HbA/A)}$ = 3 µM (Fig.3.13.B.). What plasmodial NSM activity has been detected so far resides at the parasite plasma membrane or in domains of the parasite vacuolar membrane – tubovesicular membrane network (PVM - TVM) [526]. However, the sensitivity of parasites grown in HbA/S RBCs towards NSM inhibitors was increased compared to parasites grown in HbA/A RBCs.

3.2.5 *P. falciparum* infection induces a higher Ca^{2+} permeability in HbA/S RBCs than in HbA/A RBCs

PS exposure in infected HbA/S erythrocytes might be enhanced due to stronger activation of the infection-induced NSC channel. This channel is permeable for Ca^{2+} [243] and can be activated by PGE_2 [499,440,437]. The following measurements were performed to determine whether accelerated PS exposure of *P. falciparum* infected HbA/S erythrocytes might be due to an enhanced activation of the NSC channel: cytosolic free Ca^{2+} concentration (Fig. 3.14.), Ca^{2+} permeability (Fig. 3.15.) and formation of PGE_2 (Fig. 3.16.) of non-infected and infected HbA/A and HbA/S RBCs were measured.

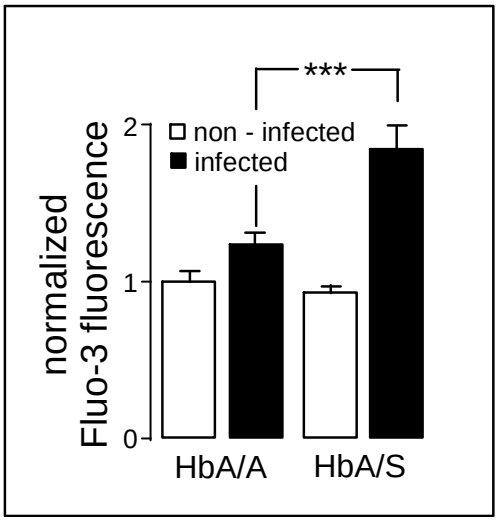


Figure 3.14. Free cytosolic Ca^{2+} in *P. falciparum*-infected human HbA/A and HbA/S erythrocytes.

Mean Fluo-3 fluorescence (n = 15, ± SE) reflecting the free Ca^{2+} concentration in non-infected (open bars) and infected (closed bars) HbA/A and HbA/S erythrocytes. ***: $p \leq 0.001$; ANOVA.

The free cytosolic Ca^{2+} concentration (free $[Ca^{2+}]_i$) was determined by Fluo-3 fluorescence. Free $[Ca^{2+}]_i$ was calculated from the geometrical means of the fluorescence histograms and normalized to the value of non-infected HbA/A RBCs. Infected HbA/S RBCs had an elevated free $[Ca^{2+}]_i$ around 1.5 fold of that in infected HbA/A RBCs, whereas free $[Ca^{2+}]_i$ was similar in non-infected HbA/A and HbA/S RBCs (Fig. 3.14.).

$^{45}Ca^{2+}$ uptake was determined in infected (corrected for parasitemia) and control non-infected HbA/A and HbA/S RBCs (Fig 3. 15.) as a means of investigating the Ca^{2+} permeability of the putative NSC channel in infected HbA/A and HbA/S RBCs. The difference in $^{45}Ca^{2+}$ uptake between infected HbA/A and HbA/S RBCs was particularly apparent in infected RBCs synchronized to ring stage of *P. falciparum*. In contrast, in later stages the differences in Ca^{2+} permeability between infected HbA/A and HbA/S disappeared (data not shown). The 30 min values of the $^{45}Ca^{2+}$ uptake measurements were pooled (Fig. 3.15.B.). Infected HbA/S erythrocytes, but not infected HbA/A erythrocytes markedly enhanced their Ca^{2+} permeability during the early ring phase of the parasite blood cycle.

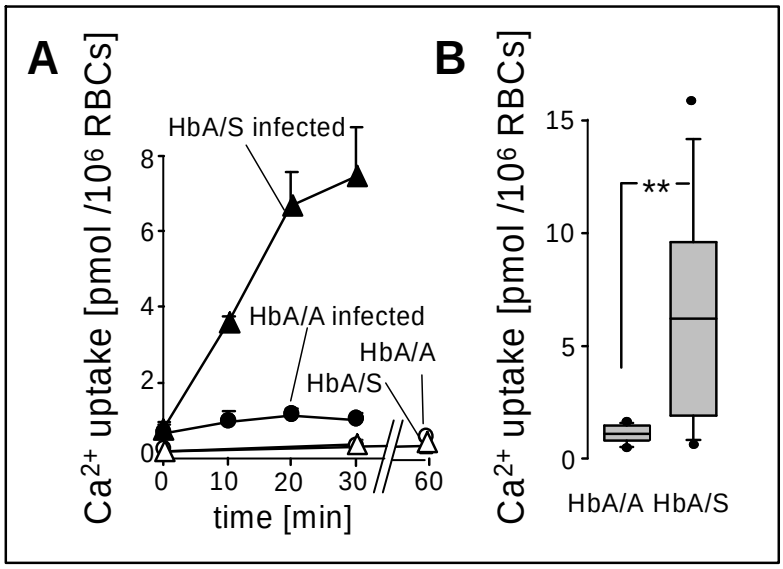


Figure 3.15. Ca^{2+} permeability in *P. falciparum*-infected human HbA/A and HbA/S erythrocytes.

(**A**) Uptake of $^{45}Ca^{2+}$ by non-infected and ring-stage-synchronized infected HbA/A and HbA/S erythrocytes in individual blood samples (in quadruplicate). Non-infected HbA/A RBCs: open circles, n = 4, ± SE; non-infected HbA/S RBCs: open triangles, n = 4, ±SE; infected HbA/A: closed circles, n = 4, ± SE; infected HbA/S: closed triangles, n = 4, ± SE. (**B**) Uptake of $^{45}Ca^{2+}$ by ring-stage-synchronized infected HbA/A and HbA/S erythrocytes; pooled data represented by box-whisker blot (30 min values, n=12). Box-whisker plots show medians and the 25th and 75th percentiles. Whiskers denote the 10th and 90th percentiles. All values are shown. **: $p \leq 0.01$; student's two-tailed t-test.

As PGE_2 is known to stimulate NSC channel activity [499,294,517,437], PGE_2 release of ring-stage synchronized *P. falciparum* infected HbA/A and HbA/S RBCs was measured over a time period of 48 h.

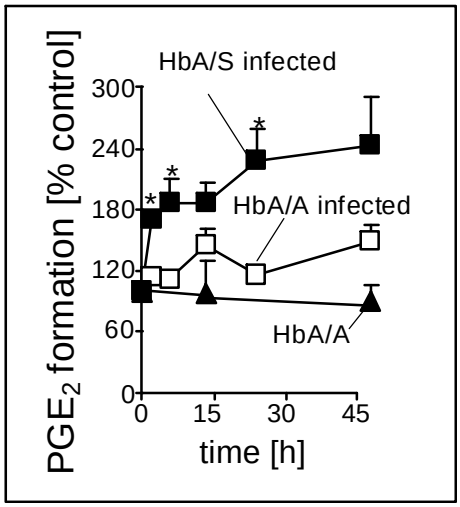


Figure 3.16. PGE_2 formation in *P. falciparum*-infected human HbA/A and HbA/S erythrocytes.

Average time-dependent PGE_2 release (n=8, ±SE) from non-infected HbA/A (closed triangles), infected HbA/A (open squares) and infected HbA/S (closed squares) RBCs given as % of initial values (98 ± 26 pg or 127 ± 32 pg PGE_2 secreted by 10^9 infected HbA/A or HbA/S RBCs, respectively; 26 ± 2 pg/10^9 non-infected HbA/A RBCs). *: P≤0.05, ANOVA between infected HbA/S and HbA/A erythrocytes. Samples were taken at 0 h, 1h, 3 h, 6 h, 12 h, 24 h and 48 h after synchronization.

Infected HbA/S RBCs released significantly more PGE_2 (normalized to the initial values at 0 h) than infected HbA/A RBCs, in particular during the first 24 hours after ring synchronization (Fig. 3.16.). Non-infected control HbA/A erythrocytes incubated under the same culturing conditions as infected RBCs for 48 h did not show enhanced PGE_2 release.

These results indicate a higher activity of the infection-induced NSC channel in ring-stage *P. falciparum* infected HbA/S RBCs as compared to ring-stage infected HbA/A RBCs, resulting in higher intracellular free Ca^{2+} concentrations in the former. The NSC channel might be stimulated through increased PGE_2 release in infected sickle-trait erythrocytes. This most probably leads to enhanced PS exposure in infected HbA/S RBCs.

4 Discussion

4.1 Functional significance of altered cation permeability for the intraerythrocytic amplification of *Plasmodium falciparum*

An obvious function of the infection-induced cation permeability is the replacement of K^+ by Na^+ in the host erythrocyte cytosol [796]. In the present study, parasite growth was significantly inhibited in a concentration-dependent manner when the culture medium of trophozoite-stage parasites was replaced for 8 hours (*i.e.* 24 – 32 h after synchronization) by mixtures of NaCl (100 % = control conditions) and N-Methyl-D-Glucamine-Chloride (NMDG-Cl) or KCl containing test solutions of different component ratios . In contrast to Na^+ ions, $NMDG^+$ ions are unable to permeate the red blood cell (RBC) membrane. Our experiments indicate that the inhibition of parasite growth cannot be attributed to a toxic effect of NMDG *per se*, as replacement of 50 % of the NaCl by raffinose in the test solution had the same effect on parasite growth as 50 % replacement by NMDG-Cl. This indicates an inhibitory effect of decreasing extracellular [Na^+] on parasite growth. Parasite growth was not inhibited when the NaCl test solution containing 126.7 mM [Cl^-] was substituted by Na-gluconate test solution containing Cl^- in a reduced concentration of 6.7 mM. This indicates that low extracellular Cl^- concentrations are sufficient to maintain parasite growth.

When NaCl was replaced by NMDG-Cl, half-maximal parasite growth (IC_{50}) was reached at a residual NaCl concentration of 24 mM NaCl (24 mM NaCl + 112 mM NMDG-Cl). When NaCl was replaced by KCl, parasites were around twice as sensitive towards the decrease in Na^+ concentration. Parasite growth was half-maximal (IC_{50}) at 58 mM NaCl and 65 mM KCl. These results indicate an additive inhibitory effect of the increasing K^+ concentration on parasite growth. These results suggest that the parasite requires not only an inwardly directed Na^+ gradient but also an outwardly directed K^+ gradient across its plasma membrane. The increase in [Na^+] and the decrease [K^+] in the host cytosol appear to benefit the parasite particularly during the second half of its intraerythrocytic development [796]. Moreover, inhibition of the Na^+ / K^+ pump by ouabain allows parasites to develop well in RBCs. This suggests that *P. falciparum* might also prefer high [Na^+] and low [K^+] within the RBC cytosol during the first half of its blood cycle [326,812].

Plasmodium falciparum trophozoites have high cytosolic [K^+] [528]. Moreover, they build up a plasma membrane potential by two discrete pump mechanisms that lead to

electrogenic extrusion of H^+. One is a V-type H^+ pump (H^+-ATPase) [24,731], and the other a Mg^{2+}-pyrophosphate complex-dependent H^+ pump (H^+-pyrophosphatase) [729]. Hence, both pumps generate a concentration and electrochemical gradient of protons across the parasite's plasma membrane. The K^+ leak of the RBC's membrane, *i.e.* the facilitated diffusion of K^+ out of the RBC, generates a K^+ diffusion potential across the parasite membrane that supports the electrogenic extrusion of H^+ out of the parasite [729] because the parasite plasma membrane hyperpolarizes both with decreasing erythrocytic [K^+] and increasing erythrocytic pH) [24]. Since the parasite plasma membrane depolarizes both with increasing erythrocytic [K^+] and decreasing erythrocytic pH, additional K^+ channel activity in the parasite plasma membrane would increasingly short-circuit the H^+ pump potential with increasing K^+ concentration in the host RBC cytosol [407]. Therefore, a low cytoplasmic [K^+] in the host RBC is needed to shift Nernst's K^+ equilibrium potential of the parasite's plasma membrane to negative values [407], *i.e.* to hyperpolarize the membrane. Thus, the K^+ leak generates a K^+ diffusion potential that counteracts the decrease in the erythrocytic pH generated by the H^+-pumps, thus inhibiting membrane depolarization. In this way the K^+ diffusion potential might reinforce the H^+ pump potential [24]. Since K^+ in the host RBC cytosol is replaced by Na^+, the parasite might utilize the increasing inwardly directed Na^+ gradient for Na^+-coupled transport, such as Na^+-coupled phosphate transport [732].

Invasion of the RBCs by merozoites, and parasite amplification within the erythrocyte between 20 and 26 hours after invasion are both strongly dependent on the Ca^{2+} concentration in the medium [866,868,867]. In line with these results, depletion of Ca^{2+} in the medium also halted parasite growth in the present study.

In conclusion, the parasite is dependent on Ca^{2+} influx and adapted to high Na^+ and low K^+ concentrations extracellularly. Such conditions are also encountered by the parasite's free swimming life stages, *e.g.* by merozoites in the blood of the mammalian host or by sporozoites in the hemolymphe of the mosquito vector and in human blood. On entering mammalian RBCs the parasite creates, as it were, an extracellular environment by increasing the cation permeability of the host RBC [407].

4.1.1 Characterization of infection-stimulated nonselective cation conductance

Ca^{2+} accumulates within the parasite in infected RBCs [118,531,813]. Trophozoites induce a 7-8 fold increase in cation conductance of the RBC, as demonstrated by patch-clamp

experiments [243]. Thus, amplification is facilitated by the infection-induced Ca^{2+}-permeable nonselective cation (NSC) conductance of the erythrocyte membrane (G_{cat}) supplying the parasite with Ca^{2+} ions. The infection-induced nonselective cation (NSC) currents exhibit a permselectivity for monovalent cations in the rank order: $Cs^+ \approx K^+ > Na^+ > Li^+ >>$ NMDG; $P_K/P_{Na} \sim 2$. The channel is significantly permeable to Ca^{2+} and is inhibited by amiloride, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), $GdCl_3$, and artemisinine, with sensitivity being highest to EIPA [244,243,242]. Removal of extracellular Cl^- activates G_{cat}. After activation, G_{cat} is 2.0 ± 0.3 nS (n = 32) in infected RBC, while in non-infected control RBC it is 0.3 ± 0.05 nS (n=16). This points to an induction of G_{cat} during infection [244]. Moreover, the NSC channel is activated by oxidative stress [507]. Oxidative stress imposed by the parasite on the erythrocyte during infection might stimulate G_{cat} [405]. The permselectivity, permeability and pharmacology of the *P. falciparum* infection-induced cation conductance are similar to those of the cation conductance induced in oxidized non-infected RBCs. This strongly suggests that the conductances in oxidized and infected RBCs are determined by one and the same channel type. However, in the present study, ring-stage infected normal (HbA/A) RBCs showed only a slightly increased Ca^{2+} permeability during patch-clamp recordings (data not shown).

EIPA is a very potent blocker of G_{cat}, as demonstrated by patch-clamp recordings [243]. In non-infected RBCs EIPA inhibits G_{cat} upon Cl^- removal and prevents eryptosis [512]. At the same time EIPA blocks Na^+/H^+ exchangers of the host cell [665] and possibly also of the parasite [126]. In the present study, the IC_{50} of EIPA observed in patch-clamp experiments on infection-induced cation conductance was 0.75 µM. In order to test for its effect on parasite growth, EIPA was applied to the normal culture medium for 48 h. EIPA hindered parasite development after synchronization with an IC_{50} of 3 µM free EIPA. This suggests a contribution of G_{cat} to parasite growth. The remaining difference in the IC_{50} values might be explained by the fact that in patch-clamp experiments the instantaneous NSC currents for mature trophozoites were measured upon application of EIPA. By contrast, in the growth experiments we investigated the influence of EIPA throughout the parasite's asexual life cycle. Moreover, the growth experiments permit no distinction between the influence of EIPA on Na^+/H^+ exchangers and that on the putative NSC channel.

Taken together, the effect of EIPA on parasite growth is no proof of a contribution of the infection-induced cation conductance to the induced cation exchange within the host cytosol, since EIPA also inhibits the Na^+/H^+ exchangers. Therefore, inhibition of host or parasite Na^+/H^+ exchangers may be an alternative explanation for the growth inhibition by EIPA [407]. Conversely, such an involvement of the NSC conductance would have had to be

excluded, if EIPA had failed to inhibit parasite growth.

In sharp contrast to non-infected cells, G_{cat} of infected RBC is inhibited by new permeation pathway (*NPP*) blockers furosemide and 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), as measured in patch-clamp experiments [243]. These blockers also inhibited parasite growth dose-dependently (with an IC_{50} (NPPB) = 60 µM and an IC_{50} (furosemide) around 100 µM, data not shown). The observed sensitivity to the anion channel inhibitors furosemide and NPPB suggests a unique channel that generates anion and cation currents in infected RBCs. This broad specificity channel might preferentially conduct cations when the concentration of permeable anionic substrates is decreased. However, infection-induced anion and cation currents studied in patch-clamp experiments differ in their EIPA sensitivity and their dependence on extracellular Cl^-. The removal of extracellular Cl^- (*i.e.* in the bath solution) stimulates the cation currents, but not the anionic inward currents carried by the efflux of Cl^- from the infected RBCs into the bath solution. Hemolysis and tracer flux data demonstrate a similar permselectivity for infected RBCs as the infection-induced cation currents measured by patch clamp whole cell recordings, namely in the rank order of $Cs^+ > K^+ > Na^+ \approx Li^+$. Specifically, the K^+ over Na^+ permeability ($P_K/P_{Na} \sim 2$) observed in tracer flux studies of infected RBCs [465,796] matches that of the NSC currents determined by patch clamp.

The data strongly suggest that the Cl^--dependent NSC currents are the electrophysiological correlate of the infection-induced permeability for Ca^{2+}, Na^+ and K^+. The NSC channel activity accounts for the dramatic changes observed in host cation concentrations. The characteristics of NSC permeability are very similar in non-infected oxidized and in infected RBCs. These similarities point to the possibility that the infection-induced cation permeability might develop through modification of endogenous RBC cation channels, *i.e.* through oxidative stress conferred by the parasite. Mature RBCs are continuously exposed to high oxidative stress throughout their 120 day life span [459]. As the NSC channel is activated by oxidative stress, the NSC channel might sense RBC age or injury *in vivo* [407]. In any case, the NSC channel is a key factor in erythrocyte programmed cell death, also referred to as eryptosis [509], and plays a key role during *P. falciparum* infection.

