Receptors in the Regulation of Suicidal Erythrocyte Death

der Fakultät für Biologie
der **EBERHARD KARLS UNIVERSITÄT TÜBINGEN**

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

von
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aus
Chuadanga, Bangladesch

vorgelegte
Dissertation
2009

Tag der mündlichen Prüfung: 23.10.2009

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1. ABBREVIATIONS

ACPA	(R,S)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid
Akt	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CMKRL1	Chemoattractant-receptor-like-1
CNQX	6-cyano-7-nitroquinoxaline-2, 3-Dione
CNS	Central nervous system
COX	Cyclooxyginase
Ctr	Control
cysLT	Cysteinyl-leukotriene receptor
DNQX	6,7-dinitro-quinoxaline-2,3-dione
EDTA	Ethylenediaminetetraacetic acid
EGTA	glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EIPA	Ethylisopropylamiloride
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothyocyanate
FL	Fluorescence channel
FLAP	5-lipoxygenase activating protein
FSC	Forward scatter
g	gram
GluA	Glutamate receptor A
GLUT	Glucose transporter
GSH	Glutathion
h	hour

Hb	Hemogblobin
HCT	Hematocrit
HEPES	32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acids
HPETE	Hydroperoxyeicosatetraenoic acids
iGluRs	Ionotropic glutamate receptors
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IONO	Ionomycin
IRAK	IL-1R-associated kinase
JAK	Janus kinase
K^+	Potassium
KCC	K+/Cl- cotransporter
LT	Leukotriene
mM	Milimolar
MyD88	Myeloid differentiation factor-88
Na^+	Sodium
NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide
NF	Nuclear factor
NHE	Na^+/H^+ exchanger
NMDA	N-methyl-D-aspartate
NMDG	N-methyl-D-glucamine
NO	Nitric oxide
PAF	Platelate activating factor
PAMP	Pathogen-associated molecular patterns
PARP	Poly(ADP-ribose) polymerase 1
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E_2
PGN	Peptidoglycan
PLA	Phospholipase A
PMSF	Phenyl methyl sulfonyl fluride
PNQX	1,4,7,8,9,10-hexahydro-9-methyl- 6 nitro-pyrido[3,4-f]-quinoxaline-2,3-dione
PPAR	Peroxisome proliferators activated receptor
PS	Phosphatidylserine

S.aureus	Staphylococcus aureus
SAG	Saline, adenine, glucose
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SM	Sphingomyelin
SOD	Super oxide dismutase
SSC	Side scatter
t-BHP	Tert-butylhydroperoxide
TLR	Toll like receptor

2. SUMMARY

Eryptosis is characterized by cell shrinkage, cell membrane scrambling with subsequent exposure of phosphatidylserine at the erythrocyte surface. Activation of the erythrocytes Ca^{2+} permeable cation channel triggers suicidal erythrocyte death. Therefore eryptosis may contribute to physiological limitation of erythrocyte survival and there is strong evidence between excessive stimuli of eryptosis and the presence of clinical signs of anemia.

Sepsis is paralleled by anemia, an effect resulting from eryptosis, the suicidal death of erythrocytes. Pathogen-induced eryptosis may partially result from interaction of bacterial cell wall components such as lipoproteins with the erythrocyte cell membrane. The first study explored, whether the synthetic lipopeptide Pam3CSK4 mimicking the acylated amino terminus of bacterial lipoproteins triggers eryptosis. According to annexin-V-binding in FACS analysis, Pam3CSK4 (1 μg/ml) stimulated phosphatidylserine exposure, an effect significantly blunted in the nominal absence of Ca^{2+}. According to Fluo3 fluorescence, Pam3CSK4 increased cytosolic Ca^{2+} activity and moderately stimulated erythrocytic ceramide formation, both are considered to be major triggers of eryptosis. In conclusion, bacterial lipoproteins participate in the stimulation of erythrocyte cell membrane scrambling by bacterial cell wall components. Thus, lipoprotein-dependent suicidal erythrocyte death may contribute to the pleiotropic effects of sepsis.

The second study explored, whether GluA1 is expressed in human erythrocytes and whether the pharmacological inhibition of the AMPA receptor modifies Ca^{2+} entry and suicidal death of human erythrocytes. GluA1 protein abundance was determined by confocal microscopy; PS exposure was estimated from annexin V-binding, cell volume from forward scatter, cytosolic Ca^{2+} concentration from Fluo3 fluorescence by FACS analysis, and channel activity by whole cell patch clamp recordings. GluA1 was indeed found to be expressed in the erythrocyte cell membrane. The AMPA receptor antagonist NBQX (1, 2, 3, 4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide) inhibited the suicidal cation channels activated by iso-osmotic cell shrinkage following Cl^- removal and the eryptosis following Cl^- removal or energy depletion. The present study reveals a novel action of AMPA receptor antagonists and raises the possibility that GluA1 or a pharmacologically related protein participates in the regulation of Ca^{2+} entry into and suicidal death of human erythrocytes.

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The third study explored the involvement of leukotrienes in the regulation of eryptosis. Western blotting was employed to detect the cysteinyl-leukotriene receptor cysLT1 and a competitive immune assay to determine leukotriene release from erythrocytes, Fluo3 fluorescence to estimate cytosolic Ca^{2+} concentration, forward scatter to analyse cell volume and annexin V-binding to disclose phosphatidylserine exposure by Facs analysis. As a result, erythrocytes expressed the leukotriene receptor CysLT1. Glucose depletion (24 hours) significantly increased the formation of the cysteinyl-leukotrienes $C_4/D_4/E_4$. Leukotriene C_4 (10 nM) increased Ca^{2+} entry, decreased forward scatter, activated caspases 3 and 8, and stimulated annexin V-binding. Glucose depletion similarly increased annexin V-binding, an effect significantly blunted in the presence of the leukotriene receptor antagonist cinalukast (1 μM) or the 5-lipoxygenase inhibitor BW B70C (1 μM). In conclusion, upon energy depletion erythrocytes form leukotrienes, which in turn activate cation channels, leading to Ca^{2+} entry, cell shrinkage and cell membrane scrambling. Cysteinyl-leukotrienes thus participate in the signaling of eryptosis during energy depletion.

Taken together, the study displays the functional significance of some receptors like GluA1 and CysLT1 which directly or indirectly regulate the suicidal erythrocyte death and survival.

3. ZUSAMMENFASSUNG

Eryptose, der suizidale Zelltod von Erythrozyten, ist gekennzeichnet durch Zellschrumpfung und Externalisierung von Phosphatidylserin auf der Erythrozytenoberfläche. Die Aktivierung eines erythrozytären, Ca^{2+} durchlässigen Kationenkanals löst den suizidalen Erythrozytentod aus. Daher könnte die Eryptose einen Beitrag zu einer physiologischen Befristung des Erythrozytenüberlebens leisten. Darüber hinaus gibt es starke Anhaltspunkte, dass exzessive Auslösung der Eryptose mit der Entwicklung einer Anämie einhergehen.