4.2 HbA/S erythrocytes do not inhibit *P. falciparum in vitro* growth

Parasite prevalence and densities are usually similar in HbA/A and HbA/S carriers with mild *P. falciparum* infection [581]. Moreover, sickling of infected HbA/S RBCs does not

occur upon invasion. Thus, sickling cannot be the mechanism of protection [251,524,523]. The inhibition of parasite growth in culture observed in several studies by Friedmann *et al.* and Pasvol *et al.* [298,300,669,667] might have been be an artifact caused by the culturing conditions used [17]. For example, Friedman *et al.* [298,300] did not add hypoxanthine to the culture medium. However, hypoxanthine is essential for parasite growth. Therefore, the culture medium of the present study was supplemented with 0.2 mM hypoxanthine. Moreover, the RBCs used were old. This can have influence on parasite growth (own observations). $[Ca^{2+}]_i$ is known to accumulate with age [21], thus triggering cell shrinkage through Ca^{2+}-activated K^+ channels (GARDOS effect). As the membrane resting potential is at – 10 mV, which is close to the Cl^- reversal potential, loss of K^+ leads to hyperpolarization. This drives Cl^- out of the RBC, followed by osmotically obliged water loss [313,776,829,542]. It is only in the presence of the Ca^{2+} ionophore A23187 that the Gardos channel is activated in infected HbA/A RBCs [485,795,829]. Friedman *et al.* [301] have shown that inhibition of parasite growth in HbA/S RBCs at 3% O_2, caused by increased leakage of K^+ ions from the infected RBC, is reversed in a K^+-enriched medium [631]. These results suggest that the Gardos effect might have caused the inhibition of parasite growth as increase of extracellular $[K^+]$ limits hyperpolarization by altering the reversal potential for K^+ [333,501,515,625].

Pasvol *et al.* [669,667] used the more appropriate medium 199 containing in particular 0.3 mg/l hypoxanthine and 10 mg/l adenine [17]. The raw data of Pasvol *et al.* [669] (Table 1) should demonstrate that under ambient oxygen tensions, parasites invade and grow similarly in HbA/S and HbA/A RBCs, whereas at reduced oxygen levels (5%) invasion and, to some degree, growth is inhibited [669,667]. However, these results are not statistically significant, as has already been noted elsewhere [17,523]. This indicates that the hypothesis supporting the view of *P. falciparum* growth inhibition in HbA/S RBCs has not been experimentally substantiated to date.

The parasite growth rate in HbA/S RBCs in a low oxygen environment (3 %) is comparable to that in HbA/S RBCs cultured with the candle-jar method. This has been tested for a time period of at least one month [655]. Therefore, both methods were applied in this study. Consistent with Ayi *et al.* [66], who have shown no significant difference in *P. falciparum* growth in HbA/A and HbA/S RBCs under optimized culture conditions even over three asexual cycles, we, too, were unable to detect different parasite growth rates in HbA/A and HbA/S RBCs.

4.3 Functional significance of ceramide production

4.3.1 Increased ceramide production in blood of *P. berghei* infected mice

The development of *Plasmodium spp.* changes the lipid composition of the host cell membrane [774,775]. The erythrocyte membrane of *P. falciparum* infected RBCs contains altogether less sphingomyelin (SM) (28.0% in non-infected RBCs *versus* 14.6% in infected RBCs), more phosphatidylcholine (PC; 31.7 % in non-infected RBCs *versus* 38.7% in infected RBCs) and more phosphatidylinositol (PI; 0.8% in non-infected RBCs *versus* 2.1% in infected RBCs) [404]. In the outer membrane leaflet of trophozoite-infected RBCs PI, phosphatidylethanolamine (PE), and phosphatidylserine (PS) content increases, while PC content decreases. Moreover, schizont-infected RBCs show a further decrease in SM and increase in PS content of the outer membrane leaflet [575,574]. The cholesterol/phospholipids ratio decreases by 55% because the parasite uses cholesterol [404]. The unsaturation index of phospholipids in infected RBCs is considerably lower than in non-infected RBCs (107.5 *versus* 161.0) [404]. This is due to the decrease in arachidonic acid (C20:4; from 17.36 % to 7.85 %) and docosahexaenoic acid (C22:6; from 4.34 % to 1.8 %), and increase in palmitic acid (C16:0) (from 21.88% to 31.21%), linoleic acid (C18:2) and oleic acid (C18:1) [404,858]. This makes the fatty acid compositon of the infected erythrocyte membrane very similar to that of the parasite's membrane [404,858].

P. falciparum infected RBCs have a single Mg^{2+}-dependent, membrane bound, parasitic neutral sphingomyelinase (NSM) [371], probably located in the parasitophorous vacuolar membrane (PVM), which is continous with the tubovesicular membranes (TVM) [526]. The observed decrease in SM in the outer membrane leaflet of infected RBCs [404,858] suggests that SM might be hydrolysed to ceramide and phosphocholine by a sphingomyelinase of erythrocytic and / or parasitic origin. In mammalian cells, ceramide derived from NSM activity is thought to be involved in modulating phospholipase A_2 (PLA_2) activity, arachidonate mobilization, and other enzymes involved in arachidonate metabolism including cyclooxgenase (COX) [69]. Mobilization of arachidonate and stimulation of COX might result in PGE_2 secretion. This leads to increased NSC channel activity in RBCs and influx of Ca^{2+}, thus contributing to PS exposure [517]. On the other hand**,** arachidonate increases SM hydrolysis and ceramide production in mammalian cells [427]. Ceramide is a signaling molecule involved in the second known pathway of eryptosis [519] that merges with the first one, increasing PS exposure. Ceramide sensitizes the scramblase to the effects of Ca^{2+} [509]. Increase in

erythrocytic $[Ca^{2+}]_i$ activates the scramblase [94,218,900,913,926], which enhances PS exchange within the membrane bilayers [780,900,899]. The scramblase acts antagonistically to an ATP-dependent translocase which transports PS from the outer to the inner membrane leaflet [61,101,184,233,913,922]. Increased $[Ca^{2+}]_i$ inhibits the translocase [108,780,832,923], thus ultimately stimulating PS exposure on the outer leaflet of the membrane [935].

In order to investigate the contribution of erythrocytic acid sphingomyelinase (ASM) activity to ceramide formation in infected RBCs ASM wildtype (ASM +/+) and ASM knockout (ASM -/-) mice were infected with *Plasmodium berghei* ANKA and then examined for parasitemia, survival rate and ceramide production.

ASM+/+ and ASM-/- mice infected with *P. berghei* ANKA showed a significant increase in ceramide production. This suggests that parasitic NSM activity most probably contributed to the increased ceramide level in both types of mice. The ceramide concentration in infected ASM+/+ RBCs was significantly higher than in infected ASM-/- RBCs. This suggests that the host ASM contributed significantly to enhanced ceramide levels in infected RBCs. ASM is secreted by leukocytes as mouse erythrocytes do not have any own ASM activity [520]. Probably the enhanced ceramide concentration resulted in enhanced PS exposure in infected ASM+/+ mice. This assumption is supported by the fact that after postincubation *ex vivo* for 24 h at 4°C PS exposure is significantly higher in infected ASM+/+ blood than it is in infected ASM-/- blood. Freshly taken blood samples do not show any annexin-binding [734]. This is probably due to the elimination of PS exposing RBCs within the organism.

We investigated whether this pathway could also contribute to the observed enhanced PS exposure in *P. falciparum* infected HbA/S RBCs as compared to that in infected HbA/A RBCs. A twofold increase in ceramide production was measured in non-infected HbA/S as compared to HbA/A RBCs. The ceramide concentration in infected HbA/S and HbA/A RBCs was on average higher than in the respective non-infected RBCs and it was slightly higher in infected HbA/S than in infected HbA/A RBCs. However, in contrast to infected mouse RBCs the difference between non-infected and infected human RBCs was not significant, because ceramide concentration between individual RBC samples varied enormously, particularly in infected RBCs. As repeat measurements showed good within-sample consistency, the variation might be due to the differences in genetic background [445]. Moreover, differences in ceramide production between infected mice and humans might also result from different sphingolipid compositions in the blood [205].

4.3.2 Ceramide is needed for *Plasmodium* membrane synthesis

The survival rate of infected ASM-/- mice was higher than that of their wild-type littermates, but not significantly different. Reduced parasitemia in ASM-/- mice between the 10th and the 21st day post infection as compared to that in their wild-type littermates correlated with a significantly decreased ceramide production in ASM -/- mice. This is possibly caused by a lack of ceramide in ASM-/- mice, since parasite membrane synthesis requires ceramide [371].

As judged from available results, the parasite uses membrane components like ceramide and cholesterol from the host erythrocyte for its own membrane synthesis including the TVM [371]. The TVM allows the parasite access to nutrient solutes [527], delivers transmembrane proteins to the PVM [525], and is thought to play a role in the metabolism and sorting of membrane lipids [308]. The tubular structures contain a Golgi-specific SM synthase which is active in regions, where ceramide accumulates [260,261,367,663]. The non-infected host erythrocyte itself is incapable of SM synthesis [663]. Attempts to detect *de novo* ceramide synthesis in infected RBCs by metabolic labeling with [^{14}C] serine and [^{14}C] palmitate were not successful to date [258,860]. However, a recent study was successful in demonstrating *de-novo* synthesis of glycosphingolipids by *P. falciparum* using metabolic labeling with triated ([^{3}H]) serine and [^{3}H] glucosamine [323]. Although well-known inhibitors of *de-novo* ceramide biosynthesis affected the synthesis of glycosphingolipids, they were unable to arrest the intraerythrocytic development of the parasites in culture [323]. This suggests that *de novo* synthesized ceramide does not serve as a major substrate for the SM synthase in developing the TVM [526], as is the case with ceramide derived from hydrolysis of host sphingomyelin and glycosphingolipids [404,525,574].

4.3.3 Effect of neutral and acid sphingomyelinase inhibitors on parasite growth

This study showed that the NSM inhibitors spiroepoxide and epoxyquinone effectively inhibited *P. falciparum* growth *in vitro* in HbA/A and HbA/S RBC. The IC_{50} for parasite growth was lower for infected HbA/S RBCs. This difference was significant for spiroepoxide ($IC_{50\ (HbA/A)}$ = 5 µM *versus* $IC_{50\ (HbA/S)}$ = 3 µM) but not for epoxyquinone ($IC_{50\ (HbA/A)}$ = 1.2 µM *versus* $IC_{50\ (HbA/S)}$ = 1.0 µM). The parasite growth inhibition was consistent with results

showing that scyphostatin, a mammalian NSM inhibitor, decreases parasite growth in HbA/A RBCs in a dose-dependent manner, as judged by [^{3}H] hypoxanthine incorporation, with an IC_{50} value of 7 µM [372]. Scyphostatin has in particular been found to inhibit the maturation from trophozoite to schizont stage, a phase coinciding with *P. falciparum* NSM expression [372].

In the present study, parasite growth was more sensitive to NSM inhibitors in HbA/S RBCs than it was in HbA/A RBCs, while the ASM inhibitor 2,4-DC had the same effect on parasite growth in both RBC types when applied in normal culturing conditions. Moreover, application of NSM inhibitors totally stopped parasite growth, whereas 2,4 DC inhibited parasite growth by up to one third when applied up to a concentration of 200 µM.

As judged by these results, parasitic NSM degrades SM from the host erythrocyte for its own membrane synthesis. This is essential for the parasite's survival, as inhibition of NSM activity results in parasite death. Beyond this, ASM of host origin contributes significantly to ceramide production. It appears that ASM activity is not as important as NSM activity is for parasite development, since neither genetic knock-out of ASM nor the ASM inhibitor 2,4-DC were able to entirely stop *Plasmodium* growth.

4.3.4 Exogenously added C6-ceramide and excess ceramide production inhibits growth of *P. falciparum in vitro*

Activation of ASM by hyperosmotic shrinkage results in ceramide formation, a process involved in programmed cell death [213,344,360,510,533,820]. Consistent with this, in the present study NaCl (IC_{50} = 128 mM, *i.e.* 25 mM above the regular level), raffinose (IC_{50} = 50 mM) and urea (IC_{50} = 140 mM), each inhibited parasite growth to a similar degree in HbA/A and HbA/S RBCs (data not shown). Ginsburg *et al.* [326] has shown that this is not due to parasite dehydration. In the present experiments significant PS exposure was detected both in infected HbA/S and HbA/A RBCs but not in non-infected RBCs cultured for 48 h with 153 mM NaCl, *i.e.* 50 mM above the regular level. Moreover, at 50 mM additional NaCl ceramide content in the outer membrane leaflet was significantly enhanced in *P. falciparum* infected HbA/S RBCs compared to that in non-infected RBCs of the same parasite culture. When applied at a concentration of 200 µM to HbA/A RBCs the ASM inhibitor 2,4-DC partially but significantly reversed the *P. falciparum in vitro* growth inhibition that had previously been induced by adding 50 mM NaCl. No significant inhibition reversal was observed in HbA/S

RBCs, however.

When added for 48 h, short-chain RBC membrane permeable C6-ceramide inhibited *P. falciparum* growth in both HbA/S and HbA/A RBCs, but more so in HbA/S RBC ($IC_{50(HbA/A)}$ = 10 µM; $IC_{50(HbA/S)}$ = 8 µM). Consistent with the results of the present study, Pankova-Kholmyansky *et al.* [663] has shown that a membrane permeable analogue of ceramide C6, added for 6 h to an unsynchronized *P. falciparum* culture, results in inhibition of parasite growth in HbA/A RBCs with an IC_{50} value of 60 µM ceramide. Addition of ceramide decreases glutathione levels in infected RBCs. The antimalarial drugs artemisinine and mefloquine increase ceramide concentration in *P. falciparum* infected RBCs. Inhibitors of *de novo* synthesis of ceramide cannot counteract the antimalarial action of these two drugs. Conversely, low concentrations of sphingosine-1- phosphate reverse parasite growth inhibition for any of the three inhibitors ceramide, mefloquine and artemisinine [663]. Sphingosine-1-phosphate, a metabolite of ceramide, is an antagonist of short chain ceramide in mammalian cells and also in RBCs [183,204,339,374,473,587,645,693].

In the present study, C6-ceramide added in concentrations up to 10 µM did not induce PS exposure in either infected HbA/A or HbA/S RBCs. At higher concentrations, ceramide induced PS exposure both in *P. falciparum* infected HbA/S and HbA/A RBCs to a similar degree. However, the basal PS exposure at control conditions, *i.e.* normal culturing conditions, was significantly higher in HbA/S RBCs than in HbA/A RBCs.

Taken together, these data suggest that inhibition of parasite growth under hyperosmotic conditions is due to ceramide production by erythrocytic ASM. Infected HbA/S RBCs might be more vulnerable to stress stimuli like osmotic stress than infected HbA/A RBCs, resulting in enhanced ceramide formation in infected HbA/S RBCs. Hence, additional stress involving increased ceramide formation would result in stronger inhibition of parasite growth in HbA/S RBCs than in infected HbA/A RBCs. This was confirmed in the present study by the finding that under similar hyperosmotic conditions inhibition of parasite growth in HbA/S RBCs could not be reversed by 200 µM of the ASM inhibitor 2,4 DC whereas in HbA/A RBCs it could. In line with this assumption, the IC_{50} value of ceramide for parasite growth was lower in HbA/S than in HbA/A RBCs.

In conclusion, although ceramide is needed for membrane synthesis of the parasite, in excess it acts as a lipid second messenger in inducing parasite cell death. These lethal effects on infected RBCs can be reversed at signal transduction level. Considering that *P. falciparum* grew normally in HbA/S RBCs, enhanced ceramide production cannot be the cause of the enhanced PS exposure on infected HbA/S RBCs observed in the present study.

4.4 Accelerated eryptosis in *P. falciparum* infected HbA/S erythrocytes

4.4.1 PGE_2 formation of *P. falciparum* infected HbA/S RBCs is enhanced compared to that of infected HbA/A RBCs

Prostaglandins (PGs) are important mediators of macrophage activity, vascular permeability, fever, erythropoiesis, and proinflammatory responses to infection. They play an important role in protective immunity [676].

P. falciparum itself also produces PGD_2, PGE_2, and PGFα, as measured by enzyme immunoassay and gas chromatography/selected ion monitoring [460]. Addition of arachidonic acid to the parasite cell culture markedly increases the ability of the parasite cell homogenate to produce PGs. Moreover, after addition of arachidonic acid, PGs are secreted from the infected RBCs into the culture medium. PGD_2 and PGE_2 accumulate at the trophozoite and schizont stages more actively than at the ring stage. PG production in the parasite homogenate is not affected by the mammalian COX inhibitors aspirin or indomethacin and is partially heat resistant. By contrast, mammalian PG synthesis is completely inhibited by both drugs and by heat treatment, suggesting that the parasite uses other enzymes to synthesize PGs than its mammalian host [483]. These plasmodial PGs are thought to be pyrogenic and injurious to host defenses [460].

Children with malaria anemia in consequence of *P. falciparum* infection have significantly lower plasma concentrations of bicyclo-PGE_2 (a stable end product of PGE_2 metabolism) and leukocyte cyclooxygenase (COX_2) gene expression than children with asymptomatic parasitemia [677]. PGE_2 (β-hydroxyketone) is synthesized from arachidonate via PGG_2 and PGH_2 by cyclooxygenase (prostaglandine G/H synthase) that contains two distinct activities: the cyclooxygenase bisdioygenates arachidonic acid PGG_2, which is then reduced by the peroxidase to form the hydroxyl endoperoxide PGH_2. Endoperoxide E isomerase (prostaglandine E_2 synthase) act on PGH_2 to produce PGE_2 [88,599]. Children with asymptomatic parasitemia have the highest bicyclo-PGE_2 / creatinine plasma concentration among *P. falciparum* infected children [677].

In vivo, PGE_2 induces preferential synthesis of fetal hemoglobin (HbF). An increase in HbF, one of the mechanisms of hydroxyurea treatment [373,658,685], has been shown to be beneficial in sickle cell disease due to the high O_2 affinity of HbF, hindering polymerization

of HbS/S RBCs**.** An investigation of the effects of PGE_2 in association with erythropoietin on the synthesis of fetal and adult Hb in peripheral blood-derived erythroid burst colonies from normal adults and from patients with sickle cell anemia, has shown a preferential synthesis of fetal Hb (HbF) after PGE_2 treatment [206]. In addition, there was an increase in the overall Hb synthesis in PGE_2 treated colonies. Furthermore, the reactivation of HbF production by PGE_2 is more pronounced when adherent cells are included in the culture dishes [206]. Thus, enhanced PGE_2 production in *P. falciparum* infected sickle trait carriers might also counteract the effects of the accelerated destruction of erythrocytes during malaria infection. Increased HbF synthesis resulting in higher O_2 affinity of the RBCs might reverse some of the consequences of anemia.

In the present study, PGE_2 release from *P. falciparum* infected HbA/S RBCs was enhanced for a period of 24 hours after ring synchronization as compared to infected HbA/A RBCs. The observed decrease in arachidonic acid in the erythrocyte membrane of infected RBCs [404] suggests that prostaglandin (PG) is synthesized by the parasite and / or the host. It has been previously shown that human erythrocytes synthesize PGE_2 after Cl^- removal [517]. This indicates that PGE_2 is, at least in part, synthesized by the erythrocyte.

Addition of PGE_2 during patch clamp experiments stimulates the NSC conductance in erythrocytes [517] as well as in nucleated cells [294]. Culturing of these cells with PGE_2 increases PS exposure [294,517]. Gene silencing of the Ca^{2+}-permeable transient receptor potential cation channel TRPC7, a NSC channel and member of the canonical subfamily of transient receptor potential (TRPC) channels, significantly blunts PGE_2-induced triggering of PS exposure and DNA fragmentation in the human leukemia cell line K562 [294].

PS exposure of infected HbA/S erythrocytes involves COX activity [443]. Treatment with diclophenac, a COX inhibitor, results in decreased PS exposure of early-stage infected HbA/S RBCs [443]. Thus, PGE_2 formation in sickle cell trait carriers may promote PS exposure, accelerating elimination of the parasite harboring erythrocytes. The present results might give a mechanistic explanation for the clinical observation that a milder clinical course of *P. falciparum* malaria in children correlates with enhanced PGE_2 plasma concentrations [677,676].

The present observations, however, do not rule out other mechanisms contributing to the partial resistance of sickle cell trait carriers to malaria. Platelet COX, platelet and endothelial cell activation could be involved, resulting in release of other prostaglandins, prostacyclins, thromboxanes, besides other products of platelet and endothelial cell metabolism [147,165,295,343,399,418,487,597,650]. Together with PGE_2 these substances might influence erythrocyte morphology and adhesion [48,120,140,248,333,349,356,430,431,430,444,443,548,651,661,683,769,785,902].

COX_2-mediated PGE_2 dependent phagocytosis of PS-exposing cells should similarly be sensitive to treatment with aspirin or other nonsteroidal anti-inflammatory drugs [48,278,349]. In any case, PGE_2-generating COX activity is enhanced in infected HbA/S RBCs. The increase in PGE_2 formation plays a decisive role for the course of the infection.

4.4.2 Calcium permeability is increased in ring-parasitized HbA/S as compared to HbA/A erythrocytes

Flufenamic acid, a Ca^{2+} channel inhibitor, reduces PGE_2-stimulated Ca^{2+} influx in erythrocytes, measured as free intracellular Ca^{2+} concentration with Fluo3 [443]. Flufenamic acid also inhibits eryptosis and intraerythrocytic growth of *P. falciparum* [444]. Consistent with these findings it was shown in the present study that increased PGE_2 formation paralleled a significantly stimulated Ca^{2+} permeability in ring-stage synchronized *P. falciparum* infected HbA/S RBCs, measured by enhanced $^{45}Ca^{2+}$ uptake, as compared to ring-infected HbA/A RBCs. Moreover, ring-infected HbA/S RBCs had a 1.5 fold increase in free Ca^{2+} concentration compared to ring-infected HbA/A RBCs. At later developmental stages these differences disappeared. It remains to be determined whether P_{sickle}, the deoxygenation-induced increase in calcium permeability in HbS/S RBCs (as an extreme of HbA/S RBCs) [141,432,436,545,539,540,801], also plays a role in infected HbA/S RBCs. Calcium influx was preferentially stimulated by PGE_2 in HbS/S RBC ghosts but not in HbA/A RBC ghosts, resulting in differing patterns of sodium and calcium transport [694,837,838]. Hence, the enhanced $^{45}Ca^{2+}$-uptake in ring-infected HbA/S RBCs observed in the present study might be due to enhanced NSC and possibly P_{sickle} permeabilty.

4.4.3 Ca^{2+} mediated phosphatidylserine externalization

Non-infected erythrocytes have a minute total pool of exchangeable Ca^{2+} of about 0.1 µmol per cell [208,262,547], as compared to an extracellular $[Ca^{2+}]_o$ of about 1 mM [735]. This results in a Ca^{2+} entry rate of about 30 - 55 µmol * (liter RBCs * h)$^{-1}$ [225,795]. The huge Ca^{2+} gradient across the plasma membrane is maintained by a Ca^{2+} pumping mechanism (ATPase) with an extrusion capacity of around 45 µmol * (liter RBCs * h)$^{-1}$ [547], activated by Ca^{2+} in the submicromolecular range. Thus, RBCs have exceedingly low levels of $[Ca^{2+}]_i$, in the range of 20 – 50 nM [14,547,538,720]. The $[Ca^{2+}]_i$ found in erythrocytes varied depending on the applied

non-fluorescent or fluorescent method. Moreover, the high intrinsic autofluorescence of RBCs and serum required appropriate corrections [14,73,547,538,549,720]. Nevertheless, except for the oldest, densest fraction, all values remained below 150 nM $[Ca^{2+}]_i$ [14]. This is consistent with the observation that only the 5 – 10 % densest cells of old RBCs showed a threefold increase in ionic Ca^{2+} [720]. This increase has been mainly attributed to deficiencies in Ca^{2+} pumping, as inferred from a 50 % reduction of the maximal extrusion capacity in the oldest cells [718]. This suggests that the GARDOS effect [313,776,829] occurs only in old HbA/A RBCs. An even greater accumulation of Ca^{2+} in HbS/S RBCs results in irreversibly sickled cells on account of the Gardos effect [22,248,333,661]. Increasing the Ca^{2+} influx above 0.7 mmol / liter ($\approx 10^{13}$) RBCs per h is sufficient to trigger the Gardos effect, reaching its maximum at a Ca^{2+} influx of 1.2 mmol * (10^{13} RBCs * h)$^{-1}$ [829]. PS exposure is stimulated both by the Gardos effect and the increase in $[Ca^{2+}]_i$ *per se* [502,515].

PS is present only in small amounts in the outer leaflet of fresh unseparated RBCs (about 300 sites per cell) [114,309,562,809]. This is consistent with the low annexin-binding observed as a measure of PS exposure [44,92,151,321,753,876] on non-infected control co-cultured RBCs in the present study. Treatment with the Ca^{2+} ionophore A23187 leads to an increase in PS exposure to a level of 300,000 sites per RBC [309]. This is consistent with the present finding that treatment with the Ca^{2+} ionophore ionomycin induced PS exposure in a high proportion of RBCs. This might be the result of the synergistic action of the two known eryptosis signaling pathways: an increase in $[Ca^{2+}]_i$ *per se* activates the scramblase and inhibits the translocase [507,510,934]. Moreover, the Ca^{2+}-induced Gardos effect activates the sphingomyelinase, resulting in ceramide formation and further stimulation of the scramblase [507,515].

De Jong *et al.* [211] have shown that oxidation of the erythrocyte itself did not induce exposure of PS on the membrane surface. In fact, when inflicted in excess**,** this oxidative damage was even able to prevent Ca^{2+}-induced PS scrambling [211]. It might be that the excessive oxidation destroyed the lipid transporters responsible for PS migration. This would explain the discrepancy with our own observation that non-infected oxidized RBCs predominantly showed PS exposure, comparable in degree to ionomycin-treated RBCs. Moreover, oxidation does not interfere with Ca^{2+} uptake [211,422].

Kuettner *et al.* [485] have revealed that non-infected RBCs pre-treated with either very high levels of $CaCl_2$ or with low levels of $CaCl_2$ (10 – 50 µM) in the presence of the ionophore A23187 and subsequently exposed to a peroxide generating system show an up to two-fold increase in lipid peroxidation in comparison to untreated RBCs, as measured by a

malonyldialdehyde assay. Lipid antioxidants and EGTA have both been found capable of preventing a significant increase in PS and PE exposure [422]. Zwaal *et al.* [934] have shown that the Ca^{2+}-mediated breakdown of phospholipid asymmetry in platelets is partly reversed by treating activated platelets with reducing agents. The treatment with reducing agents removes PS from the outer leaflet, where it was previously exposed during platelet activation [934]. These studies suggest that an increase in $[Ca^{2+}]_i$ results in oxidative stress, and that these two effects might synergistically enhance PS exposure on RBCs.

Usually, during parasite development the erythrocytic free cytosolic calcium concentration, $[Ca^{2+}]_i$, is maintained at low levels, namely between 40 and 100 nM [312]. The unidirectional influx of Ca^{2+} into infected HbA/A RBC cultured in glucose-containing media with vanadate-induced inhibition of the Ca^{2+} pump is within same the range as the influx observed in non-infected RBCs of the same culture, *i.e.* 30-55 µmol * (10^{13} RBCs * h)$^{-1}$ [795]. Without inhibition of the Ca^{2+}-ATPase, glucose-fed infected HbA/A RBCs show a slightly elevated $^{45}Ca^{2+}$ uptake rate [15 ± 1.2 µmol * (10^{13} RBCs * h)$^{-1}$] as compared to co-cultured non-infected RBCs [4 ± 1 µmol * (10^{13} RBCs * h)$^{-1}$] [795]. The elevated $^{45}Ca^{2+}$ uptake rate is consistent with other studies on glucose-fed or serum-incubated infected RBCs [119,224,477,482,813].

The Ca^{2+} concentration in infected RBCs rises progressively during parasite development and reaches values that are 10 to 20-fold greater in schizont-infected RBCs than in non-infected RBCs [119,531,813]. However, free $[Ca^{2+}]_i$ remains low in infected RBCs, as the increased Ca^{2+} influx is mainly sequestered within the parasite and thus buffered [126,312,311,481,579,813,811,830]. $[Ca^{2+}]_i$ rises only transiently during the trophozoite / schizont stage [6,311]. Together with subsequent oxidative stress this might stimulate PS exposure on infected RBCs.

Only infected RBCs bathed in glucose-free solution exhibit a significant increase in Ca^{2+} permeability. In glucose-free media, the Ca^{2+} influx in infected RBCs increases to about 1 mmol * (10^{13} RBCs * h)$^{-1}$ at approximately 30 h post-invasion and is inhibited by Ni^{2+}. This elevated influx of Ca^{2+} into glucose-deprived infected RBC is almost an order of magnitude higher than the Ca^{2+} influx seen in non-infected erythrocytes under equivalent conditions [795]. Several studies have shown an increased influx of Ca^{2+} into *P. falciparum* infected RBCs [224,477,482], reaching a net Ca^{2+} entry into infected RBCs that is 18 times faster than into non-infected RBCs [224]. Rather than being attributable to the NPPs the increase in Ca^{2+} permeability after glucose or ATP depletion probably results from the oxidation of energy-depleted infected RBCs. Glucose or ATP depletion leads to oxidative stress in RBCs, as the fueling with NADPH via the pentose phosphate pathway is impaired. In neither case is Ca^{2+}

conductance inhibited by blockers of the *NPP*s, *e.g.* furosemide [795]. Ca^{2+}-permeable NSC conductances of non-infected RBCs, induced by oxidative stress, are similarly insensitive to *NPP* inhibitors, *e.g.* NPPB, as measured by patch-clamp whole-cell recordings [244]. This oxidation-induced conductance of non-infected RBCs is comparable to the increased conductance of infected RBCs measured in glucose-free media.

In contrast, the cation conductance of infected RBCs (G_{cat}) is inhibited by *NPP* blockers (furosemide and NPPB), as measured by patch-clamp recording [243]. This data suggests that not all of the observed infection-induced increase in Ca^{2+} permeability can be attributed to the pathway developing in glucose or ATP-depleted cells. In the present study, heavily infected HbA/A RBCs exhibited a large proportion of PS exposing cells. PS exposure was half of that in oxidized or ionomycin-treated non-infected RBCs. These results indicate that PS exposure resulting from the infection-induced Ca^{2+} uptake may occur *in vivo*.

Attempts to show a significant percentage of *Plasmodium* infected RBCs harboring PS at their surface have yielded inconclusive results. While some studies found no such indication [363,613,819,854], others demonstrated a breakdown of the phospolipid asymmetry in *P. falciparum*-infected human [434,574,747,765,762] and *P knowlesi*-infected rhesus monkey RBCs [362,434]. This conflict in results was not due to the use of different methods for detecting changes in membrane composition, as there was some overlap for both positive and negative results. The performed assays were either enzymatic (PLA_2 [434,362,574,747,854], Sphingomyelinase C [574,854], PC-transfer [854], prothrombin-assay [271,574,613]); fluorescent (fluorescamine labeling [574,854], Merocyanine 540 [434], (FITC-labeled) annexin V [91,474,363,583,765,819]); or chemical (2,4,6-trinitrobenzene sulphonic acid (TNBS) [362,747]. A monoclonal antibody against PS was very sensitive [574]. However, several studies [434,435,758] that obtained results pointing towards a dramatic change in the phospholipids asymmetry exhibited one common feature: the absence of glucose in the media in which the erythrocytes were kept during experimental handling [774].

Sherman and Prudhomme have detected only a low proportion (10%) of *P. falciparum*-infected erythrocytes showing Ca^{2+}-dependent annexin-binding [765]. They have concluded that parasitemias higher than 25 % and multinucleate forms of the parasite are necessary to obtain statistical differences for PS exposure [858]. In the present study, the increase in annexin V-binding correlated weakly with the increase in parasitemia. However, PS exposure was high for *P. falciparum* cultures with high parasitemia, in particular for infected RBCs with high DNA content, indicating a late parasite stage. This finding is consistent with the study of Sherman and Prudhomme.

Hence, although the infection-induced Ca^{2+}-permeable NSC conductance is important

for parasite growth,, it facilitates breakdown of the phospholipid asymmetry of the host cell membrane [238,513,512] at mature parasite stages. Together with parasite-induced oxidative stress, transient increases in free [Ca^{2+}] might induce PS scrambling. This appears plausible because PS exposure occurs especially under conditions of high parasitemia, which is associated with increased oxidative stress and ATP depletion [77,684,188,229,809]. This breakdown in PS asymmetry suggests phospholipid scramblase activation and translocase inactivation during the course of infection.

4.4.4 Functional significance of PS exposure in infected HbA/A RBCs

Loss of phospholipid asymmetry and exposure of PE and PS on malaria-infected RBCs [362,434,574,747,765,762] are signals for phagocytosis [562,748,600]. Phagocytosis mechanisms can be distinguished according to whether or not they involve opsonization-, *i.e.* antibody responses.

The majority (75%) of adult patients with uncomplicated *P. falciparum* and *P. vivax* malaria are positive for anti-phospholipid antibodies (aPLA) [267]. Facer *et al.* [267] have demonstrated this by ELISA using a panel of anionic and cationic phospholipids. IgG and IgM binding is highest to the anionic phospholipids PS, phosphatidic acid (PA), and cardiolipin (CL), whereas against phosphatidylinositol (PI) only low antibody levels have been detected. Comparison of the average concentration of IgG and IgM aPLA shows a trend for anti-PA > CL > PS > PC > PE > PI [267]. IgG antibodies, referred to as “opsonins”, bind to the infected RBCs with their Fab region leaving the Fc region out. Macrophages interact with opsonized RBCs via their Fc γ receptors and finally internalize them.

Schizont- and trophozoite-infected normal RBCs are recognized in part by opsonization-independent mechanisms which possibly involve PE / PS exposure. These account for about 15 – 20 % of total phagocytosis [52]. A small but significant inhibition of monocyte phagocytosis of trophozoite-infected HbA/A RBCs *in vitro* is effected by PS, but not by PC liposomes [844]. In conclusion, PS exposure directly fosters clearance of infected RBCs by the innate immune system, *e.g.* macrophages [137,271,268,269,270,283,459,660,756,853,856].

At mature parasite stages, however, PS exposure contributes to cytoadherence: endothelial membrane receptors such as CD36, intercellular adhesion molecule ICAM-1 [393,394,644], chondroitin sulphate, and secreted proteins such as thrombospondin (TSP) interact with erythrocyte surface molecules of infected RBCs such as clustered, modified host membrane band 3 (AE1) protein, adhesive neoantigens, *e.g.* PfEMP1 [78,863], and exofacial PS

[250,755,762,763]. PS is involved in erythrocyte-endothelium adhesion in a Ca^{2+}-dependent manner [179]. Hence, enhanced PS exposure might promote cytoadherence of human HbA/A RBCs infected with mature *P. falciparum* trophozoite and schizont stages to endothelial cells lining the post-capillary venules [762]. The endothelial ligand TSP and in particular CD36 are major sequestration receptors for PS [250,755]. PS is externalized in a time-dependent manner depending on the intracellular development of the parasite [250,575,574]. PS externalization coincides with the oxidization of membrane lipids in trophozoite and schizont-infected RBCs [348].

Thus, PS exposure during mature trophozoite and schizont stage plays a major role in microvessel sequestration in the deep tissues. In this way the parasite evades the immune system and avoids destruction by the spleen [394,762]. Cytoadherence favors parasite development in a low oxygen pressure microenvironment [763]. At the same time microcirculatory obstruction through hemostatic plug formation leads to hypoxia, metabolic disturbances, and multiorgan failure, all of which are detrimental to the host [394].

4.4.5 Functional significance of early detection of infected HbA/S RBCs

In vivo, early identification and selective removal of early ring-stage *P. falciparum* parasites by splenic macrophages result in fewer parasites reaching schizogony. This is thought to be a prerequisite for a mild course of malaria tropica in sickle cell trait carriers. During primary and subsequent secondary infections the immune system of sickle cell trait carriers might therefore be exposed to more antigens of the early parasite stages [624] rather than to neoantigens [334], which are exclusively or predominantly expressed at late trophozoite and schizont stages [82]. Subsequent alterations in antigen processing, presentation and recognition might explain the observed differences in T-cell responses between infected trait carriers and normal individuals. T lymphocyte proliferation and IL-1 production by monocytes / macrophages is suppressed in general during malaria infection [39]. Addition of IL-1 partially reverses depression responses of T-lymphocytes cultured together with other blood mononuclear cells from patients infected with *P. falciparum* and *P. vivax* [39]. This is probably because in T-lymphocytes IL-1 enhances the expression of the IL-2 receptor and the production of other cytokines, *e.g.* IL-2 [353]. The antiproliferative effect of PGE_2 on cultured T-lymphocytes from infected individuals in turn is minimized [39]. In line with these observations, indomethacin, a COX inhibitor, enhances *in vitro* T-cell responses to several malaria-specific antigens [710]. In *P. falciparum* infected sickle trait carriers the T-cell response

might be so adjusted as to lead to a balanced TH1/TH2 response promoting protection [83,752].

Macrophages that phagocytose ring-stage infected RBCs are less intoxicated by hemozoin than those that phagocytose late-stage infected RBCs. *Plasmodium* parasites detoxify the free heme of digested Hb as polymerized ß-hematin (the pigment hemozoin) in their food vacuoles [252,654]. Hemozoin has different ferromagnetic properties than hemoglobin [51]. This makes it possible to separate non-infected and ring-stage infected RBCs from trophozoite and schizont infected RBCs with an efficacy up to 96 – 98 % by magnetic assisted cell sorting (MACS) [846]. When monocytes/macrophages or dendritic cells phagocytose late-trophozoite infected RBCs with high hemozoin levels they undergo functional alterations [51,618,751,749]. Ingested hemozoin impairs macrophage motility [2,63,62,584,591] and decreases cellular immunity [51,84,618,752,845]. Moreover, macrophages (isolated from peritoneal exudate cells) of *P. c. chabaudi* infected mice co-cultured with antigen-specific T-cell hybridomas suppress IL-2 production by antigen-activated T-cell hybridomas [51,84,618,752,845]. Macrophage ingestion of hemozoin from infected RBCs contributes to the cytokine-mediated pathology of malaria, thus aggravating the disease [63,156,426,618]. AT-rich malaria DNA, bound to hemozoin, and presented to the Toll-like receptor 9, an endosomal receptor in cells of the innate immune system (in particular in dendritic cells), is highly proinflammatory, whereas synthetic ß-hematin is ineffective in this regard [666]. The inflammatory response is increased by modulation of macrophages' cytokine and chemokine expression [51,164,416,425,426,424,643,690,767,817].

The early removal of infected RBCs would prevent dysregulation of monocyte / macrophage function [751] and endothelial cell dysfunction [816]. Finally, if ring-stage parasites are removed, there are no mature infected erythrocytes that adhere to myeloid dendritic cells and macrophages. Hence, the innate and aquired immune responses would not be down-regulated [595,850,851].