Sepsis geht mit Mikrozirkulationsstörungen einher, was zumindest teilweise auf die Stimulation von Eryptose zurückzuführen sein dürfte. Durch Erreger hervorgerufene Eryptose dürfte sich teilweise aus der Wechselwirkung zwischen bakteriellen Zellwandbestandteilen, wie Lipoproteinen, mit der Zellmembran der Erythrozyten ergeben. Die erste Studie untersuchte, ob das künstliche Lipoprotein Pam3CSK4, das den azylierten N-Terminus von bakteriellen Lipoproteinen nachahmt, Eryptose auslöst. Die durchflusszytometrische Untersuchung der Annexin-V-Bindung, ergab, dass Pam3CSK4 (1 µg/ml) die Phosphatidylserinexposition humaner Erythrozyten auslöste. Dieser Effekt wurde signifikant durch die Abwesenheit von Ca^{2+} abgeschwächt. Messungen der Ca^{2+} abhängigen Fluo3-Fluoreszenz ergaben, dass Pam3CSK4 die zytosolische Ca^{2+}-Aktivität erhöht und die Zeramidbildung in Erythrozyten mäßig stimuliert. Beide Veränderungen sind typische Auslöser der Eryptose. Zusammenfassend kann gesagt werden, dass bakterielle Lipoproteine an der Stimulation der Skramblase mit nachfolgender Phosphatidylserinexposition in Erythrozyten durch bakterielle Zellwandkomponenten beteiligt sind. Demzufolge könnte der lipoproteinabhängige suizidale Erythrozytentod zu den pleiotropen Effekten der Sepsis beisteuern.

Die zweite Studie erforschte, ob GluA1 in menschlichen Erythrozyten exprimiert wird und ob die pharmakologische Hemmung von AMPA-Rezeptoren die Ca^{2+}-Aufnahme und den suizidalen Erythrozytentod verändert. Die Expression des GluA1ä-Proteins wurde durch konfokale Mikroskopie, Phosphatidylserinexposition, durch Annexin-V-Bindung, das Zellvolumen durch das Signal des Vorwärtsstreulichts, die zytosolische Ca^{2+}-Konzentration durch Fluo3 Fluoreszenz jeweils duchflusszytometrisch und die Kanalaktivität durch elektrophysiologische Messungen mit der Membranfleckklemme bestimmt. Demzufolge wird GluA1 tatsächlich in der Zellmembran von Erythrozyten exprimiert. Der AMPA-Rezeptoranatagonist NBQX (1, 2, 3, 4-Tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxalin-7-

sulfonamid) hemmte die suizidalen Kationenkanäle, die durch isoosmotische Zellschrumpfung in Folge von Cl^--Wegnahme aktiviert wurden, und die Eryptose als Folge von Cl^--Wegnahme oder Energiedepletion. Die vorliegende Studie deckt einen neuen Effekt der AMPA-Rezeptoranatgonisten auf und lässt es wahrscheinlich erscheinen, dass GluA1 oder ein pharmakologisch verwandtes Protein an der Regulation des Ca^{2+}-Eintritts in und am suizidalen Tod von menschlichen Erythrozyten beteiligt ist.

Die dritte Studie erforschte die Beteiligung von Leukotrienen an der Regulation der Eryptose. Mittels Western Blots konnte der Cysteinyl-Leukotrien-Rezeptor cysLT1 nachgewiesen werden. Durch kompetitiven Immunoassay wurde die Leukotrienfreisetzung aus Erythrozyten bestimmt. Duchflusszytometrisch wurde durch Fluo3-Fluoreszenz die zytosolische Ca^{2+}-Konzentration abgeschätzt, mit dem Signal des Vorwärtsstreulichtes das Zellvolumen analysiert und mit der Annexin-V-Bindung die Phosphatidylserinexposition ermittelt. Glukosedepletion (24 Stunden) erhöhte signifikant die Bildung von Cysteinyl-Leukotrienen C4/D4/E4. Leukotrien C4 (10 nM) erhöhte den Ca^{2+}-Einstrom, erniedrigte das Signal des Vorwärtsstreulichtes, aktivierte die Caspasen 3 und 8 und stimulierte die Annexin-V-Bindung. Die Glukosedepletion erhöhte gleichermaßen die Annexin-V-Bindung, ein Effekt, der signifikant durch die Anwesenheit des Leukotrienrezeptorantagonisten Cinalukast (1 µM) oder den 5-Lipoxygenase-Inhibitor BW B70C (1 µM) abgeschwächt wurde. Zusammenfassend ergibt sich, dass Erythrozyten bei Energiedepletion Leukotriene bilden, welche dann sinngemäß Kationenkanäle aktivieren, was dann zu Ca^{2+}-Einstrom, Zellschrumpfung und Externalisierung von Phosphatidylserin führen. Cysteinyl-Leukotrien ist daher am Signalweg der Eryptose während der Energiedepletion beteiligt.

Zusammengefaßt zeigen die Studien die Bedeutung membranöser Rezeptoren für die Regulation des suizidalen Erythrozytentodes.

4. INTRODUCTION

Premise

Erythrocytes are the most numerous types of blood cell in the human body. The Dutch Scientist Lee Van Hock, in 1674, first described these cells in human blood samples. Erythrocytes are biconcave in shape with an average diameter of 6-8µm, thickness of 1.5-1.8 µm and humans have roughly about 4-6 million erythrocytes per cubic millimeter of blood (1987). Their function is to carry O_2 from the lungs to the tissues and carry CO_2 from the tissues to the lungs. Erythrocytes are a highly specialized O_2 carrier system in the body. Erythrocytes are devoid of nucleus and mitochondria. Because of the absence of nucleus and mitochondria, all the intracellular space is available for O_2 transport and these cells do not consume any of the oxygen which they carry (Tavassoli 1978). The process of erythrocyte formation within the body is known as erythropoiesis. Erythrocyte production is stimulated by the hormone-erythropoietin (EPO), synthesized by the kidney. The life span of a mature erythrocyte is approximately 120 days (Lew and Bookchin 2005). At the end of their life, they are retained, e.g- by the spleen where they undergo physiological phagocytosis.

Composition of the erythrocyte membrane

The membrane of erythrocytes consists of a lipid bilayer, integral membrane proteins and a membrane skeleton. The lipid bilayer composed by phospholipids and cholesterol. The phospholipids are asymmetrically dispersed in the bilayer (Steck 1974). The outer half of the bilayer contains sphingomyelin, glycolipids, and phosphatidylcholine and the inner half (facing the cytoplasm) is composed of phosphatidylinositol, phosphatidylserine and phosphatidyl-ethanolamine (Palek and Lux 1983; Smith 1987; Palek 1993). The red cell membrane skeleton is a group of protein complexes formed by structural proteins including α and β spectrin, ankyrin, protein 4.1 and actin. The main function of the membrane skeleton proteins is to interact with the lipid bilayer and with transmembrane proteins and to give strength and integrity to the cell membrane (Tse and Lux 1999).

Erythrocyte Ion transport Pathway

There are several ion transport pathways that exist in erythrocytes such as (1) Ca^{2+}-ATPase (2) Na^{+}-K^{+}-ATPase (3) the Gardos channel (4) K^{+}/Cl^{-} co-transport (5) Band 3 anion exchanger (6) $Na^{+}/K^{+}/2Cl^{-}$ co-transporter (7) glucose transporter (Maher and Kuchel 2003). The ion transport pathways in the erythrocyte membrane are summarized in Fig.1.

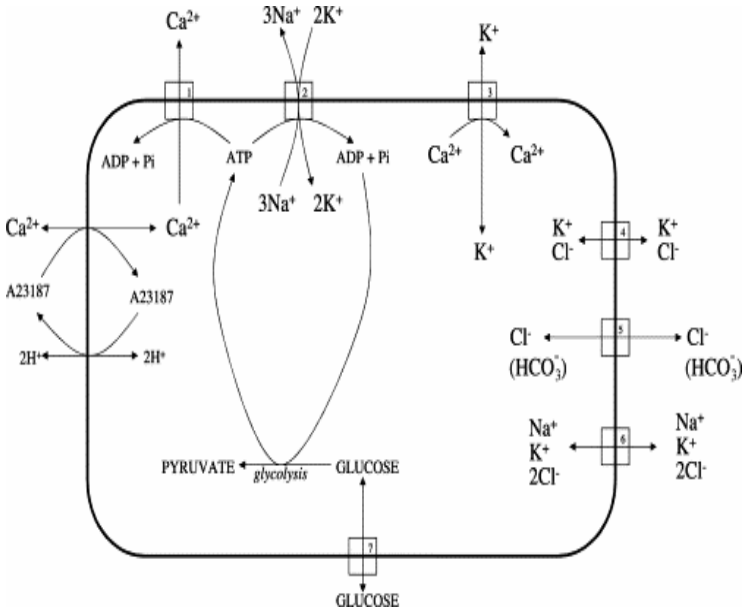

Figure 1 The ion transport pathways in the erythrocyte membrane (Maher and Kuchel 2003)

The plasma membrane Ca^{2+}-ATP-ase of erythrocytes is a transmembrane proteins which serves the removeal of cytosolic Ca^{2+}-. Since its function is to transport Ca^{2+} from the intracellular to the extracellular space, it thus regulates cellular signalling . The function of the Ca^{2+}- pump is powered by the hydrolysis of ATP. With the hydrolysis of an ATP molecule, a Ca^{2+} ion is expelled. At least in theory, impairment of the Ca^{2+}-ATPase and increase in the cytosolic Ca^{2+} activity could trigger suicidal death of erythrocytes or eryptosis (Lang, Lang et al. 2005). Vanadate (VO_4^{3-}) is a naturally occurring inhibitor of several ATPases including the Ca^{2+}-ATPase (Garrahan and Rega 1988).

The energy-dependent (ATP-dependent) pump system Na^{+}/K^{+}-ATP-ase of erythrocytes is essential for the maintenance of the Na^{+} and K^{+} concentrations across the membrane. To maintain the concentration gradients for Na^{+} and K^{+}, it is necessary to transport Na^{+} out of the cell and K^{+} back into the cell. The Na^{+}, K^{+} flux ratio of erythrocytes through this pump is 3:2 (Lew and Bookchin 2005).

Erythrocytes express Ca^{2+}-sensitive K^+ channels which are known as Gardos channels. Approximately 100-200 Gardos channels are found in the erythrocyte membrane (Lew, Muallem et al. 1982; Alvarez and Garcia-Sancho 1987; Brugnara, De Franceschi et al. 1993). Gardos channels are closed at normal erythrocyte cytosolic Ca^{2+} concentration, 20-50nM (Lew and Bookchin 2005), and they are activated due to increase in the intracellular Ca^{2+} concentration- 150 nM-2 µM (Simons 1976; Lew and Bookchin 2005). Activation of Gardos channel hyperpolarizes the erythrocytes-membrane, which leads to efflux of potassium ions resulting in a net loss of KCl and $KHCO_3$, resulting in an obliged cellular water loss and consequently shrinkage (Lew and Bookchin 2005). Charybdotoxin, clotrimazole, Tram34(1-((2-chlorophenyl) diphenylmethyl)-1H-pyrazole) are common pharmacological inhibitors of the Gardos channel (Brugnara, De Franceschi et al. 1993; Maher and Kuchel 2003).

KCl cotranspoters are abundant in erythroid precursors (Lauf, Bauer et al. 1992; Gillen, Brill et al. 1996) and the activity of the transpoters is reduced during maturation of erythrocytes (Lew and Bookchin 2005). The passive K^+ fluxes of reticulocytes are mediated by the KCl cotranspoter (Hall and Ellory 1986). The KCl cotransporter mainly mediates a strictly coupled electroneutral transport of K^+ and Cl^- independent of the membrane potential (Jennings and Adame 2001). Erythrocyte membranes also process an integral membrane protein called Band3 (AE1) that plays a pivotal role in the assembly of the membrane skeleton in developing erythroblasts (Peters, Shivdasani et al. 1996).

Apoptosis of nucleated cells

A balance in cellular homeostasis is important for the survival of living organisms and maintains a steady structural and functional integrity of the organism. One of the ways by which this is achieved is through apoptosis that counteracts the excesses of cell proliferation. Apoptosis, until recently, has been considered a hall mark of nucleated cells (Rozengurt 1992; Vaux, Haecker et al. 1994). It is a unique process distinguished from the more compromising forces of necrosis. At cellular level a wide variety of external and internal forces may lead to a triggering of the suicidal machinery. Apoptosis can be triggered by forces outside the cellular environment or may be a part of the cellular constitution (Elmore 2007). Diverse signals can induce apoptosis in a wide variety of cell types. Activation of endogenous proteases would result in cytoskeletal damage with membrane blebbing, cell shrinkage, nuclear condensation and DNA fragmentation (Reipert, Reipert et al. 1996). The intrinsic pathway and the extrinsic pathway of apoptosis are two major chains of events which for a long time have thought to be

the major players in cell death and survival. The intrinsic pathway begins within the cell and can be induced by a variety of stimuli such as stress, DNA damage or the deprivation of a growth factor. This may lead to the permeabilization of the outher membrane of the mitochondria, cytochrome C realease and the activation of caspases 9 and 3. The extrinsic pathway is more dynamic and shows the constant interaction of the cell with stimuli perceived from the external environment. Viruses, radiation, drugs may all act as stimuli. Cells are known to posses death receptors on the surface. The binding of certain ligands to these death recptors may exacerbate a chain of death inducing events. TNF receptors and TNF lingands, TRAIL and FAS-ligand are some important players. The Fas ligand has been shown to upregulate the Death-Induced signaling Pathway DISC (Lowe, Bodis et al. 1994; Riedl and Salvesen 2007). Apart from the extrinsic and intrinsic pathway, dependence receptors may trigger apoptosis in the absence of a ligand contradictory to the extrinsic pathways.