One reason for the impairment of monocyte / macrophage functions upon hemozoin ingestion is oxidative stress [751]. However, this is not due to the release of iron [348,817]. RBC lipid peroxidation is associated with the formation of toxic products such as low (submicromolar) concentrations of esterified monohydroxy derivatives of polyenoic fatty acids (OH-PUFA), hydroxyaldehydes, monohydroxy derivatives of arachidonic acid (hydroxyeicosatetraenoic acids = HETEs) and of linoleic acid (hydroxyoctadecadienoic acids = HODEs). These impair important macrophage functions such as NADPH-oxidase or iNOS via protein kinase C (PKC) inhibition [51,156,750,749]. The concentration of OH-PUFA is 1.8 µM (µmoles per liter RBC or µmoles per liter RBC equivalents) in non-infected RBCs, 11.1 µM in ring-infected erythrocytes , 35 µM in trophozoites; and ~ 90 µM in hemozoin [749]. This

oxidative stress might also change RBC deformability [86,649].

Furthermore, hemozoin ingestion by phagocytosis of infected RBCs influences PGE_2 secretion. Cultured mononuclear cells isolated from maternal placental (intervillous) blood of *P. falciparum* infected women show a decrease in PGE_2 secretion as compared to cultured intervillous blood mononuclear cells (IVBMC) of non-infected women [678]. Cultured IVBMC from women with a positive antenatal peripheral parasitemia who are negative for placental malaria at term produce the highest PGE_2 levels. In placental malaria, cultured IVBMC which have ingested medium quantities of hemozoin by phagocytosis of infected RBCs during natural infection show no signficant changes in secretion of PGE_2 or either one of the regulatory cytokines TNFα and IL-10. By contrast, cultured IVBMC which have ingested large quantities of hemozoin show depressed secretion of PGE_2, IL-10 and TNFα [678]. In cultured peripheral blood mononuclear cells (PBMCs) of *P. falciparum*-infected children, the mechanism of PGE_2 supression is not due to decreased cell viability, measured in terms of mitochondrial bioactivity, but to the inhibition of *de novo* cyclooxygenase 2 (COX_2) transcription [452]. At the same time, cultured PBMCs that have ingested hemozoin by phagocytosis of infected RBCs overproduce IL-10. Application of IL-10 antibodies to neutralize this overproduction failed to restore PGE_2 production by cultured PBMCs. Therefore, the molecular mechanisms that reduce PGE_2 secretion are probably independent of those that increase IL-10 production [452]. Moreover, the results of Keller *et al.* [452] on cultured PBMCs appear contradictory to the study of Perkins *et al.* [678] on cultured IVBMCs. However, *in vivo*, the plasma level of IL-10, known as a suppressor of COX_2 expression, is inversely correlated with the plasma level of PGE_2 [677].

Healthy *P. falciparum*-exposed children have elevated levels of bicyclo-PGE_2 / TNFα compared to children with malaria anemia [451]. Hemozoin ingested by PBMCs through phagocytosis of infected RBCs induces PGE_2 suppression, which facilitates TNFα overexpression. This is associated with enhanced malaria anemia. In line with this, cultured PBMCs that have ingested *P. falciparum* hemozoin show decreased TNFα expression when treated with PGE_2 [451]. These results, too, appear contradictory to the study of Perkins *et al.* [678] on cultured IVBMCs. In all three studies, however, PGE_2 secretion by the BMCs was reduced after ingestion of high levels of hemozoin. Moreover, children with cerebral malaria have also significant lower levels of bicyclo-PGE_2 than children with asymptomatic parasitemia [675].

Taken together, these studies indicate the importance of early removal of infected RBCs [66,152] for the patient's resistance to *P. falciparum* infection. Identification and clearance of early (*i.e.* ring) stage-infected erythrocytes by the innate immune system subsequently

lowers the hemozoin load in phagocytes, resulting in less oxidative stress [348], more PGE_2 production and a modified cytokine and chemokine release.

4.4.5.1 Band 3 (AE1) aggregation in HbA/A, HbA/S and *P. falciparum* infected erythrocytes upon "oxidative ageing" and Ca^{2+} influx

Ageing leads to changes in RBCs [136] that resemble changes observed during malaria infection. In non-infected RBCs these changes result in a moderately decreased surface area, volume and cell water loss, and thus to reduced deformability and increased RBC density [19,20,21,94,146,276,499,517,514,681,791,934]. Moreover, ageing RBC show PS exposure, increase in $[Na^+]_i$, decrease in $[K^+]_i$, and ATP depletion [240,398,763,877,912].

In addition, the decreased expression of blood group antigen in senescent RBCs is associated with a loss of up to 10 % surface sialic acid residues [41,175,181,315,563,568,855 695]. This might cause unmasking of the penultimate beta-galactosyl residues and expression of a particular antigen in ageing RBCs [93,105].

Finally, ageing in RBCs is associated with the exposure of a peptidic cryptic epitope, referred to as "senescent antigen". Exposure occurs through the cytoplasmic cleavage of band 3 protein (anion exchanger 1 = AE1). This cleavage involves a change in the tertiary structure of band 3 [448,446,447,448,449]. Band 3 is a membrane protein that not only functions as a transporter (HCO_3^- / Cl^- antiporter) but is also essential for membrane stability [680]. In this way, it is involved in controlling the size, shape, integrity, flexibility, and durability of the RBC membrane as well as such processes as cell-cell interactions and membrane fusion [136]. In RBCs undergoing oxidative stress, oxidized protein hydrolase (acylpeptide hydrolase) cleaves band 3 proteins [304,305,306]. RBCs' antioxidative defenses become weaker with ageing [237]. Senescent RBCs show alterations similar to those observed in oxidized cells [134,211,386,459,490,507,566,567,872]. Remarkably, human RBCs infected with the malaria parasite *P. falciparum* undergo an identical "oxidative ageing" process [332,763].

"Oxidative ageing" is caused by formation of hemichomes (oxidized / denaturated Hb) bound to the membrane. This leads to aggregation of band 3 (AE1) and subsequent deposition of autologous IgG antibodies and complement C3b fragments. These effects are reversed by treatment with mercaptoethanol [763]. The age- or *P. falciparum*-infection-dependent clustering of the membrane spanning region of the band 3 protein serves as a recognition site for IgG accumulation of naturally circulating anti-band 3 autoantibodies [105,456,818]. While the amount of anti-band 3 IgG is not sufficient to elicit Fc-mediated phagocytosis, these antibodies with a

high affinity for complement fragment C3 are able to activate the alternative complement pathway [332,447]. Activation of the alternative complement pathway results in deposition of C3b on the RBC membrane in a IgG dose-dependent manner. Covalently bound C3b/IgG (anti-band 3)-complexes on the RBCs surface are recognized by complement receptor type 1 (CR1) of macrophages [66,844]. Consistent with these results, inactivation of complement convertases or blockage of CR1 lead to considerable inhibition of opsonization-dependent phagocytosis [52,566,567].

Accelerated "oxidative ageing" of *P. falciparum* infected RBCs occurs in genetic disorders [412] such as sickle cell trait, ß-thalassemia trait, X-chromosomal linked G6PD-deficiency [53], and HbH disease (Hb Hammersmith, a Hb mutant where phenylalanine at CD1, adjacent to heme, is replaced by serine). Accelerated "oxidative ageing" facilitates phagocytosis of infected HbA/S and glucose-6- phosphate-dehydrogenase (G6PDH)-deficient RBCs by macrophages already at ring stage, thus indirectly limiting the intraerythrocytic lifetime of the parasite [66,152] and reducing the fatal consequences of malaria infection.

The accelerated oxidative (biochemical) changes observed by Ayi *et al.* [66] in infected HbA/S RBCs might have been potentiated by an increase in erythrocytic free $[Ca^{2+}]_i$ [21,459,719], since changes in the topology of band 3 (AE1) by cross-linking are sufficient to expose binding sites to anti-band 3 antibodies [403,705]. After treatment with the Ca^{2+} ionophore A23187 membrane proteins are crosslinked by transamidase action or by oxidation of sulphydryl groups resulting from an increase in malonyldialdehyde, which in turn is caused by peroxidation of polyunsaturated fatty [19]. Analogous changes can be observed in the dense fraction of HbS/S RBCs and to a lesser extent in dense, *i.e.* old HbA/A RBCs [700,702]. The oxidative effects observed by Allan *et al.* upon treatment with the Ca^{2+} ionophore [19] and by Rank *et al.* [700] or Raval *et al.* [702] in sickle RBCs might be due to a decrease in GSH levels. This decrease might have been indirectly facilitated by Ca^{2+}, which decreases cellular ATP levels by activation of the Ca^{2+} pump (ATPase) [21]. Decreased ATP levels might decrease the supply of the cell with NADPH (by the pentosephosphate pathway) for replenishing the GSH pool, thus weakening its antioxidative defense. In the present study, an increase in erythrocytic free $[Ca^{2+}]_i$ was shown in ring-infected HbA/S RBCs. In non-infected cells, the third exofacial loop of the 55 kDa transmembrane domain of band 3 (AE1) that contains the amino acid sequences HPLQKTY and YVKRVK, 547–553 and 824–829, respectively, and the amino acid sequence 534 – 547: YETFSKLIKIFQDH are exposed upon calcium loading, changes in ionic strength of the medium, treatment with acridine orange and ageing [763].

Amino acid residues 547-553 and 824-829 act as epitopes in senescent cells [449]. The

synthetic peptide 534-547 blocks the *in vitro* binding of HbS/S RBCs to human umbilical vein endothelial cells, and a monoclonal antibody against this peptide immunostains HbS/S RBCs [823]. The band 3 (AE1) sequences 547–553 and 824–829, and 534–547 are cryptic in intact human cells, but are exposed during intracellular development of *P. falciparum* [763]. Naturally occurring anti-band 3 autoantibodies bind to an 85 kDa and a > 250 kDa antigen of band 3 protein on the surface of *P. falciparum* infected RBCs [906,908,909].

Monoclonal and polyclonal antibodies raised against these three sequences of the transmembrane region of AE1 block the adherence of *P. falciparum*-infected RBCs [198,249,904] both *in vitro* and *in vivo.* The antibodies detect RBCs infected with *P. falciparum*, but do not react with the surface of non-infected RBCs [201,200]. Further, the synthetic peptide 534-547 blocks *in vitro* the adherence of mature-stage *P. falciparum* infected RBCs to CD36 and to human brain endothelial cells, but not to TSP [905]. Finally, *in vivo*, sera of individuals living in a malaria-endemic region (The Gambia) also recognize peptide motifs of the human erythrocyte anion transport protein (band 3). These sera react strongly with residues 534-560, 638-660, and 808-842 [199]. Thus, antibodies against modified band 3 protein play a role in protective immunity during *P. falciparum* infection [199,397,904]. The band 3 protein, in turn, might be modified earlier in infected HbA/S RBCs than in infected HbA/A RBCs because of the enhanced Ca^{2+} influx into infected HbA/S RBCs at ring stage.

4.4.5.2 Functional significance of enhanced PS exposure in infected HbA/S RBCs

PS exposure plays a major role in the recognition and removal of aged RBCs by phagocytosis, mainly by Kupffer cells in the liver [459]. Sickle as well as thalassemic RBCs and, to a lesser extent, RBCs with G6PDH deficiency are exquisitely sensitive to PS exposure promoting stimuli such as osmotic shock, oxidative stress and/or energy depletion [210,513]. Increased PS exposure of human RBCs has been shown in α and ß-thalassemia [124,492], and in another anemic condition, hereditary hydrocytosis, an uncommon variant of hereditary stomatocytosis [310]. All these traits confer a partial resistance towards malaria tropica.

In the present study, increase in Ca^{2+} permeability and in erythrocytic free Ca^{2+} concentration, which together mediate PS exposure, were particularly enhanced in ring-stage infected HbA/S RBCs. Infected HbA/S RBCs grown for 44 – 54 h after ring synchronization (with a parasitemia of around 7 %) exposed significantly more PS than did infected HbA/A or non-infected HbA/A or HbA/S RBCs. The significant difference in PS exposure between infected HbA/S and HbA/A RBCs has been confirmed for ring-stage as well as for late-stage

parasites in experiments with glucose-containing Ca-Ringer [443]. These data corroborate the results of the present study. Furthermore, infected HbA/S RBCs are cleared faster in *in vitro* phagocytosis experiments [443]. The subsequent elimination of intracellular pathogens could serve as a host defense mechanism [115,250,268,269,346,345,476,499,509,603,662,739,862]. Ionomycin-treated PS-exposing RBCs are rapidly cleared *in vitro* and *in vivo* from circulating peripheral blood and sequestrated into the spleen [443,514]. Coating of PS by annexinV delays the clearance of ionomycin-treated mouse RBCs [443]. Thus, enhanced Ca^{2+}-dependent PS exposure induced by PGE_2 formation might contribute to the enhanced early removal of *P. falciparum* infected HbA/S RBCs from the circulation at ring-stage. The infected HbA/S RBCs might be removed in a nonopsonic manner by macrophages via the monocyte / macrophage scavenger and pattern recognition receptor CD36 [65,268,272,596] in concert with previously reported mechanisms of phagocytosis. These other mechanisms, *i.e.* amplified 'eat-me' signals, are the enhanced surface exposure of the *P. falciparum* protein PfEMP1 [273], which binds to CD36 [596], and accelerated "oxidative ageing" as described above [66,844].

Taken together, the observed significant increase in PGE_2 secretion and subsequently enhanced Ca^{2+} influx in ring-infected HbA/S RBCs results in increased free $[Ca^{2+}]_i$. This in turn facilitates an ageing and eryptotic process that has an earlier onset in infected HbA/S RBCs than in infected HbA/A RBCs – a difference that is crucial to the protection of sickle cell trait carriers against *P. falciparum* infection.

5 Conclusions

In the first part of my thesis, I investigated the activation of erythrocyte nonselective cation (NSC) permeability and of sphingomyelinase by *Plasmodium falciparum* infection. Both are involved in programmed cell death in erythrocytes, also referred to as eryptosis [499,509], and meet four requirements for parasite growth at the same time:

i) Development of an inwardly directed Na^+ and an outwardly directed K^+ ion gradient across the parasite plasma membrane, this in turn making the parasite dependent on high [Na^+] and low [K^+] extracellularly [132].

ii) The presence of Ca^{2+} ions in the culture medium and a Ca^{2+} permeability conferred by a NSC conductance [132].

iii) Increased ceramide formation (detected in the blood of *P. berghei* ANKA infected mice).

iv) Activity of parasitic neutral sphingomyelinase and host acid sphingomyelinase, probably to provide ceramide for membrane synthesis [371].

In the second part of my doctoral thesis I investigated differences in the induction of eryptosis in normal (HbA/A) and sickle trait (HbA/S) *P. falciparum* infected red blood cells (RBCs). The results were as follows:

i) *P. falciparum* did not develop differently in HbA/A and HbA/S RBCs, but infected HbA/S RBCs showed enhanced PS exposure.

ii) The host ceramide pathway played only a minor role in causing PS exposure in infected RBCs under normal culturing conditions.

iii) Although parasite growth was dependent on ceramide, extragenously applied ceramide inhibited *P. falciparum in vitro* growth, and more so in HbA/S RBCs than in HbA/A RBCs.

iv) Infected HbA/S RBCs secreted more prostaglandin E_2 (PGE_2) than did infected HbA/A RBCs, probably significantly contributing to the selective advantage of *P. falciparum* infected sickle cell trait carriers. Increased PGE_2 levels are associated with a mild course of malaria infection [677].

v) The increased PGE_2 levels enhanced the infection-induced Ca^{2+} influx into ring-stage infected HbA/S RBCs. This promoted the observed increase in PS exposure on the outer membrane leaflet of infected HbA/S RBCs as compared to infected HbA/A RBCs.

These observations show that the accelerated eryptosis of *P. falciparum* infected HbA/S RBCs is caused by enhanced activation of a NSC permeability during ring stage. Enhanced PS exposure acts as an “eat-me” signal for macrophages. Together with other signals [66], it triggers the early recognition and removal of ring-stage infected HbA/S RBCs [443].

6 Summary

Plasmodium falciparum, an intracellular protozoan parasite, causes the lethal form of malaria. It mostly affects children up to 5 years of age, with a death toll of 1 million per year or more than 2700 per day in this age group [139,888]. Thus, every 32 seconds a child dies of malaria. However, sickle cell trait (HbA/S) children are strongly protected against severe malaria, *i.e.* severe malarial anemia [227] and cerebral malaria [36,896]. The parasite is transmitted by the female *Anopheles* mosquito to the human host, where it replicates once in the liver (9-14 days) and then in erythrocytes. The early asexual erythrocyte stage (up to 15 h post invasion) is termed ring stage. This is followed by the trophozoite and the schizont stages (~ 40 h post invasion). *P. falciparum* multiplies asexually every 48 hours up to 32-fold. The parasite induces new permeability pathways (*NPP*s) in the erythrocyte membrane to meet its need for nutrients and disposal of waste products. Among these is a Ca^{2+}-permeable nonselective cation (NSC) channel [85,243,407,793]. NSC conductance is stimulated by the formation of prostaglandin E_2 (PGE_2). However, this triggers also one pathway of erythrocyte programmed cell death, also referred to as eryptosis [507]. An increase in erythrocytic $[Ca^{2+}]_i$ inhibits an ATP-dependent translocase which transports PS from the outer to the inner membrane leaflet. It also activates a scramblase which enhances phosphatidylserine (PS) exchange within the membrane bilayer, thus acting antagonistically to the translocase [499]. In effect, an increase in $[Ca^{2+}]_i$ thus leads to PS exposure on the outer leaflet of the membrane [935].

The first part of the study investigated the dependence of *P. falciparum in vitro* growth on NSC Ca^{2+}-permeable channel activity. 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a potent cation channel blocker, inhibited NSC conductance (IC_{50} ~ 0.75 µM) as well as *in vitro* growth of *P. falciparum* (IC_{50} ~ 3 µM). EGTA, a Ca^{2+} chelating agent, also inhibited parasite *in vitro* growth. To test for the significance of extracellular $[Na^+]$ or $[K^+]$ on parasite growth, replacement of the culture medium by NaCl containing test solution for 8 h during the trophozoite stage (24 to 32 h post ring-synchronization) provided the control conditions for the following experiments. When the NaCl containing test solution was replaced by mixtures of NaCl and N-Methyl-D-Glucamine-Chloride (NMDG-Cl) or KCl of different component ratios, parasite growth was inhibited, indicating an inhibitory effect of decreasing $[Na^+]$. $(NMDG)^+$ does not permeate the red blood cell (RBC) membrane. In the present study, it did not show any toxic effect *per se* on parasite growth. Replacement of NaCl by KCl inhibited parasite growth even stronger than replacement by NMDG-Cl. Replacement with KCl

resulted in half-maximal growth at a residual [NaCl] about twice as great as in the case of replacement with NMDG-Cl ($IC_{50(NaCl/KCl)}$ = 58 mM NaCl / 65 mM KCl; $IC_{50(NaCl/NMDG-Cl)}$ = 24 mM NaCl / 112 mM (NMDG)-Cl). This indicates an additive inhibitory effect of increasing [K^+] on parasite growth. Hence, the parasite requires not only an inwardly directed Ca^{2+}- and Na^+-gradient, but also an outwardly directed K^+-gradient of for its development within the RBC. The necessary ion exchange is probably accomplished by an EIPA sensitive cation channel that has a permselectivity of $P_K/P_{Na} \sim 2$ [243].

Treatment with the Ca^{2+}-ionophor ionomycin, oxidation of non-infected RBC or high parasitemia led to breakdown of PS asymmetry, as judged by annexin-binding [132].

Furthermore, the study explored whether in *Plasmodium* infected RBCs a second known signaling pathway of eryptosis is induced. This pathway involves sphingomyelinase activation that cleaves ceramide of sphingomyelin. Ceramide stimulates the scramblase and sensitizes it to the effects of Ca^{2+}, thus contributing to PS exposure on the outer RBC membrane leaflet. Interestingly, *P. berghei* ANKA infection of mice significantly increased the ceramide level in acid sphingomyelinase knock-out (ASM-/-) and wild-type (+/+) mouse blood, probably by neutral sphingomyelinase activity (NSM) of the parasite [371,372]. In mice, ASM is secreted by leukocytes [520]. The blood of infected ASM -/- mice produced significantly less ceramide than the blood of infected ASM +/+ mice. Increase in parasitemia between the 10th and 21st day post infection was significantly slower in ASM -/- mice than it was in ASM +/+ mice. This is probably due to a lack of ceramide in ASM-/- mice, since the parasite requires ceramide for its membrane synthesis [260,261,367,371,663].

In the case of normal (HbA/A) RBCs infected with ring-stage *P. falciparum*, macrophages recognize the PS-exposing infected RBCs and remove them in a nonopsonic manner via the scavenger and pattern recognition receptor CD36 [65,268,269,270]. In the case of infected HbA/S RBCs the enhanced PS exposure resulting from accelerated eryptosis could reinforce the removal.

Therefore, the aim of the study was to explore differences in the induction of eryptosis between HbA/A and HbA/S infected erythrocytes. Although ring-stage synchronized parasites grew similarly in HbA/S and HbA/A RBCs, PS exposure was significantly enhanced in infected HbA/S RBCs. Differences in sphingomyelinase activity were detected between infected HbA/S and HbA/A RBCs. However, the enhanced enzyme activity seems not to be involved in the enhanced PS exposure in infected HbA/S RBCs. Although the parasite requires ceramide for membrane synthesis [371], ceramide in the culture medium inhibits parasite growth, and more so in HbA/S than in HbA/A RBCs.

Ring-infected HbA/S RBCs showed enhanced $^{45}Ca^{2+}$ uptake and a 1.5-fold increase in free cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, compared to ring-infected HbA/A RBCs. Hence, the enhanced PS exposure in infected HbA/S RBCs results from an enhanced NSC Ca^{2+}-permeable conductance. Moreover, infected HbA/S RBCs produced more PGE_2 than did infected HbA/A RBCs. This resulted in enhanced NSC channel activity of ring-stage infected HbA/S RBCs as compared to HbA/A RBCs. Interestingly, elevated PGE_2 plasma levels are correlated with a decrease of severe malaria in children with *P. falciparum* infection [677]. Enhanced PGE_2-induced PS exposure on infected HbA/S RBCs accelerates their removal at the ring-stage of the parasite [443]. In addition, accelerated "oxidative ageing" of the host erythrocyte stimulates phagocytosis of ring-infected HbA/S RBCs [66,763]. This first-line defense of the host affords a crucial protection against the lethal consequences of *P. falciparum* infection to infected sickle cell trait carriers.

7 Zusammenfassung

Plasmodium falciparum, ein intrazellulärer einzelliger Parasit, ist Erreger der letalen Form von Malaria. Vor allem Kinder im Alter bis zu 5 Jahren sind davon betroffen mit Todesfällen von rund 1 Million im Jahr [139,888], was mehr als 2700 Todesfällen am Tag entspricht. Folglich stirbt etwa alle 32 Sekunden ein Kind an Malaria. Kinder mit heterozygoter Sichelzellanämie (HbA/S) sind hingegen gut geschützt vor den Folgen lebensbedrohlicher Malaria, d.h. schwerer Anämie [227] und zerebraler Malaria [36,896]. Der Parasit wird durch die weibliche *Anopheles*-Mücke übertragen, durchläuft zunächst ein Leberstadium (9-14 Tage) und vermehrt sich dann in den Erythrozyten. Das früheste asexuelle Erythrozytenstadium (bis zu 15 Stunden nach Invasion) wird Ringstadium genannt, die späteren Stadien sind Trophozoiten, dann Schizonten (~ 40 Stunden nach Invasion). *P. falciparum* vermehrt sich asexuell bis zu 32 mal innerhalb von 48 h. Der Parasit induziert neue Permeabilitäten (*NPP*s) in der Erythrozytenmembran, um seinen Bedarf an Nährstoffen zu decken und um Abfallprodukte zu entsorgen. Ein nicht selektiver Kationenkanal gehört zu den *NPP*s [85,243,407,793]. Die nichtselektive Kationenleitfähigkeit wird durch Bildung von PGE_2 (Prostaglandin E_2) stimuliert. Dies induziert aber auch einen Signalweg für den programmierten Zelltod von Erythrozyten, auch Eryptose genannt [507]. Ein Anstieg in der erythrozytären Kalziumkonzentration, $[Ca^{2+}]_i$, inhibiert eine ATP-abhängige Translokase, die Phosphatidylserin (PS) von der äußeren zur inneren Membranschicht transportiert, und aktiviert eine Scramblase, die den Austausch von PS zwischen den Membranschichten erhöht, und damit der Translokase entgegenwirkt [499]. Demzufolge führt ein Anstieg der $[Ca^{2+}]_i$ zu PS-Exposition in der äußeren Membranschicht des Erythrozyten [935].

Der erste Teil der Studie untersuchte die Abhängigkeit des *in vitro* Wachstums von *P. falciparum* von der nichtselektiven Ca^{2+}-durchlässigen Kationenkanalaktivität. 5-(N-Ethyl-N-Isopropyl)-Amilorid (EIPA), ein effektiver Kationenkanalblocker, hemmte sowohl die Kationenleitfähigkeit (IC_{50} ~ 0.75 µM) als auch das Parasitenwachstum (IC_{50} ~ 3µM) *in vitro*. Darüberhinaus wurde das Parasitenwachstum durch EGTA, einen Ca^{2+}-Chelatbildner, beeinträchtigt. Um die Bedeutung von extrazellulärer $[Na^+]$ oder $[K^+]$ auf das Parasitenwachstum zu überprüfen, bildete der Austausch des Kulturmediums durch NaCl-haltige Testlösung über 8 h während des Trophozoitenstadiums (24 bis 32 h nach Synchronisation) die Kontrollbedingung für die folgenden Versuche. Wurde die NaCl-haltige Testlösung durch N-Methyl-D-Glukamin-Chlorid (NMDG)-Cl oder KCl-haltige Testlösung in verschiedenen Mischungsverhältnissen ersetzt, wurde das Parasitenwachstum inhibiert, was

auf einen inhibitorischen Effekt von abfallender [Na^+] hindeutet. $(NMDG)^+$ durchdringt die Erythrozytenmembran nicht. In dieser Studie wirkte es selbst nicht toxisch auf das Parasitenwachstum. Der Austausch von NaCl durch KCl inhibierte das Parasitenwachstum stärker als die Substitution von NaCl durch (NMDG)-Cl. Beim Austausch durch KCl war die verbleibende NaCl-Konzentration, die für halbmaximales Parasitenwachstum benötigt wurde, ungefähr doppelt so hoch wie beim Austausch durch NMDG-Cl ($IC_{50(NaCl/KCl)}$ = 58 mM NaCl / 65 mM KCl; $IC_{50(NaCl/NMDG-Cl)}$ = 24 mM NaCl / 112 mM (NMDG)-Cl). Das deutet auf einen zusätzlichen inhibitorischen Effekt der ansteigenden [K^+] auf das Parasitenwachstum. Demzufolge benötigt der Parasit nicht nur einen einwärts gerichteten Ca^{2+} und Na^+-Gradienten, sondern auch einen auswärts gerichteten K^+-Gradienten zur Entwicklung innerhalb des Erythrozyten. Der dafür notwendige Ionenaustausch wird wahrscheinlich bewerkstelligt durch einen EIPA-sensitiven Kationenkanal, der eine Permselektivität von P_K/P_{Na}~2 besitzt [243].

Behandlung mit dem Kalziumionophor Ionomycin bzw. Oxidation von nicht infizierten Erythrozyten oder eine hohe Infektionsrate, führte zum Zusammenbrechen der PS Asymmetrie, wie aus der gemessenen Annexinbindung geschlossen werden konnte [132].

Weiterhin untersuchte die Studie, ob in *Plasmodium*-infizierten-Erythrozyten ein zweiter bekannter Signalweg der Eryptose induziert ist. Dieser Signalweg führt zu einer Sphingomyelinaseaktivierung, die Ceramid aus Sphingomyelin freisetzt. Ceramid stimuliert die Scramblase und erhöht ihre Aktivierung durch Ca^{2+}. Damit trägt Ceramid zur PS-Exposition in der äußeren Membranschicht des Erythrozyten bei. Interessanterweise steigerte eine *P. berghei* ANKA-Infektion sowohl signifikant den Ceramid-Gehalt im Blut von saurer-Sphingomyelinase-defizienten- (ASM -/-) als auch im Blut von Wildtyp-(ASM +/+) Mäusen wahrscheinlich durch die neutrale Sphingomyelinase-Aktivität des Parasiten [371,372]. In Mäusen wird ASM von Leukozyten sezerniert. [520]. Das Blut infizierter ASM-/- Mäuse enthielt signifikant weniger Ceramid als das Blut von infizierten ASM +/+ Mäusen. Der Anstieg der Parasitämie zwischen dem 10. und dem 21. Tag nach Infektion in ASM -/- Mäusen war signifikant verringert im Vergleich zum Anstieg der Parasitämie in ASM +/+ Mäusen. Dies ist höchstwahrscheinlich durch einen Ceramidmangel verursacht, da der Parasit zur Membransynthese Ceramid benötigt [260,261,367,371,663].

Im Fall von *P. falciparum*-infizierten normalen (HbA/A) Erythrozyten im Ringstadium erkennen Makrophagen die PS-exponierenden Erythrozyten mit dem sog. Scavenger-Rezeptor oder Mustererkennungsrezeptor CD36 und phagozytieren diese Zellen [65,268,269,270]. Erhöhte PS-Exposition aufgrund beschleunigter Eryptose in infizierten HbA/S

Erythrozyten könnte diesen Mechanismus verstärken.

Deshalb war das Ziel dieser Studie, Unterschiede in der Induktion der Eryptose zwischen HbA/A und HbA/S *P. falciparum*-infizierten-Erythrozyten aufzudecken. Obwohl sich im Ringstadium-synchronisierte-Parasiten in HbA/S- und HbA/A-Erythrozyten in gleichem Maße vermehrten, war die PS-Exposition in infizierten HbA/S-Erythrozyten signifikant erhöht. Unterschiede in der Sphingomyelinaseaktivität zwischen infizierten HbA/S- und HbA/A-Erythrozyten wurden beobachtet. Jedoch scheint die erhöhte Enzymaktivität nicht ursächlich die erhöhte PS-Exposition von infizierten HbA/S-Erythrozyten zu bedingen. Obwohl der Parasit Ceramid zur Membransynthese benötigt [371], hemmt Ceramid im Kulturmedium das Parasitenwachstum, und zwar verstärkt in HbA/S-Erythrozyten.

Ring-infizierte-HbA/S-Erythrozyten zeigten erhöhte Ca^{2+}-Aufnahme und einen 1,5-fachen Anstieg der freien zytosolischen Ca^{2+}-Konzentration, $[Ca^{2+}]_i$, im Vergleich zu Ring-infizierten-HbA/A-Erythrozyten. Demzufolge resultiert die erhöhte PS-Exposition in infizierten HbA/S-Erythrozyten aus einer erhöhten nichtselektiven Ca^{2+}-durchlässigen Kationenleitfähigkeit. Darüberhinaus war die PGE_2-Bildung von infizierten HbA/S-Erythrozyten höher als die von infizierten HbA/A-Erythrozyten. Demnach führen erhöhte PGE_2-Konzentrationen zu erhöhter nichtselektiver Kationenkanalaktivität von Ring-infizierten-HbA/S-Erythrozyten. Interessanterweise korrelieren erhöhte PGE_2-Plasmaspiegel mit einem Rückgang von schwerer Malaria bei Kindern, die mit *P. falciparum* infiziert sind [677]. PGE_2-induzierte PS-Exposition in infizierten HbA/S Erythrozyten bewirkt deren effektive Beseitigung schon im Ringstadium des Parasiten [443]. Zusätzlich erhöht beschleunigtes „oxidatives Altern“ des Wirtserythrozyten die Phagozytose von Ring-infizierten HbA/S Erythrozyten [66,763]. Diese Abwehrmechanismen des Wirts bedeuten einen entscheidenden Schutz gegenüber den tödlichen Konsequenzen einer *P. falciparum*-Infektion für infizierte Sichelzellgen-Überträger.

8 Appendix

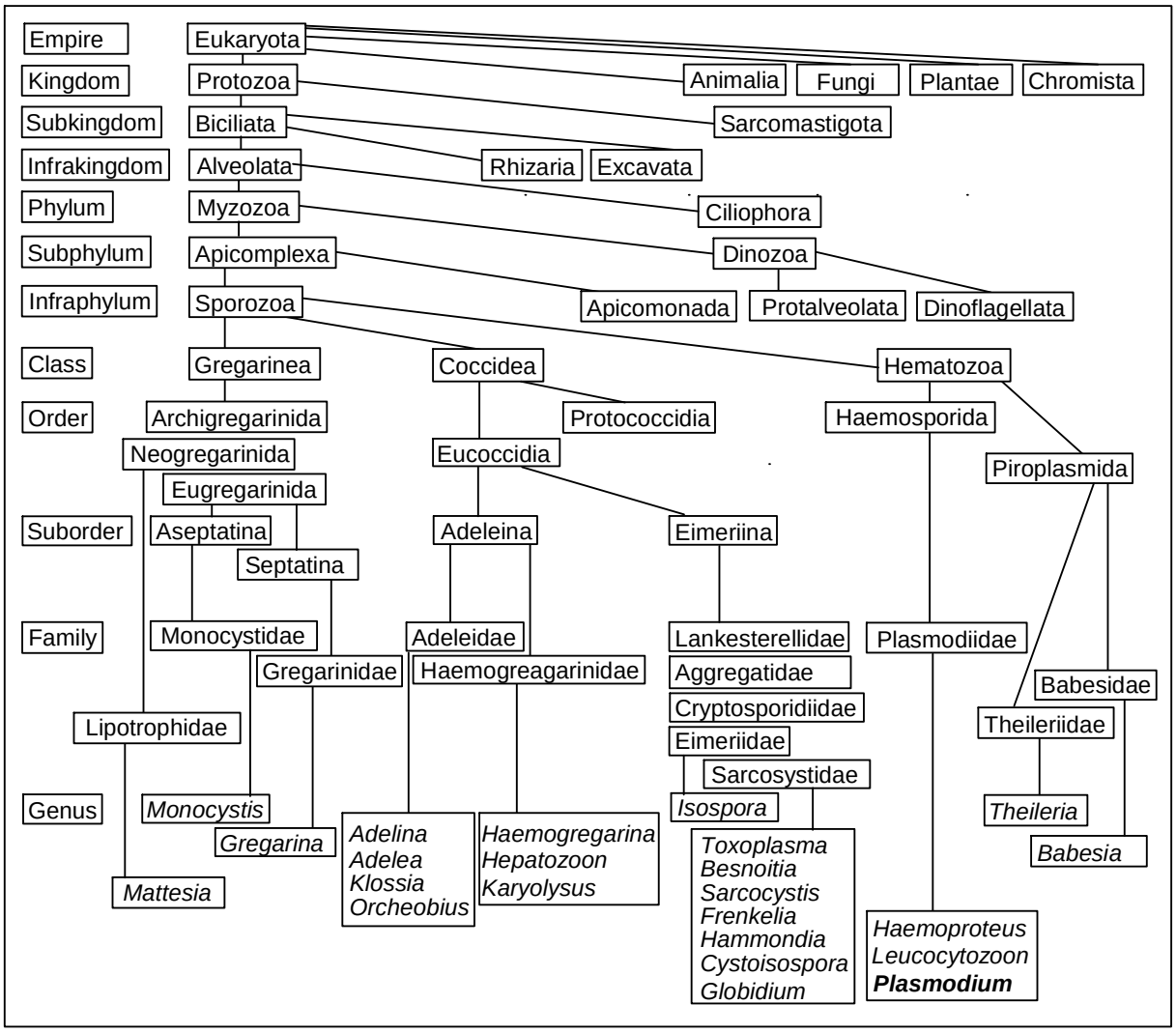


Figure 8.1. Classification of *Plasmodium* [157,158,159,160,161,162,534,535]**.**

The infraphylum sporozoa (about 4,000 known species) (Fig.8.1.) belongs to the subphylum apicomplexa. Sporozoa are characterized by an apical complex (see parasgraph 1.4.) with a conoid. The conoid is a funnel of rods, composed of two electron dense polar rings and criss-cross spiral of trough-shaped tubulin rods that extend subpellicularly from the polar rings parallel to the long axis of the cell. The funnel of rods functions presumably as a support for the cell - except in the genus *Babesia* and *Theileria* (former Aconoidasida). Furthermore, sporozoa are characterized by dioecious sex and gliding motility. This is to say that motile forms of sporozoa crawl along the substratum in a non-ameboid fashion as they do not have flagella or pseudopods or cilia and lack any visible means of locomotion beside flagellated gametes [162].

The phylum myzozoa (myzocytotic = sucking life) consists of the subphyla apicomplexa and dinozoa. Myzozoa and the phylum ciliophora belong to the infrakingdom of

alveolata [160]. Located just underneath their plasma membrane all myzozoa have a diminished mouth termed the micropore, two inner membranes and flattened vesicle-like structures termed cortical alveolae. These cortical alveolae support the outer pellicle, forming a semi-rigid pellicle. The ancestral alveolate most probably had a colorless plastid, called the apicoplast in apicomplexa. The plastid has an approximately 35 kb genome with an operon organization very similar to red algae plastid genomes [594]. The apicoplast is probably inherited from a common sporozoan and chromist ancestor, a chromalveolate [159,277,376]. This would mean that the apicoplast has a common origin with the chloroplasts in dinoflagellates. Such an apicoplast is present in eimeriid coccidea [241], piroplasmida [342] and *Plasmodium* [903] and might well be present in some apicomonads. In the other main sporozoan clade that includes both *Cryptosporidium* and gregarines the apicoplast appears to be lost [928].