Erythrocyte suicidal death

The term "eryptosis" was coined to denote the structural and functional state of apoptosis or suicidal death in erythrocytes. Eryptosis is characterized by cell shrinkage, cell membrane blebbing and membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface. Macrophages are equipped with receptors specific for phosphatidylserine. Phosphatidylserine-exposing erythrocytes are recognized by macrophages, engulfed, degraded and thus cleared from circulating blood (Lang, Lang et al. 2006).

Triggers of eryptosis

Several conditions are now known to trigger eryptosis and include osmotic shock (Lang, Duranton et al. 2003), oxidative stress (Bracci, Perrone et al. 2002; Lang, Roll et al. 2002; Barvitenko, Adragna et al. 2005), energy depletion (Lang, Roll et al. 2002), activation of the death receptor CD95/Fas (Mandal, Mazumder et al. 2005), ligation of specific surface antigens, such as glycophorin-C (Head, Lee et al. 2005) or the thrombospondin-1 receptor CD47 (Head, Lee et al. 2005). Further stimulators of erythrocyte suicidal death include ceramide (Lang, Myssina et al. 2004), prostaglandin E_2 (Lang, Kempe et al. 2005), platelet activating factor (Lang, Kempe et al. 2005), methyldopa (Mahmud, Foller et al. 2008), cisplatin (Mahmud, Foller et al. 2008), hemolysin from Vibrio parahaemolyticus (Lang, Kaiser et al. 2004), listeriolysin (Foller, Shumilina et al. 2007), amantadine (Foller, Geiger et

al. 2008), retinoic acid (Niemoeller, Foller et al. 2008), valinomycin (Schneider, Nicolay et al. 2007), vanadium (Foller, Sopjani et al. 2008).

Signaling involved in eryptosis

Eryptosis parallels the apoptotic death machinery and is regulated by different channels like Cl^- channels, Ca^{2+}-permeable cation channels and Ca^{2+}-sensitive K^+ channels etc. Signaling pathways involved during eryptosis increase in the cytosolic Ca^{2+} concentration by activation of a Ca^{2+}-permeable cation channel and the activation of a phospholipase A_2 leading to the release of platelet activating factor (PAF), which in turn activates a sphingomyelinase resulting in the formation of the proapoptotic sphingolipid ceramide (Foller, Huber et al. 2008).

Cl^- channels:

Cl^- channel activation plays a decisive role in the eryptosis machinery. Activation of such channels depolarizes the cell membrane due to exit of Cl^- ion. This leads to release of KCl from the cells due to enhancement of the the driving force for K^+ exit. The loss of osmotically active KCl is paralleled by loss of osmotically obliged water and thus by cell shrinkage, one of the hallmarks of eryptosis (Lang, Lang et al. 2006). A typical feature of apoptotic cells is the activation of Cl^- channels that lead to HCO_3^- exit and thus facilitate cytosolic acidification. The acidification during apoptosis may also be due to the inhibition of the Na^+/ H^+-exchanger which is at least partially due to caspase-dependent degradation of the carrier protein NHE1 (Gottlieb, Gruol et al. 1996; Thangaraju, Sharma et al. 1999; Lang, Madlung et al. 2000; Waibel, Kramer et al. 2007)

Ca^{2+} permeable cation channels

Activation of monovalent and divalent cation-permeable channels are called nonselective cation channels results in elevation of cytosolic Ca^{2+}. This, in turn, promotes activation of Gardos channels which mediate K^+ loss together with Cl^- via parallel anion pathways. Together, these events result in osmotic water loss and account for cell shrinkage (Lang, Huber et al. 2007). In parallel, raised intracellular Ca^{2+} activates a membrane scramblase which increases the abundence of phosphatidylserine in the outer leaflet of the erythrocyte membrane (Lang, Huber et al. 2007).

Oxidative stress and caspase activation triggers eryptosis

Energy depletion or oxidative stress are among the major triggers of eryptosis (Barvitenko, Adragna et al. 2005). Energy depletion activates the non-selective cation channels (Duranton, Huber et al. 2002; Lang, Roll et al. 2002) and impairs the replenishment of glutathione and thus interferes with the antioxidative defense of the erythrocytes (Mavelli, Ciriolo et al. 1984; Damonte, Guida et al. 1992; Bilmen, Aksu et al. 2001). Caspases have been shown to be expressed in erythrocytes (Bratosin, Estaquier et al. 2001; Mandal, Moitra et al. 2002). Oxidative stress further activates caspases (Bracci, Perrone et al. 2002; Matarrese, Straface et al. 2005) and has been shown to cleave the anion exchanger band 3 (Mandal, Baudin-Creuza et al. 2003) and to stimulate phosphatidylserine exposure (Mandal, Moitra et al. 2002). Cl^- channels are further activated by oxidative stress which are required for erythrocyte shrinkage and thus participate in the triggering of eryptosis (Huber, Uhlemann et al. 2002; Myssina, Lang et al. 2004; Tanneur, Duranton et al. 2006). Caspases activation is not required for ionomycin or hyperosmoticshock induced eryptosis (Weil, Jacobson et al. 1998; Berg, Engels et al. 2001; Lang, Myssina et al. 2004).

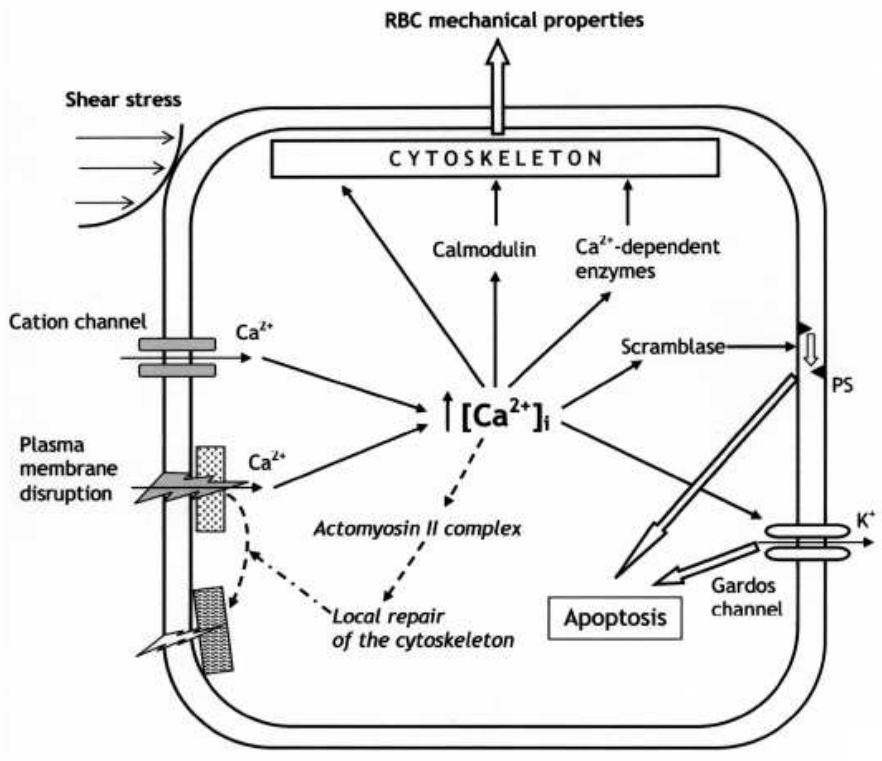

Figure 2 Scheme illustrating shear stress-induced Ca^{2+}-dependent mechanisms in RBCs (Barvitenko, Adragna et al. 2005)

Cycloxygenase activation, PGE_2 formation, and activation of Cation channels

Prostaglandin E_2 (PGE_2) is released upon hyperosmotic shock and Cl^- removal which activates nonselective cation channels (Kaestner and Bernhardt 2002; Lang, Kempe et al. 2005) and increases the cytosolic Ca^{2+} concentration (Kaestner, Tabellion et al. 2004; Lang, Kempe et al. 2005), stimulates phosphatidylserine exposure at the erythrocyte surface (Lang, Kempe et al. 2005), and triggers cell membrane vesiculation. Oxidative stress (Duranton, Huber et al. 2002) can also activate similar channels which similarly stimulates Ca^{2+} entry (Lang, Duranton et al. 2003) and triggers phosphatidylserine exposure at the cell surface. An increase in the cytosolic Ca^{2+} concentration stimulates the Ca^{2+}-sensitive "Gardos" K^+ channels (Bookchin, Ortiz et al. 1987; Brugnara, de Franceschi et al. 1993; Franco, Palascak et al. 1996) in erythrocytes. The subsequent hyperpolarization of the cell membrane drives Cl^- in parallel to K^+ out of the cell. The cellular loss of KCl favors cell shrinkage. Moreover, the cellular loss of K^+ (Lang, Warskulat et al. 2003) contributes to the triggering of eryptosis.

PGE_2 further activates the Ca^{2+}-dependent cysteine endopeptidase calpain. Amiloride (Lang, Duranton et al. 2003) and ethylisopropylamiloride (EIPA) (Lang, Myssina et al. 2003) are inhibitors of non-selective cation channel. The activation of the cation channels by Cl^- removal is pharmacologically abolished by the cyclooxygenase inhibitors, diclophenac and acetylsalicylic acid and by the phospholipase-A_2 inhibitors, quinacrine and palmitoyl-trifluoromethyl-ketone (Lang, Kempe et al. 2005).

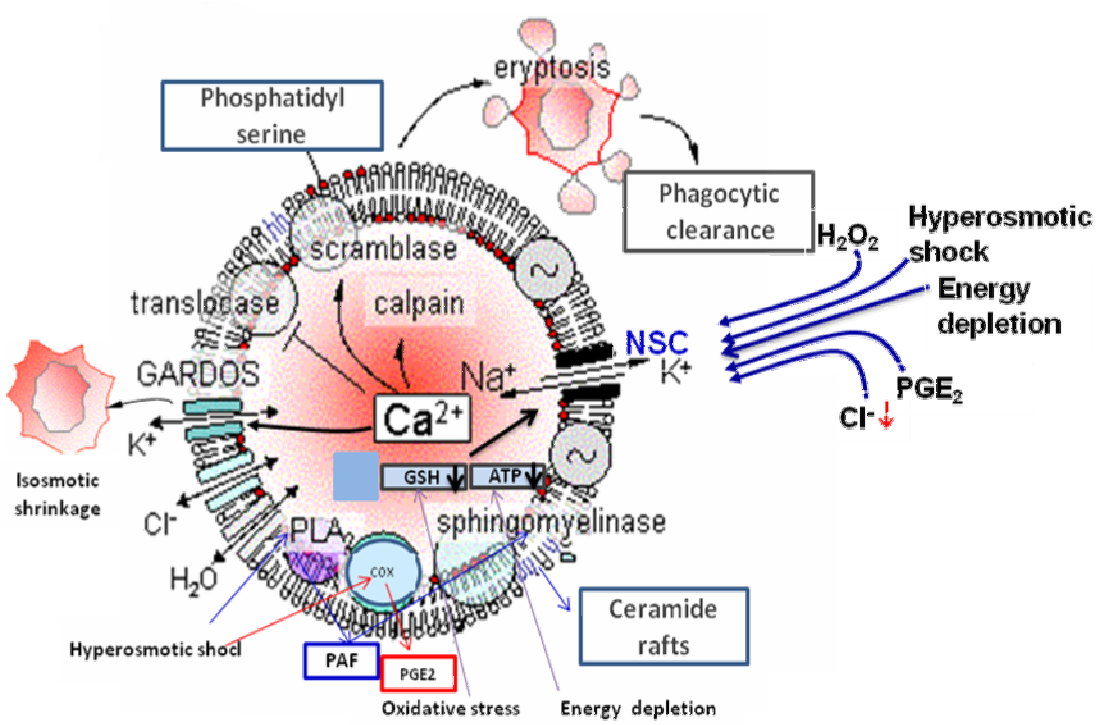

Figure 3 Synopsis of the mechanisms involved in eryptosis (Lang, Lang et al. 2006).

COX, cyclooxygenase; PAF, platelet activating factor; PGE_2, prostaglandin E2; PLA_2, phospholipase A_2; GSH, glutathione and NSC, nonselective cation channel.

Platelet activating factor stimulates sphingomyelinase

The phospholipid mediator platelet activating factor (PAF) is released due to cell shrinkage. PAF in turn stimulates the breakdown of sphingomyelin and formation of ceramide in erythrocytes (Lang, Kempe et al. 2005). PAF further triggers cell shrinkage and phosphatidylserine exposure of erythrocytes. Thus, PAF participates in the stimulation of sphingomyelinase activation and -"eryptosis"-. PAF further activates Ca^{2+}-sensitive K^+ channels (Gardos channels) in the erythrocyte cell membrane (Garay and Braquet 1986) by sensitizing them for the stimulating effects of cytosolic Ca^{2+} (Lang, Lang et al. 2006).