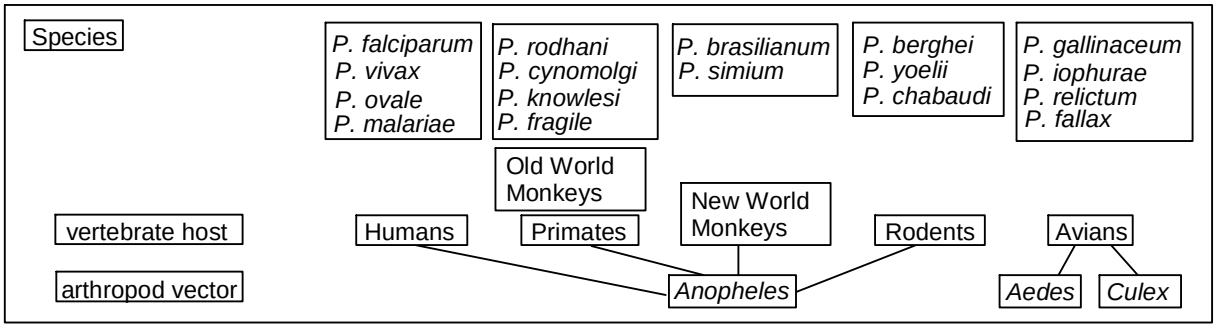


Figure 8.2. *Plasmodium spp*., their vertebrate and arthropod hosts.

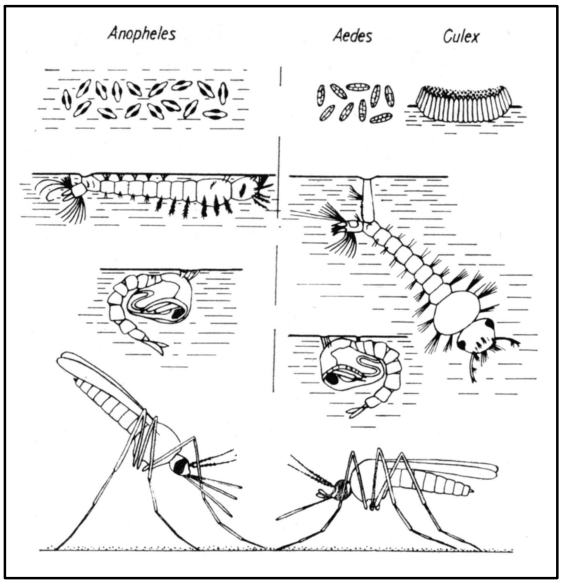


Figure 8.3. Eggs, larvae, pupae, adult from *Anopheles* in comparison to *Aedes* and *Culex* [833].

	Malaria tropica	**Malaria tertiana**	**Malaria tertiana**	**Malaria quatarna**
Species	***P. falciparum***	***P. vivax***	***P. ovale***	***P. malariae***
Incubation days (range)	6 - 14 d	8-20 d or up to 6-12 months	12 - 20 d or longer	14 – 40 d or longer
Exoerythrocytic cycle (days)	5.5 -7 d	6 -8 d	9 d	12 -16 d
No. of merozoites per liver cell	(2^{15}) 40,000	(2^{13}) 8, 000 - 10,000	(2^{14}) 15,000	(2^{11}) 2,000
Erythrocytic cycle (hours)	48 h (irregular)	42 - 48 h	49 - 50 h	72 h
RBC preference	younger cells, but can invade cells of all ages	Reticulocytes	Reticulocytes	older cells
Relapses (years)	No (no late relapses)	Yes (up to 3 -4 years)	Yes (up to 4.5 years)	regular up to 20 years (even up to 52 years)
Fever periodicity (hours)	48 h	48 h	48 h	72 h
Febrile duration (hours)	16 - 36 h or longer	8 – 12 h	8 – 12 h	8 – 10 h
Severity of primary attack	severe in non-immune individuals	mild to severe	mild	mild
Mortality	high	low	low	low
Drug Resistance	++	+	-	-
Distribution	worldwide in the tropics	worldwide in the tropics, subtropics, and temperate zone	Western coast of Africa	rarest species, in the (sub)tropics, endemic, within restricted areas

Table 8.1. Most important characteristics of human malaria.

	P. berghei	***P. falciparum***
Merozoites per blood schizont	12 -18	8 -32
Reticulocyte preference	Yes	No
Synchronous blood cycle	No	Yes
Mean diameter of schizonts in µm	27	45
Sporozoite size in µM	11	12
Sequestration	Yes	Yes

Table 8.2. *P. berghei, P. falciparum* - Quick comparison.

COMPOUND	**ASSOCIATED PROBLEMS**
Quinolines	
Quinine (Fig.8.5.A.; Trade names: Nivaquine, Malaraquine, Aralem, etc.) Quinine sulphate Quinidine gluconate (dextroisomer)	Drug of last resort (originally from cinchona tree). Side-effects: tinnitus; ineffective due to parasite resistance [601]. Limited availability; requires cardiac monitoring [601].
Mefloquine (quinoline-methanol derivative) (Fig.8.5.B.; Trade names: Lariam; Mephaquine)	Side-effects: psychoses; ineffective due to parasite resistance in Indochina, Africa [601]. Undergoing Phase IV clinical trials in 1998 [601].
8-Aminoquinolines (Fig.8.4) Primaquine Pamaquine (no longer available) (First synthetic antimalarial drug)	Undergoing preclinical trials in 1998 [601]. Eliminate hypnozoites of *P. vivax* or *P. ovale* after 14 days treatment or more; narrow therapeutic index; not used in G6PD-deficient patients [601]; ineffective due to parasite resistance.
4-Aminoquinolines Chloroquine (Trade name: Aralen) (Fig. 8.6.)	Ineffective due to parasite resistance worldwide except in Central America [878,915]; prior to resistance development it was the drug of choice

Amodiaquine (Trade name: Camoquine)	for treating non-severe or uncomplicated malaria and for chemoprophylaxis. Relatively widely available compound.
Halofantrine (Phenanthrene-methanol) (Trade name: Halfen). Desbutylhalofantrine	Cardiotoxicity[637]; poor resorption; sporadic ineffective due to parasite resistance [601]. Undergoing Phase IV clinical trials in 1998 [601]. Undergoing Phase I clinical trials in 1998 [601].
Antifolates	
<u>Anti-dihydrofolate synthase (DHFR)</u> (Fig. 8.7.A.) Proguanil (Paludrine) (Prodrug) Chlorproguanil Cycloguanil Proguanil analogs Pyrimethamine Trimethoprim WR99210 [847] WR238605	Causing rapidly developing resistance of *Plasmodium*, if used alone [354]. Side effects: mouth ulcers; ineffective due to parasite resistance [601]. Undergoing preclinical trials in 1998 [601]. Undergoing Phase III clinical trials in 1998 [601].
<u>Anti-Dihydroopterate synthase (DHPS)</u> (analogues of para-aminobenzoic acid) (Fig. 8.7.B.) Sulfadoxine Dapsone	
<u>Combinations</u> Chlorproguanil/dapsone (Lap-dap) Sulfamethaxole/trimethoprim (Co-trimoxazole) Sulfalene/pyrimethamine (Metakelfin) Sulfadoxine/pyrimethamine (SP, Fansidar)	If used in combination, drugs act synergistically, possibly overcoming individual resistances; furthermore, a higher cure rate, a lower likelihood of inducing parasite resistance, pharmacokinetic and pharmacodynamic advantages over the individual components. [112,871]. Severely allergenic; ineffective due to parasite resistance worldwide [601].

Atovaquone (hydroxynapthoquinone, an analogue of ubiquinone) Combination: Malorone: 250 mg atovaquone + 100 mg proguanil [113,293,560,697]	Used for the treatment of opportunistic infections in immunosuppressed patients; effective against chloroquine-resistant parasites; parasite resistance develops rapidly if used alone (polymorphism in the gene for cytochrome b [792]); usually given in combination with proguanil [560,697].
Endoperoxides (Sesquiterpene lactone) Artemisinine (Fig.8.8.A.; qinghaosu; shrub *Artemisia annua*) [709,886] Artemisinic acid, Artemether Artelinic Acid Arteether Artesunate (Fig. 8.8.B.)	Neurotoxic; if given alone development of resistance of *P. falciparum*; high recrudescence rates. Undergoing Phase I clinical trials in 1998 [601]. Less neurotoxic than artemether; undergoing Phase III clinical trials in 1998 [601]. Undergoing Phase II clinical trials in 1998 [601].
Artemisinine-based combination therapy Artemether-lumefantrine (fluoro-methanol) (Co-artem; Alamet) [607,821] Artesunate-mefloquine Artesunate-amodiaquine Artesunate-sulfadoxine/ pyrimethamine Chlorproguanil hydrochloride / dapson artesunate	Combination with long half-life drug mandatory, combination slows development of drug resistance of *P. falciparum*; responsible for decreased malaria transmission levels in South-East Asia [887,637].
Synthetic peroxides Trioxane; Tetraoxane	Undergoing preclinical trials in 1998 [601].
Antibiotics [593,699,698] Tetracycline; Doxycycline (Cotrifazid) (Fig. 8.9.) Clindamycin Isoniazid; Rifampicin Azithromyin (analog of erthromycine)	Improved cure rates in combination with quinine [112]. Doxycycline is phototoxic; not used in pregnant women and children; gastrointestinal intolerance [601]. Undergoing Phase IV clinical trials in 1998 [601]. Parasites respond slowly; high recurrence rates [479,478]. Limited use; efficacy remains to be defined. Undergoing Phase II clinical trials in 1998 [601].

Acridine analogues Pyronaridine (Acridine type benzonaphthyridine) Dihydroacridinedione;Quinacrine Floxacrine analogs	100 % effective in one trial in Cameroon [711], 63 % and 88 % effective in Thailand [559]. Undergoing Phase II clinical trials in 1998 [601]. Undergoing preclinical trials in 1998 [601].

Table 8.3. Treatment of malaria.

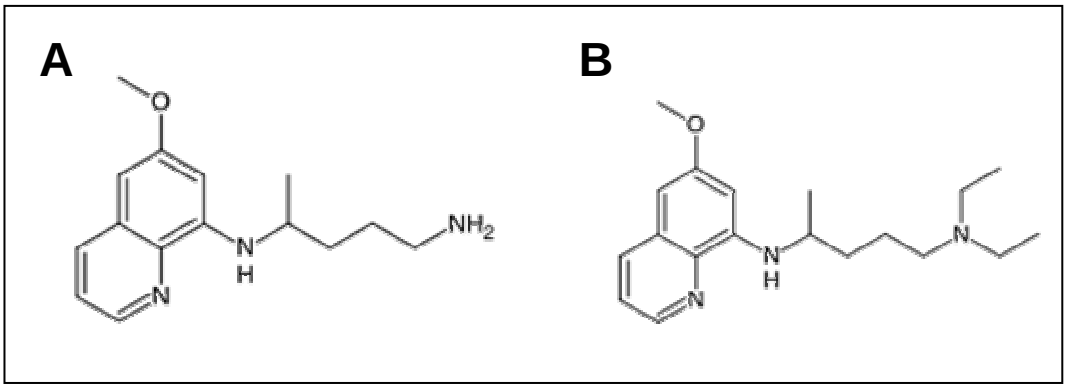


Figure 8.4. (A) Primaquine. *N*-(6-methoxyquinolin-8-yl) pentane-1,4-diamine. **(B) Pamaquine.** *N*,*N*-diethyl-*N'*-(6-methoxyquinolin-8-yl)pentane-1,4-diamine) was the first synthetic antimalaria drug. It is no longer available. Like primaquine, pamaquine causes hemeolytic anemia in patients with G6PDH deficiency.

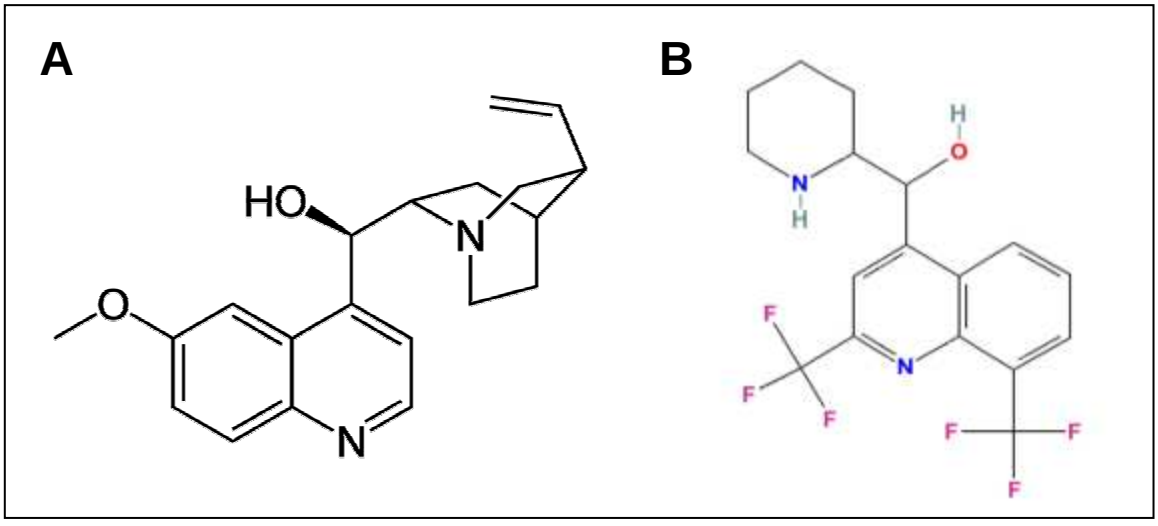


Figure 8.5. (A) Quinine. (2-ethenyl-4-azabicyclo[2.2.2]oct-5-yl)- (6-methoxyquinolin-4-yl)-methanol. **(B) Mefloquine.** 2,8-bis(trifluoromethyl)quinolin-4-yl]-(2- piperidyl)methanol.

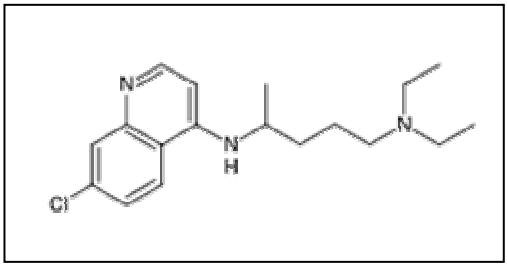


Figure 8.6. Chloroquine. *N'*-(7-chloroquinolin-4-yl)-*N*,*N*-diethyl-pentane-1,4-diamine.

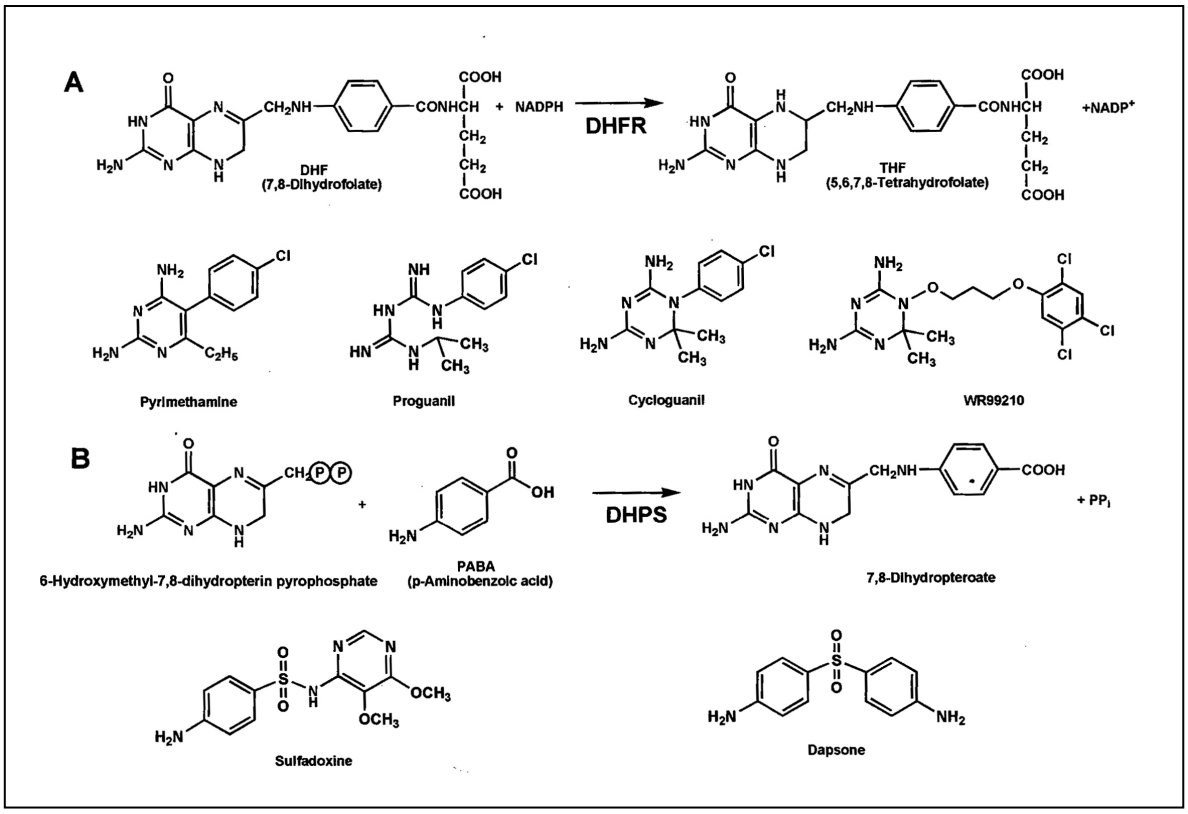


Figure 8.7. (A) DHFR and (B) DHPS inhibitors. (**A**) Chemical reaction catalyzed by dihydrofolate reductase (DHFR) and structures of selected antimalarial DHFR inhibitors. The prodrug proguanil is converted to the DHFR inhibitor cycloguanil. (**B**) Chemical reaction catalyzed by dihydropteroate synthase (DHPS), together with the structures of the DHPS inhibitors sulfadoxine and dapsone [847].

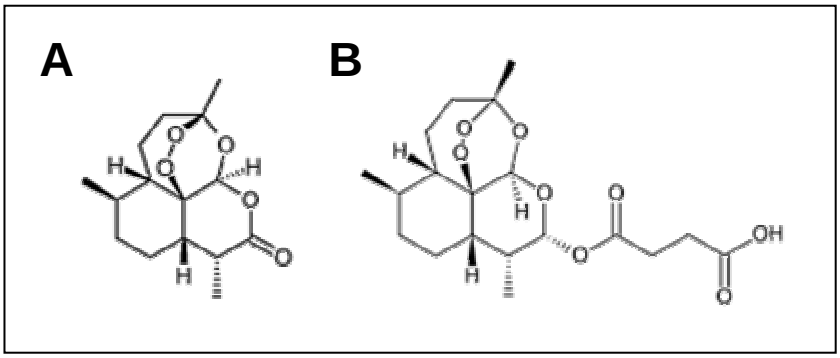


Figure 8.8. (A) Artemisinine. (3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10(3*H*)-one [480,642]. **(B) Artesunate.**

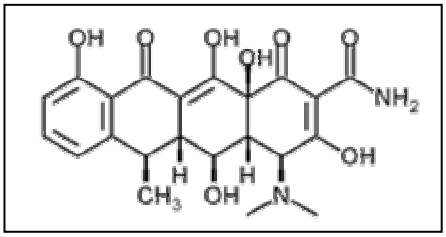


Figure 8.9. Doxycycline. (2-(amino-hydroxy-methylidene)-4-dimethylamino-5,10,11,12a-tetrahydroxy-6-methyl- 4a,5,5a,6-tetrahydro-4H-tetracene-1,3,12-trione.