Physiological function of eryptosis

Eryptosis is the key process by which injured erythrocytes are removed from the circulation prior to hemolysis. Phosphatidylserine-exposing defective erythrocytes are equipped with phosphatidylserine receptors which are recognized, engulfed and degraded by macrophages (Boas, Forman et al. 1998) and cleared from the circulating blood prior to hemolysis. The pathways described above i.e. increase in the intracellular calcium concentration or the formation of ceramide leads to eryptosis. Excessive and unbalenced eryptosis may lead to anemia (Lang, Lang et al. 2005). All the stimuli of eryptosis described earlier are associated with the development of anemia. Therefore eryptosis may contribute to the physiological limitation of erythrocyte survival (Lang, Lang et al. 2005). The function of the Na^+/K^+-ATP-ase is impaired by energy depletion, leakiness of the cell membrane enhances the accumulation of Na^+, Cl^- and osmotically obliged water in the cell and leads to subsequent cell swelling (Lang *et al.*, 1998a). The Na^+ entry is initially followed by cellular loss of K^+ thus decreasing the equilibrium potential of K^+ leading to gradual depolarization and favouring the entry of Cl^-. Swelling of erythocytes may cause rupture and the release of intacellular hemoglobin into the circulation, which may be filtered in the glomeruli. When eryptosis precedes this, hemolysis is prevented (Lang, Lang et al. 2005). Circulating free hemoglobin may precipitate within the renal tubles and lead to acute renal failure due to excessive hemolysis (Sillix and McDonald 1987; Lang, Lang et al. 2005).

Eryptosis may further limit the intracellular growth of *Plasmodium falciparum* and remains an important host response benificially influencing the course of malaria (Foller, Huber et al. 2008). Malaria-induced eryptosis may be caused by oxidative stress which the pathogen

imposes onto the erythrocyte. Macrophages clear the eryptotic erythrocytes and limit the life span of infected cells (Lang, Lang et al. 2004). The activation of the cation channel by *Plasmodium falciparum* allows the uptake of nutrients, Na^+ and Ca^{2+} which may also be beneficial for the intra erythrocytic survival of malaria parasites (Kirk 2001).

Lipopeptides

Characteristics

Lipopeptides are a family of proinflamatory cell wall components which are produced by a large variety of bacteria from different genera such as *Bacillus*, *Lactobacillus*, *Streptococcus* and *Pseudomonas* (Busscher, van Hoogmoed et al. 1997; Lindum, Anthoni et al. 1998; Bender, Alarcon-Chaidez et al. 1999; Velraeds, van de Belt-Gritter et al. 2000; Mireles, Toguchi et al. 2001; Huber, Riedel et al. 2002). They are composed of a peptide moiety that can be cyclized to form a lactone ring between two amino acids in the pepetide chain and a fatty acid chain at the N-terminal amino acid. Different lipopeptides may show different properties including antifungal activity, phyto-toxicity and regulation of biofilm formation (Nielsen, Christophersen et al. 1999; Huber, Riedel et al. 2002). The biosynthesis of lipopeptides occurs non-ribosomally via multifunctional proteins (Kleinkauf and von Dohren 1995). Bacterial lipoproteins from eubacteria use a signal peptidase II cleavage system that adds a diacylglycerol moiety to the free thiol group of an amino terminal cysteine and cleaves the signal peptide to generate the mature protein (von Heijne 1989). Many gram-negative bacteria and mycobacteria, add another acyl chain to the amino terminal cysteine's primary amine, resulting in triacylated lipoproteins whereas for some eubacteria, such as gram-positive cocci and mycoplasma, the cysteine undergoes no further modification, resulting in lipoproteins with amino terminal diacylcysteines. These lipid-modified proteins are present in the cell membranes of bacterial cell walls and can be processed into lipopeptides (Le Henaff, Chollet et al. 2001). Bacterial cell wall containing lipoprotein/peptides are classified into two groups, diacylated lipopeptides and triacylated lipopeptides. Lipopeptides are known to be strong modulators of the immune system. Diacylated lipopeptides are recognized by TLR2/TLR6-heteromers whereas triacylated lipopeptides are assumed to be recognized by TLR2/TLR1 for signaling (Buwitt-Beckmann, Heine et al. 2006).

Pam3CSK4

Pam3CSK4 (Pam3CysSerLys4) is a synthetic triacylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins (Aliprantis, Yang et al. 1999; Ozinsky, Underhill et al. 2000). The chemical name of Pam3CSK4 is N-Palmitoyl-S-[2,3 bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S] lysine (Fig.4)

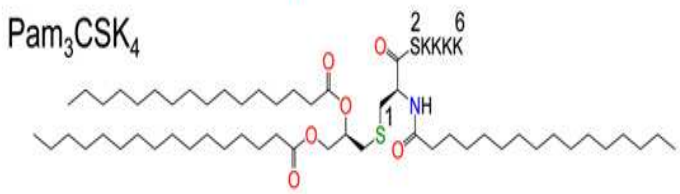

Figure 4 Chemical structures of triacylated lipoproteins-synthetic Pam3CSK4 (Jin and Lee 2008)

Lipoproteins and lipopeptides are amphiphilic components and found in the outer membrane of microorganisms (Yang, Alani et al. 2003; Bubeck Wardenburg, Williams et al. 2006; Gioffre, Caimi et al. 2006; Hashimoto, Tawaratsumida et al. 2006; Kataoka, Yasuda et al. 2006). It was shown that synthetic lipopeptides are corresponding to N-terminal partial structures of bacterial lipoproteins that defined the chemical prerequisites for their biological activity. Like native lipopeptides, synthetic lipopeptides also show a variety of biological activities based on the highly conserved lipopeptide moiety of Braun's lipoprotein derived from *Escherichia coli* (Braun and Wolff 1970). Lipopeptides are recognized by type I transmembrane proteins of the Toll-like receptor family. The triacylated lipopeptide (Pam3CSK4) shows higher activity, whereas diacylated lipopetide (Pam3CS) shows lower activity (Bessler, Cox et al. 1985; Prass, Ringsdorf et al. 1987).

TLR1-TLR2-Pam3CSK4 interaction

For TLR2, it is essential to associate with TLR1 or TLR6 for recognizing bacterial lipoproteins and lipopeptides. Lipopeptides induce strong proinflammatory responses by anchoring with the cell membrane via lipid chains attached to conserved N termini (Chambaud, Wroblewski et al. 1999). Diacylated lipopeptides are recognized by both, the TLR1-TLR2 and TLR2-TLR6 complexes (Takeuchi, Kawai et al. 2001; Buwitt-Beckmann, Heine et al. 2005). The triacylated lipopeptides such as, Pam3CSK4 can activate the TLR1-

TLR2 complex (Takeuchi, Sato et al. 2002; Shimizu, Kida et al. 2007). The dimer formation between two TLRs is made by the lipid chains of Pam3CSK4. Two of the three lipid chains of Pam3CSK4 interact with a pocket in TLR2, and the remaining amide-bound lipid chain is inserted into a hydrophobic channel in TLR1. The location of the ligand-binding pockets of TLR1 and TLR2 is at the boundary of the central and C-terminal domain in the convex region and the flexible loops at the domain boundaries are separated, forming crevices that are connected to large internal pockets. The ligand, Pam3CSK4 makes the bridge between the pockets of TLR1 and TLR2 and therefore forms a long continuous hydrophobic pocket (Jin and Lee 2008) (Fig.5).

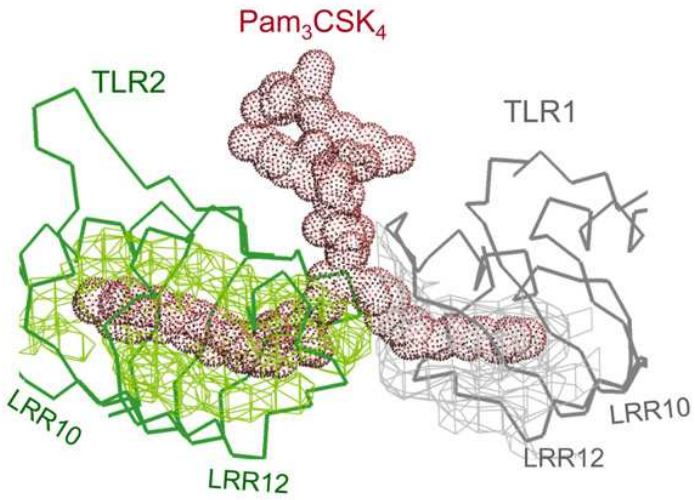

Figure 5. TLR1, TLR2, and ligand interaction (Jin and Lee 2008)

TLR1-TLR2-Pam3CSK4 signaling

The Nobel laureate Christiane Nüsslein-Volhard and her colleagues originally described Toll as one of a series of mutations in the fruit fly *Drosophila melanogaster* (van Duin and Shaw 2007). The mammalian TLRs are homologues of *Drosophila* Toll and they are the key molecules for recognizing bacterial components to evoke inflammatory response (Hemmi, Takeuchi et al. 2000). TLRs are type I transmembrane glycoproteins composed of extracellular, transmembrane and intracellular signaling domains (Gay and Gangloff 2007; Jin and Lee 2008). TLR signaling includes the activation of a cascade of intermediates, e.g. myeloid differentiation factor-88 (MyD88), Toll-interleukin (IL)-1 receptor–associated-protein (TIRAP), Toll receptor–associated activator of interferon, and Toll receptor–associated molecule (O'Neill and Bowie 2007; van Duin and Shaw 2007). Upon stimulation

of TLRs, myD88 recruits a death domain–containing serine/threonine kinase, the IL-1R-associated kinase (IRAK). IRAK is then activated by phosphorylation and associates with TRAF6 that leads to activation of two distinct signaling pathways, JNK and NF-κB (Muzio, Ni et al. 1997; Wesche, Henzel et al. 1997; Burns, Martinon et al. 1998; Medzhitov, Preston-Hurlburt et al. 1998; Muzio, Natoli et al. 1998; Takeda, Kaisho et al. 2003) (181–185). Bacterial lipopeptides are strong immune modulators that induce signaling in cells of the immune system through TLR2–TLR1 or TLR2–TLR6 heteromers (Buwitt-Beckmann, Heine et al. 2005; Lombardi, Van Overtvelt et al. 2008). An important triacylated lipopeptide, Pam3CSK4, is a potent activator of the proinflammatory signaling which is recognized by TLR2 and cooperates with TLR1 through their cytoplasmic domain whereas diacylated lipopeptides are recognized through TLR2–TLR6 heteromers and induce the signaling cascade leading to the activation of NF-κB (Takeda, Kaisho et al. 2003) (Fig.6).

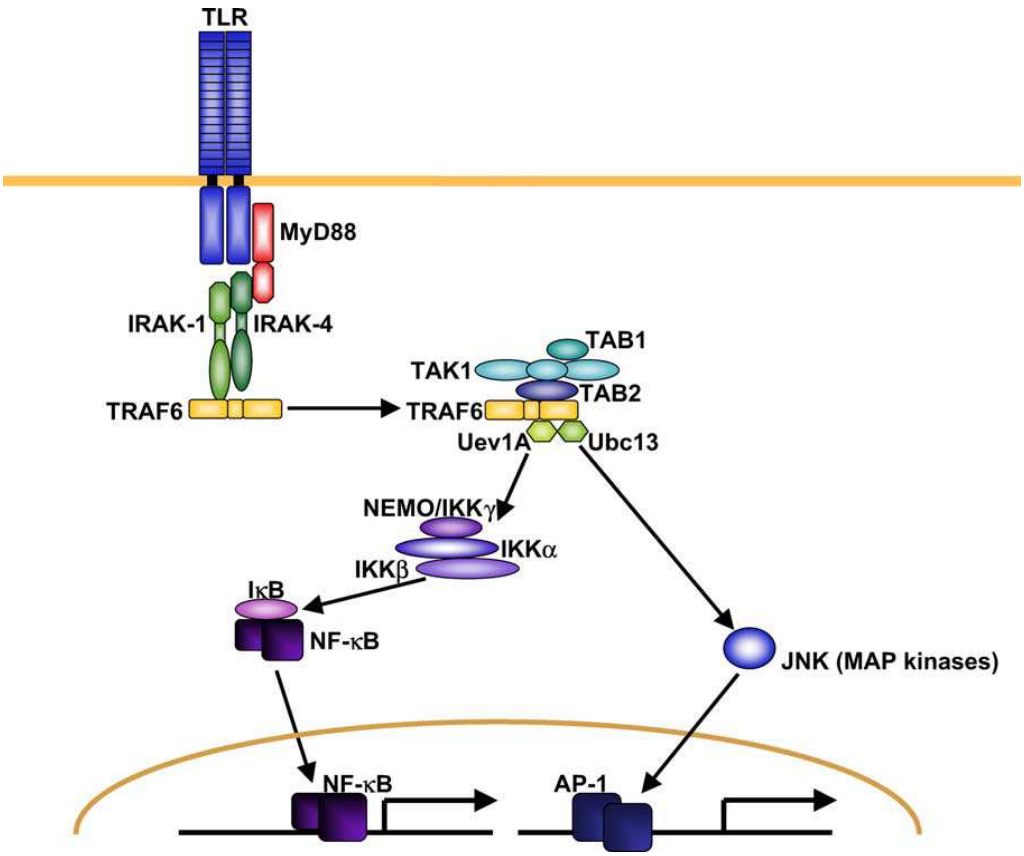

Figure 6 TLR-mediated MyD88-dependent signaling pathway (Takeda and Akira 2004)