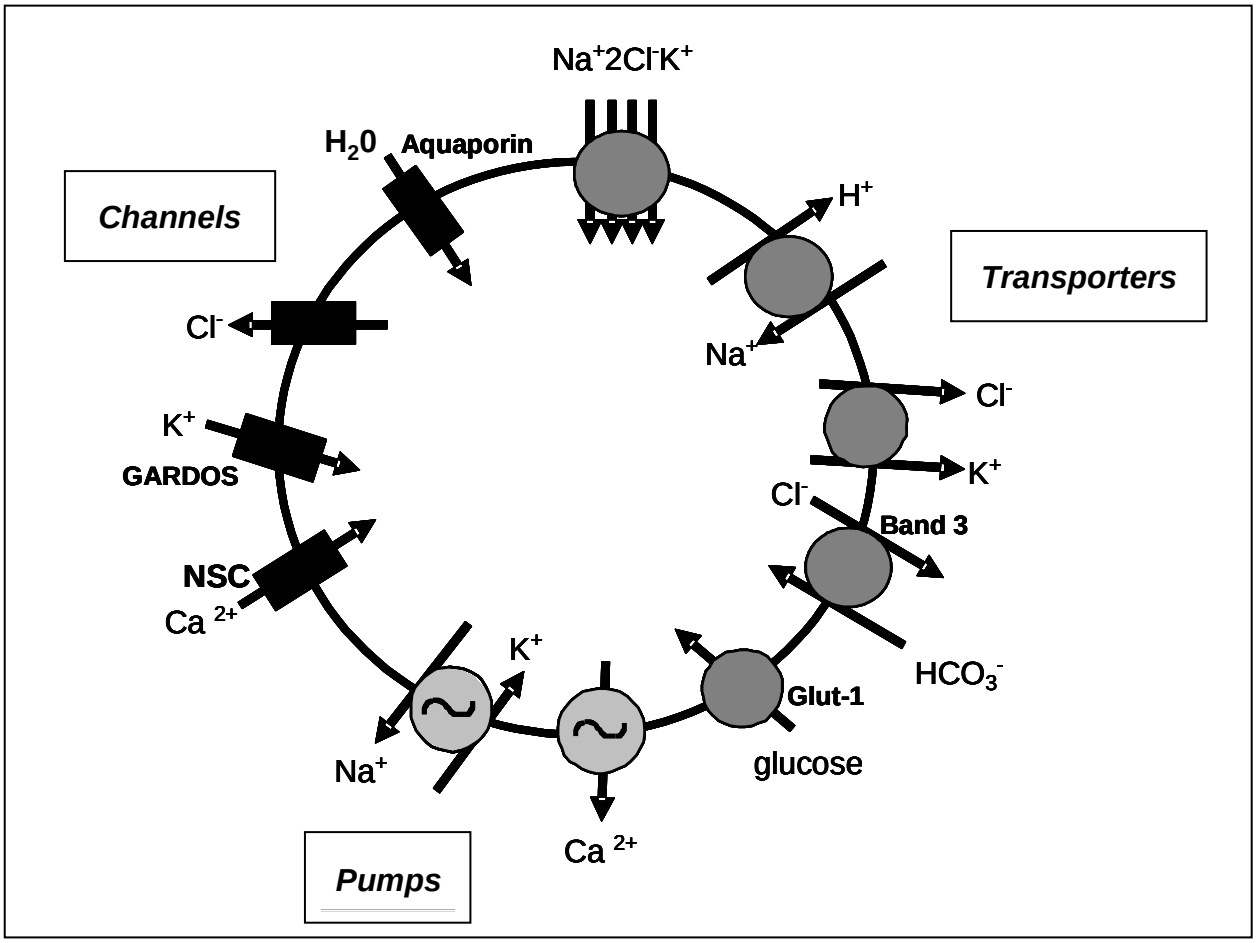
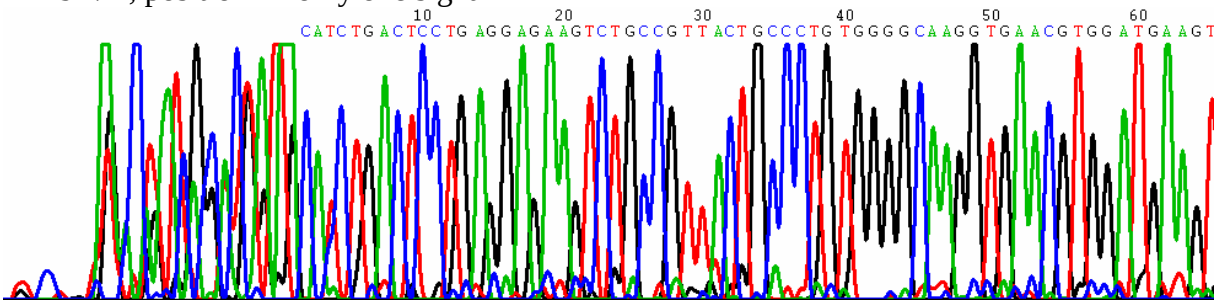
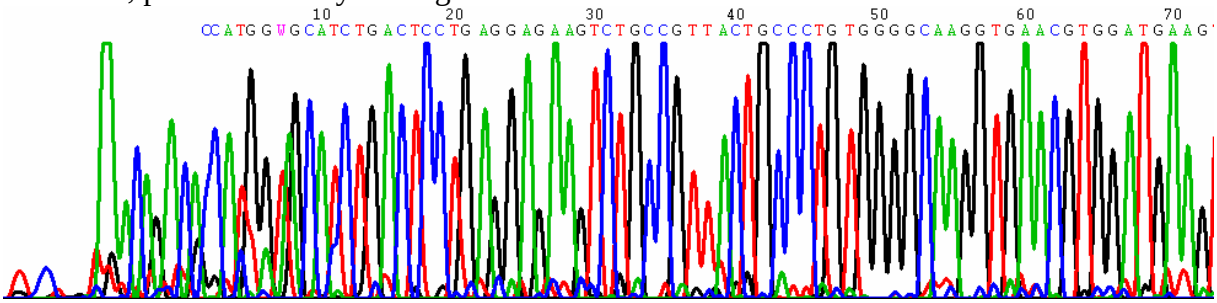


Figure 8.10. Principal pumps, channels and transporters of human erythrocytes. With permission modified from Tanneur *et al.* [814].

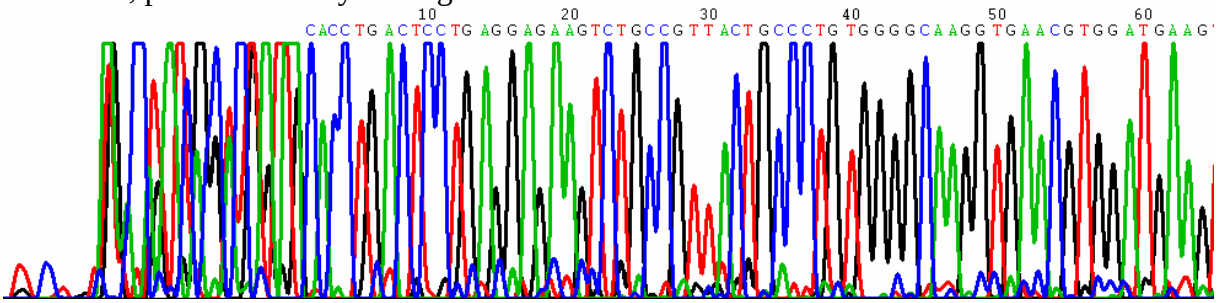
A HbA/A, position 14 only one signal A



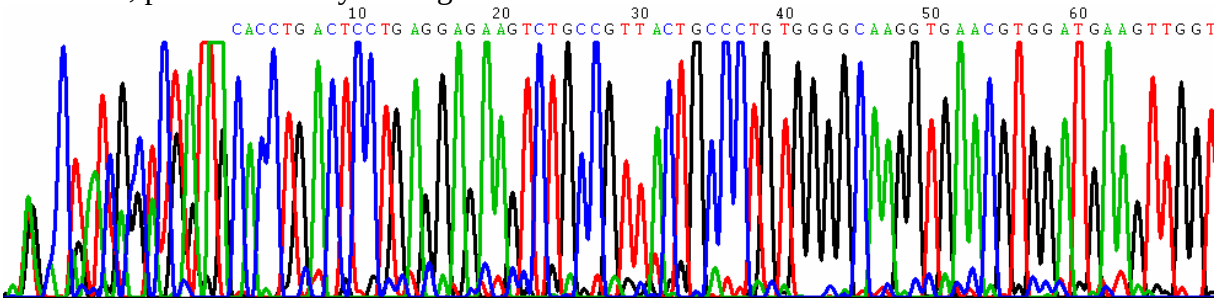
B HbA/A, position 22 only one signal A



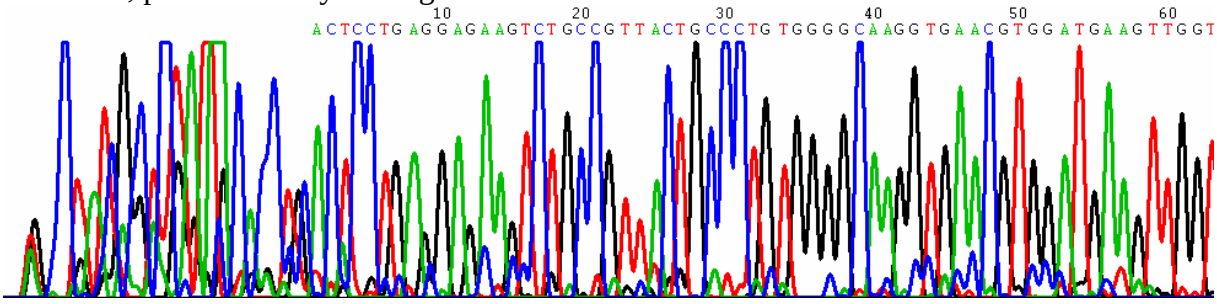
C HbA/A, position 14 only one signal A



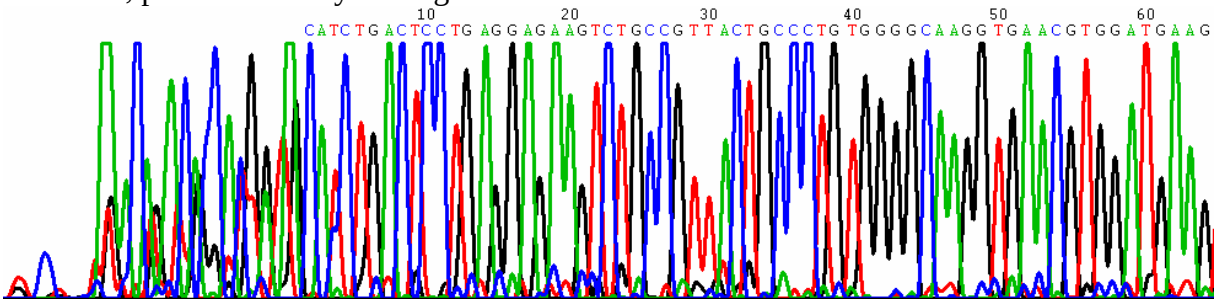
D HbA/A, position 14 only one signal A



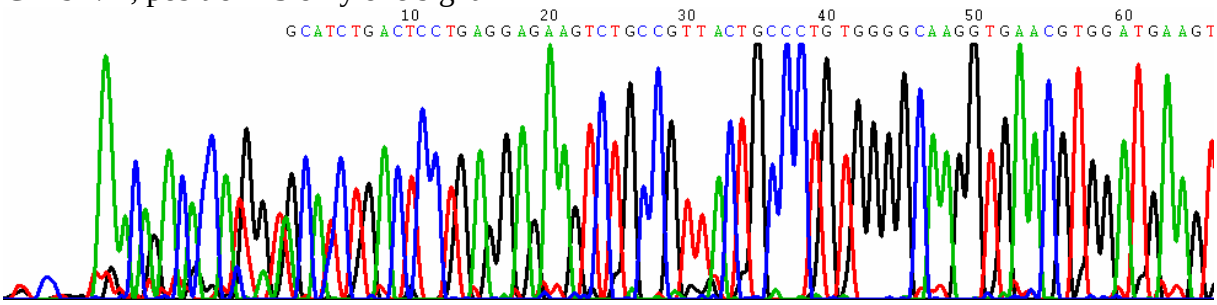
E HbA/A, position 8 only one signal A



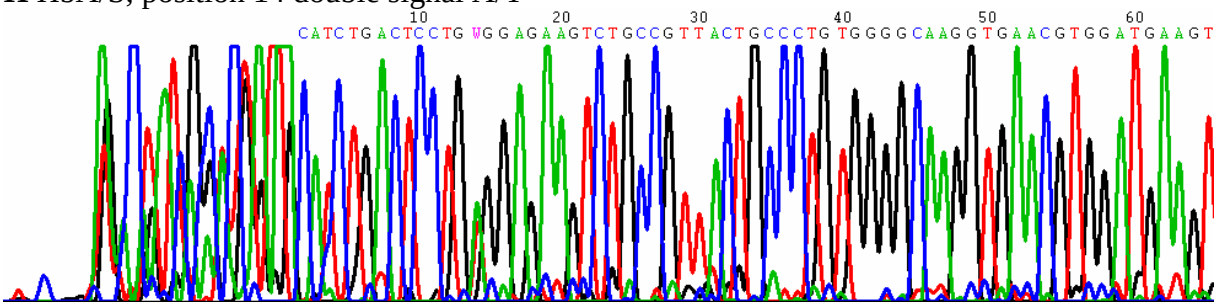
F HbA/A, position 14 only one signal A



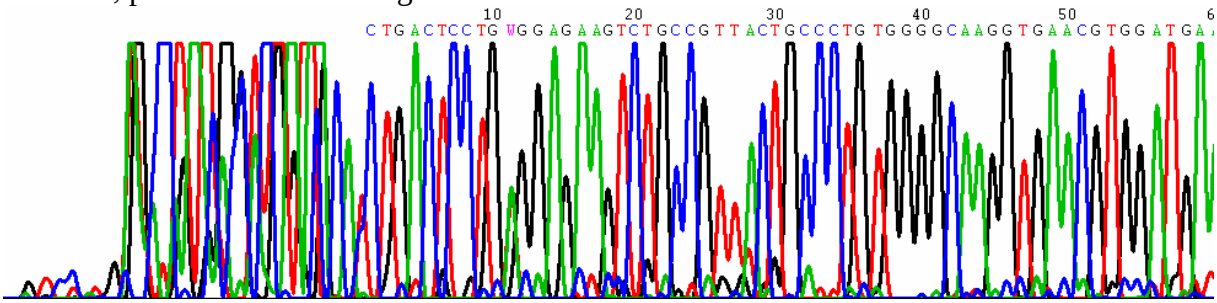
G HbA/A, position 15 only one signal A



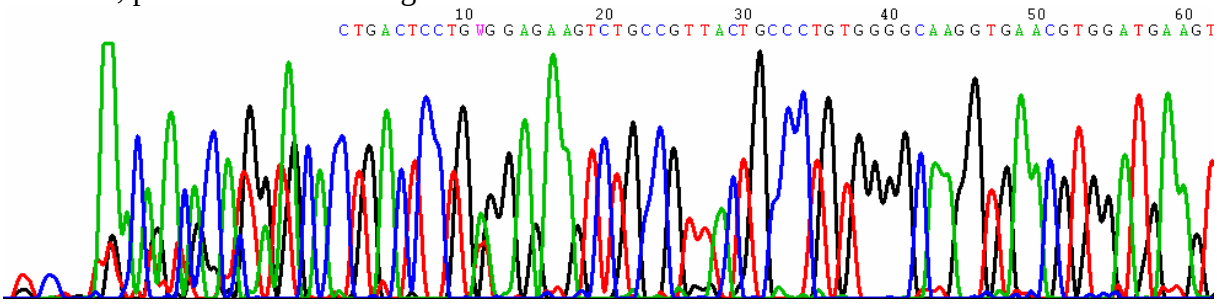
H HbA/S, position 14 double signal A/T



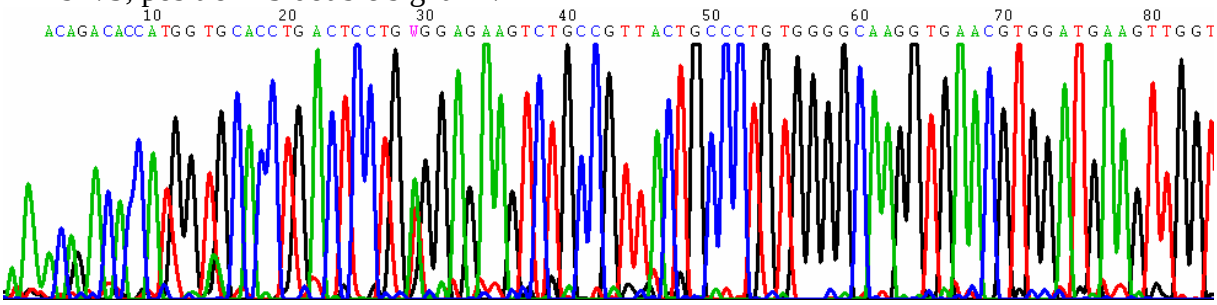
I HbA/S, position 11 double signal A/T



J HbA/S, position 11 double signal A/T



K HbA/S, position 29 double signal A/T



L HbA/S, position 0 double signal A/T

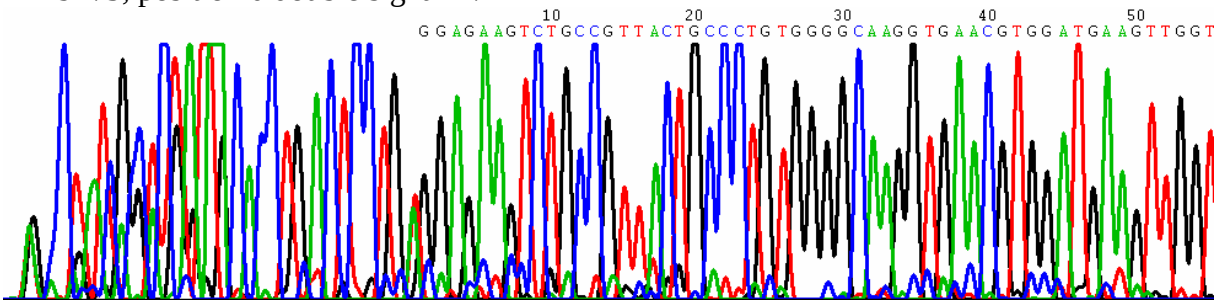


Figure 8.11. Confirmation of the genotype of the blood samples used. Sequencing results of an amplified DNA fragment of healthy and sickle cell trait individuals, containing the codon 6 of the β-hemoglobin chain.

A-L. Sequences of an amplified DNA fragment containing the codon 6 of the β-hemoglobin chain. Genomic DNA, extracted from whole blood of healthy (HbA/A) and sickle cell trait (HbA/S) individuals were PCR amplified and sequenced. See Material and Methods for details. Normal hemoglobin (HbA) has the code GAG (position 13-15 **A**) for glutamine (**A - G**), while sickle cell hemoglobin (HbS) has the code GTG for valine. The DNA from sickle trait (HbA/S) RBCs (**H – L**) therefore shows a double signal A/T at position 14 (**H**).

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CURRICULUM VITAE

Dr. Verena Beatrice Brand ♦ Gimbacher Weg 56 ♦ D-65779 Kelkheim ♦ verena.brand@uni-tuebingen.de

BERUFSERFAHRUNG	
2005 - 2006	Wissenschaftliche Mitarbeiterin, MARINE BIOLOGICAL LABORATORY, Bay-Paul Centre, Woods Hole, USA: Global infectious disease program, Biosynthese und Funktion der DNA-Glykosylierung in Afrikanischen Trypanosomen.
2001 - 2005	Wissenschaftliche Mitarbeiterin, EBERHARD-KARLS-UNIVERSITÄT, Physiologisches Institut, Tübingen, Deutschland: Ionenkanäle und Malaria.
1991 - 1992	Biologielaborantin, BATTELLE-INSTITUT, Frankfurt am Main, Deutschland: Molekulare und Zelluläre Biologie, Terrestrische Ökotoxikologie.
1988 - 1991	Biologielaborantin (Ausbildung), HOECHST AG, Frankfurt-Hoechst, Deutschland: Stoffwechsel, Virologie, Biotechnologie, Pflanzenschutz, Pharmakologie.

LEHRERFAHRUNG	
2002 - 2004	Kurse und Seminare für Medizinstudenten in der Physiologie des Sehens, Hörens und der Muskulatur, EBERHARD-KARLS-UNIVERSITÄT, Physiologisches Institut, Tübingen, Deutschland.
2001 - 2005	Anleitung von Medizinstudenten und Biologielaboranten, EBERHARD-KARLS-UNIVERSITÄT, Physiologisches Institut, Tübingen, Deutschland.

UNIVERSITÄTS- UND BERUFSAUSBILDUNG	
2001 - 2007	Doktorarbeit, EBERHARD-KARLS-UNIVERSITÄT, Physiologisches Institut, Tübingen, Deutschland. Promotionsthema: Programmierter Zelltod in *Plasmodium* infizierten HbA/A und HbA/S Erythrozyten. Abschluss: Dr. rer. nat. (Note: m a g n a c u m l a u d e).
1999 - 2001	Diplomarbeit (Note: sehr gut), MAX-PLANCK-INSTITUT FÜR PHYSIOLOGIE, Physikalische Biochemie, Dortmund, Deutschland: Multiphotonphotometrie, Detektion von Sauerstoffradikalen. Abschluss: Diplom-Biochemikerin (Note: gut).
1995 - 1999	Biochemie-Studium, UNIVERSITÄT WITTEN / HERDECKE, Deutschland. Diplompüfungen in Biochemie (Note: gut).
1994 - 1995	Biochemie- und Französisch-Studium (ERASMUS-Austauschstudentin), UNIVERSITE DE BOURGOGNE, Dijon, Frankreich.
1992 - 1994	Biologie-Studium, JOHANNES-GUTENBERG-UNIVERSITÄT, Mainz, Deutschland. Vordiplom in Biologie (Note: sehr gut).
1988 - 1991	Ausbildung zur Biologielaborantin, HOECHST AG, Frankfurt-Hoechst, Deutschland. Abschluß: Biologielaborantin (Note: gut).

SCHULAUSBILDUNG	
1985 - 1988	Gymnasiale Oberstufenschule IMMANUEL KANT, Kelkheim im Taunus, Deutschland. Abschluß: Abitur (Note: sehr gut).
1978 - 1985	Gymnasium ST. ANGELA-Schule, Königstein im Taunus, Deutschland.
1974 - 1978	Grundschule PESTALOZZI, Kelkheim im Taunus, Deutschland.

METHODEN-SPEKTRUM

MOLEKULARBIOLOGIE / MIKROBIOLOGIE

- Untersuchung differentieller Genexpression (RNAse protection assay zur mRNA Bestimmung).
- Klonieren, Erstellung und Expression von Plasmiden *in vitro* und in Säugetierzellen.
- Durchführung von RT-PCR, PCR, Agarosegel-Elektrophorese und Southernblots.
- Vorbereitung von Proben zum Sequenzieren und Analyse der Sequenzierngsergebnisse.
- Gute Grundkenntnisse in Mikrobiologie.

BIOCHEMIE / PROTEOMIK

- Chromatographische Reinigung und Auftrennung von Antikörpern und Proteinen.
- Analyse aufgereinigter Proteine von Geweben, Organen und rekombinanter Proteinexpression durch Westernblots und Immunodotblots.
- Etablierung eines radioenzymatischen Ceramidassays.

BIOPHYSIK

- Multiphoton-konfokale Laserscanning-Mikroskopie; Messung von Point-Spread-Function und 3D Rekonstruktion.
- UV / VIS Absorptions- und Fluoreszens-Spektrophotometrie.

ZELLBIOLOGIE / PARASITOLOGIE

- Zellkultur von über 20 verschiedenen, z.T. transfizierten Zellinien, Krebszellinien, Endothelzellen, von *Plasmodium falciparum* und*Trypanosoma brucei*.
- Bestimmung der Wachstumsabhängigkeit von *P. falciparum in vitro* vom Zellvolumen, Redoxstatus des Erythrozyten, Ionenkonzentration, Wirtszellsignaling und Programmierten Zelltod in heterozygoten Sichelzell- und normalen Erythrozyten (FACS, Tracer flux, ELISA, Hämolyse, Membranaufbereitung).
- 3D-Kollagenmatrix-Kultur von T-Zellen mit verschiedenen Antikörperen; Computer-gestützte Analyse der Zellwanderung und Statistik.

IMMUNOLOGIE

- Entwicklung einer neuen Methode der Immunhistologischen Färbung von Paraffinschnitten mit unterschiedlichen Antikörpern gegen Endothelzellen.
- Magnet-gestützte Zellsortierung (MACS), um T-Zellen zu isolieren und unterschiedliche asexuelle Entwicklungsstadien von *P. falciparum* aufzutrennen, Reinheitskontrolle durch FACS Analyse.

WEITERE METHODEN

- Infektion gendefizienter Mäuse mit *Plasmodium berghei* ANKA.
- Dokumentation gemäß der Guten Laborpraxis (GLP), professioneller Umgang mit Versuchstieren und Radioaktivität.
- Kenntnis in Arbeitsschutz und Umweltschutz.

ZUSAMMENFASSUNG DER KOMPETENZEN UND QUALIFIKATIONEN

- Deutsch (Muttersprache), Französisch, Englisch: Sehr gut in Wort und Schrift.
- EDV-Kenntnisse: Windows XP, MacIntosh, Adobe Photoshop, Canvas.
- Selbstständige Etablierung und Entwicklung wissenschaftlicher Methoden.
- Kompetenz in fortgeschrittenen Methoden in Malaria, Biochemie, molekularer Zellbiologie, Immunologie und Biophysik.
- Wissenschaftliche Präsentationen in verschiedenen Ländern in Deutsch, Englisch, Französisch.
- Teamfähig und erfolgreich in eigenständiger Arbeit.
- Problemlösung mit sehr guten analytischen Fähigkeiten.

FORSCHUNGSPRAKTIKA	
1999	MAX-PLANCK-INSTITUT FÜR PHYSIOLOGIE, Strukturelle Biologie, Dortmund, Deutschland: Kleine GTP-bindende Proteine Ras, Betreuer: RH Cool.
1997	IGBMC (Institut de Génétique et Biologie Moleculaire et Cellulaire), Département de biologie cellulaire et de transduction du signal, Illkirch, Straßbourg, Frankreich: Einfluß von Retinsäure auf den Zellzylus von Krebszellen, Betreuer: H Gronemeyer.
1997	UNIVERSITÄT WITTEN / HERDECKE, Institut für Biochemie, Deutschland: Analyse des Pikrinsäureabbaus in Mikroorganismen, Betreuer: K Heesche-Wagner.
1996 - 1997	UNIVERSITÄT WITTEN / HERDECKE, Institut für Immunologie, Deutschland. Studentische Hilfskraft: Wanderung von T-Zellen in Extrazellulärer Matrix, Betreuer: P Friedl.
1996	UNIVERSITÄT WITTEN / HERDECKE, Institut für Phytochemie, Deutschland: Isolierung von anti-Viscotoxin Antikörpern aus Pleuraergüssen, Betreuer: U Füller.

STIPENDIEN	
2005 - 2006	ELLISON-Forschungsstipendium, MARINE BIOLOGICAL LABORATORY, Bay-Paul Centre, Woods Hole, USA: Global infectious disease program.
2000	DAAD-Stipendium, INTERNATIONALE FRAUENUNIVERSITÄT (in Englisch), Fachhochschule Nordost-Niedersachsen, Suderburg, Deutschland: Projektbereich Wasser.
1997	Stipendium der UNIVERSITE DE L´EUROPE, Paris, Frankreich für ein Forschungspraktikum in molekularer Zellbiologie am IGBMC, Illkirch, Frankreich.
1994 - 1995	ERASMUS-Stipendium, Biochemie und Französisch-Studium an der UNIVERSITE DE BOURGOGNE, Dijon, Frankreich.

PROFESSIONELLE AKTIVITÄTEN UND MITGLIEDSCHAFTEN	
Seit 2005	BRITISH SOCIETY FOR PARASITOLOGY
Seit 2003	VEREINIGUNG TÜBINGER BIOCHEMIKER (Mobbel e.V.), Schriftführerin des Vorstands
2002	Kongreß der deutschen, skandinavischen und britischen PHYSIOLOGISCHEN GESELLSCHAFT in Tübingen 2002: Mitglied des Organisationskomitees.

REFERENZEN

Prof. Dr. med. Florian Lang, Direktor des Physiologischen Instituts (Doktorvater)

Eberhard-Karls-Universität, Physiologisches Institut, Gmelinstr. 5, D-72076 Tübingen, Deutschland

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http://www.physiologie.medizin.uni-tuebingen.de/DepI

Privatdozent Dr. rer. nat. Stephan Huber, Oberassistent (Laborleiter: Ionenkanäle und Malaria)

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stephan.huber@uni-tuebingen.de, Telefon: + 49 7071 297 2194
http://www.physiologie.medizin.uni-tuebingen.de/DepI/Researchgroups/huber

Professor Dr. rer. nat. Susanne Ullrich (ehemalige Kollegin)

Eberhard-Karls-Universität, Medizinische Klinik IV, Otfried-Müller-Strasse 10, D-72076 Tübingen, Deutschland

susi_ullrich@yahoo.com, Telefon: + 49 7071 298 7599

INTERNATIONALE VERÖFFENTLICHUNGEN (PEER-REVIEWED)

Tanneur V, Duranton C, **Brand VB**, Sandu CD, Akkaya C, Kasinathan RS, Gachet C, Sluyter R, Barden JA, Wiley JS, Lang F, Huber SM. **2006**. Purinoceptors are involved in the induction of an osmolyte permeability in malaria-infected and oxidized human erythrocytes. *FASEB J*. 20:133-135.

Duranton C, Akkaya C, **Brand VB**, Tanneur V, Lang F, Huber SM. **2005**. Artemisinin inhibits cation currents in malaria-infected human erythrocytes. *Nanomedicine: Nanotechnology, Biology and Medicine*. 1:143-149.

Duranton C, Tanneur V, **Brand V**, Sandu CD, Akkaya C, Huber SM, Lang F. **2005**. Permselectivity and pH-dependence of *Plasmodium falciparum*-induced anion currents in human erythrocytes. *Pflügers Arch*. 450:335-344.

Huber SM, Duranton C, Henke G, Van de Sand C, Heussler V, Shumilina E, Sandu CD, Tanneur V, **Brand V**, Kasinathan RS, Lang KS, Kremsner PG, Hubner CA, Rust MB, Dedek K, Jentsch TJ, Lang F. **2004**. *Plasmodium* induces swelling-activated ClC-2 anion channels in the host erythrocyte. *J Biol Chem*. 279:41444-41452.

Duranton C, Huber SM, Tanneur V, **Brand VB**, Akkaya C, Shumilina EV, Sandu CD, Lang F. **2004**. Organic osmolyte permeabilities of the malaria-induced anion conductances in human erythrocytes. *J Gen Physiol*. 123:417-426.

Lang F, Lang PA, Lang KS, **Brand V**, Tanneur V, Duranton C, Wieder T, Huber SM. **2004.** Channel-induced apoptosis of infected host cells-the case of malaria. *Pflügers Arch*. 448:319-324.

Lang KS, Myssina S, **Brand V**, Sandu C, Lang PA, Berchtold S, Huber SM, Lang F, Wieder T. **2004**. Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ*. 11:231-243.

Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, Lang F. **2003**. Dependence of *Plasmodium falciparum in vitro* growth on the cation permeability of the human host erythrocyte. *Cell Physiol Biochem*.13:347-356.

Duranton C, Huber S, Tanneur V, Lang K, **Brand V**, Sandu C, Lang F. **2003**. Electrophysiological properties of the *Plasmodium falciparum*-induced cation conductance of human erythrocytes. *Cell Physiol Biochem*. 13:189-98.

Porwol T., W. Ehleben, **V. Brand**, H. Acker. **2001**. Tissue oxygen sensor function of NADPH oxidase isoforms, an unusual cytochrome aa3 and reactive oxygen species. *Respir Physiol*. 128:331-348.

ANDERE VERÖFFENTLICHUNGEN

Lang PA, Kasinathan RS, **Brand VB**, Duranton C, Lang C, Koka S, Shumilina E, Kempe DS, Tanneur V, Akel A, Lang KS, Foeller M, Kun J, Kremsner PG, Wesselborg S, Gulbins E, Laufer S, Clemen CS, Herr C, Noegel A, Wieder T, Gulbins E, Lang F, Huber SM. Accelerated clearance of malaria-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. In Vorbereitung.

Kieft R*, **Brand V***, Ekanayake DK, Sweeney K, DiPaolo C, Reznikoff WS, Sabatini R. JBP2, a SWI2/SNF2-like protein, regulates de-novo telomeric DNA glycosylation in bloodstream form *Trypanosoma brucei*. *Geteilte Erstautorenschaft. Vorgelegt bei *Mol Biochem Parasitol*.

Duranton C, Tanneur V, **Brand VB**, Koka S, Kasinathan RS, Dorsch M, Hedrich HJ, Baumeister S, Lingelbach K, Huber SM, Lang F. A high specificity and affinity interaction with serum albumin stimulates an anion conductance in malaria-infected erythrocytes. Vorgelegt bei *J Physiol*.

Brand, V. 2001. The localisation of reactive oxygen species in human hepatoma cells visualised by one and two photon excitation confocal laser scanning microscopy; http://edoc.mpg.de/21131 (Diplomarbeit).

ABSTRACTS, POSTERPRÄSENTATIONEN AUF WISSENSCHAFTLICHEN KONGRESSEN

BRITISH SOCIETY FOR PARASITOLOGY, Malaria meeting, 3. - 6. April **2005** in Nottingham, U.K.

Brand VB, Sandu CD, Duranton C, Huber SM, Lang F. **2005**. Dependence of *Plasmodium falciparum in vitro* growth on the cation permeablitiy of the human host erythrocyte (*British Society for Parasitology, Spring and Malaria Meeting 2005*).

DEUTSCHE PHYSIOLOGISCHE GESELLSCHAFT, Kongresse: 14. – 17. März **2004** in Leipzig; 2. - 5. März **2003** in Bochum; 15. - 19. März **2002** in Tübingen; 10. – 13. März **2001** in Berlin.

Tanneur V, Duranton C, **Brand VB**, Sandu CD, Gachet C, Lang F, Huber SM. **2005**. Involvement of purionoceptors in the induction of an oxidation-induced osmolyte permeability in malaria-infected erythrocytes. *Pflügers Arch.* 449:S131.

Huber SM, Akkaya C, Tanneur V, **Brand VB**, Duranton C, Sandu CD, Lang F. **2004**. Is the *Plasmodium falciparum*-induced anion channel the ATP release pathway of the host erythrocyte? *Pflügers Arch.* 447:S63.

Duranton C, **Brand VB**, Tanneur V, Sandu CD, Akkaya C, Baumeister S, Lingelbach K, Lang F, Huber SM. **2004**. Effects of albumin on the organic osmloyte permeability in *P. falciparum* infected erythrocytes. *Pflügers Arch.* 447:S63.

Tanneur V, Duranton C, **Brand VB**, Sandu CD, Gachet C, Sluyter R, Barden JA, Wiley J, Lang F, Huber SM. **2004**. Oxidation-induced activation of an organic osmolyte permeability in human erythrocytes involves purinoceptor signalling. *Pflügers Arch.* 447:S135

Brand VB, Lang PA, Lang K, Uhlemann AC, Sandu CD, Tanneur V, Duranton C, Lang F, Huber SM. **2004**. Ceramide- and cell shrinkage-induced inhibition of intraerythrocytic growth of *P. falciparum*. *Pflügers Arch.* 447:S135.

Sandu CD, Lang PA, Jendrossek V, Gulbins E, Tanneur V, **Brand VB**, Duranton C, Lang F, Huber SM. **2004**. Decreased suspectibility of acid sphingomyelinase-deficient mice to *Plasmodium berghei* ANKA malaria. *Pflügers Arch.* 447:S136.

Huber SM, Henke G, Duranton C, Shumilina E, Tanneur V, Sandu CD, **Brand VB** , Van der Sand C, Heussler V, Lang F. **2003**. *Plasmodium* induces swelling-activated ClC-2 anion channels in the host erythrocytes. *Pflügers Arch.* 445:S38,O15-4.

Duranton C, Akkaya C, Tanneur V, **Brand VB**, Sandu CD, Lang F, Huber SM. **2003**. Organic osmolyte and anion channels in human erythrocytes-infected with *Plasmodium falciparum*. *Pflügers Arch.* 445:S43,O19-1.

Wieder T, Lang KS, Myssina S, **Brand V**, Sandu C, Huber SM, Lang F. **2003**. Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Pflügers Arch.* 445:S43,O19-3.

Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Lang F, Huber SM. **2003**. Dependence of intraerythrocytic *Plasmodium falciparum* development on extracellular Ca and Na. *Pflügers Arch.* 445:S71,P09-2.

Sandu CD,Tanneur V, Duranton C, **Brand VB**, Van der Sand C, Heussler V, Lang F, Huber SM. **2003**. Organic osmolyte and ClC-2 channels in oxidized erythrocytes. *Pflügers Arch.* 445:S99,P17-10.

Tanneur V, Duranton C, **Brand VB**, Sandu CD, C. Akkaya C, Lang F, Huber SM. **2003**. Oxidation-induced activation of an osmolyte permeability in human erythrocytes involves purinoceptor signalling. *Pflügers Arch.* 445:S108,P19-12.

Brand V, Uhlemann AC, Kremsner PG, Huber SM, Lang F. **2002**. In vitro oxidation mimics the oxidative stress conferred by *Plasmodium falciparum* on the host erythrocyte. P 39-7. *Pflügers Arch.* 443:S325.

Uhlemann AC, **Brand V**, Kremsner P, Huber SM, Lang F. **2002**. Hyperosmolar media inhibit intraerythrocytic growth of *Plasmodium falciparum*.P 12-10. *Pflügers Arch.* 443:S256.

Brand V, Porwol T, Acker H. **2001**. 1 and 2 photon laser microscopy of dihydrorhodamine and dichlorodihydrofluorescein for localising reactive oxygen species in HepG2 cells. *Pflügers Arch.* 441(6):P47-6.