Activation of toll-like receptors may lead to pleotropic effects participating in a variety of disorders including sepsis (Han, Kim et al. 2006), atherosclerosis (Curtiss and Tobias 2007), insulin resistance and obesity-related metabolic dysfunction (Vitseva, Tanriverdi et al. 2008;

Zappulla 2008). Sequelae of overwhelming infection or sepsis include stimulation of eryptosis. Further bacterial components described to stimulate eryptosis include hemolysin from *Vibrio parahaemolyticus* (Lang, Kaiser et al. 2004), listeriolysin (Foller, Shumilina et al. 2007) and peptidoglycans (Foller, Biswas et al. 2009). Phosphatidylserine exposure in erythrocytes could further be elicited by ligation of specific surface antigens, such as glycophorin-C (Head, Lee et al. 2005), of CD47, the critical RBC antigenic marker that exhibits an inhibitory role in macrophage activation (Head, Lee et al. 2005), and of the death receptor CD95/Fas (Mandal, Moitra et al. 2002). Mechanisms underlying eryptosis may not only affect circulating erythrocytes but may contribute to the limitation of erythrocytes during storage (Kriebardis, Antonelou et al. 2007; Kriebardis, Antonelou et al. 2008).

Glutamate receptors

Overview

Glutamate receptors belong to the family of transmembrane receptors which are divided into two groups according to their activation and functions- ionotropic and metabotropic (Madden 2002) receptors. Glutamate receptors are ionotropic receptor when glutamate binds to its receptor, an ion channel opened (Kew and Kemp 2005). In the case of meatbotropic receptors ion channels may be activated indirectly. Based on their structural similarities ionotropic glutamate receptors are subdivided into three groups AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate) and kainate (2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine) receptors. AMPA receptors are composed of a four-subunit family (GluA1–4) (Rosenmund, Stern-Bach et al. 1998) and native AMPA receptors are likely heteromeric in composition. Glutamate receptors have three transmembrane domains (M1, M3 and M4) and a cytoplasm facing re-entrant-facing loop (M2). The location of the N-terminus is extracellular whereas the C-terminus is intracellular (Dingledine, Borges et al. 1999) (Fig.7).

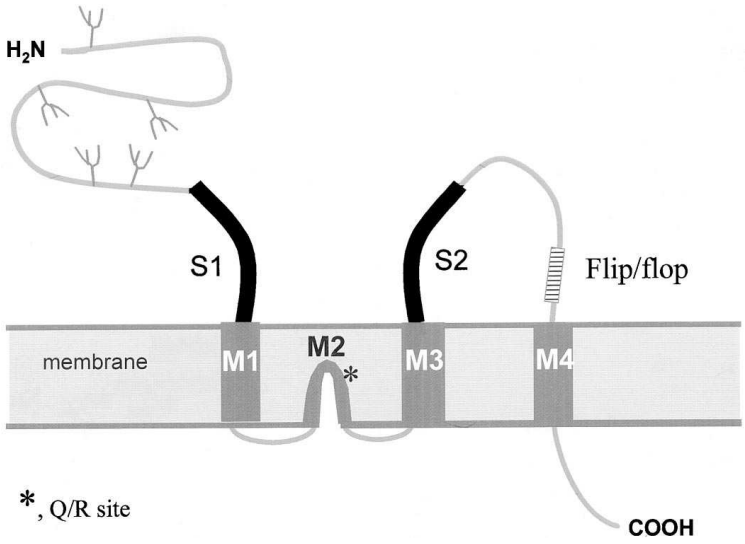

Figure 7 Structure of AMPA receptor subunits (Dingledine, Borges et al. 1999)

Ionic selectivity of glutamate receptors

With the exception of GluA2 and GluA6, glutamate receptors are permeable to cations largely excluding anions from the pore. Na^+ and K^+ are equally permeable through various glutamate receptors. Ca^{2+} is more permeable to NMDA receptors than to non NMDA receptors and Ca^{2+} permeation is more complex in the case of NMDA than of AMPA receptors (Premkumar and Auerbach 1996; Wollmuth, Kuner et al. 1998). AMPA receptors lacking the GluA2 subunit are Ca^{2+} permeable (Hollmann, Hartley et al. 1991; Hume, Dingledine et al. 1991; Burnashev, Monyer et al. 1992). The Ca^{2+} impermeability of GluA2 is conferred by an arginine (R) at a critical site in the pore loop (m2 domain) and a glutamine (Q) at the corresponding position in the other subunits (Seeburg 1996; Dingledine, Borges et al. 1999). The C-terminal splice variants of GluA2 and GluA4 encode short or long intracellular regions, which are important in intracellular protein–protein interactions and receptor clustering (Dingledine, Borges et al. 1999).

AMPA receptor agonists and antagonists

There is a large number of AMPA receptors agonists: (R,S)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl)propionic acid (ACPA), quisqualic acid and AMPA itself are AMPA analogues

(Armstrong and Gouaux 2000; Stensbol, Madsen et al. 2002; Mayer and Armstrong 2004). 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitro-quinoxaline-2,3-dione (DNQX) are competitive antagonists (Drejer and Honore 1988). 2,3-dihydroxy- 6-nitro-7-sulfamoyl-benzo(F) quinoxaline (NBQX) (Sheardown, Nielsen et al. 1990); 1,4,7,8,9,10-hexahydro-9-methyl- 6 nitropyrido[3,4-f]-quinoxaline-2,3-dione (PNQX); 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione (YM-90K) (Ohmori, Sakamoto et al. 1994); [2,3-dioxo-7-(1H-imidazol- 1-yl)-6-nitro-1,2,3,4 tetrahydro-1-quinoxalinyl]-acetic acid (YM872) (Kohara, Okada et al. 1998); and [1,2,3, 4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl) quinoxalin-1-yl]methylphosphonate (ZK200775) (Turski, Huth et al. 1998) are more selective competitive AMPA receptor antagonists. 1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466) (Solyom and Tarnawa 2002), (R)-1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-3-methylcarbomyl-2,3-benzodiazepine (GYKI 53784/ LY303070), (R)-7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo(4,5-h) (2,3) benzodiazepine (GYKI 3773/LY300164) (Hamori, Solyom et al. 2000; Kapus, Szekely et al. 2000; Ruel, Guitton et al. 2002) are non competitive AMPA receptor antagonists. There is also a number of positive allosteric modulators of AMPA receptors which do not activate the receptors when applied alone but which can modulate receptor desensitization. 1-(quinoxalin-6-ylcarbonyl)-piperidine (CX516), 1-(1,4-benzothiadiazine 1,1-dioxide (CX546) and 2H,3H,6aH-pyrrolidino [2″,1″-3′2′]1,3-oxazino[6′5′-5,4]benzo[e]1,4-dioxan-10-one (CX614), (R,S)- N-2-(4-(3-thienyl)phenyl) propyl-2-propanesulfonamide (LY 392098), N-2-[4-(4-cyanophenyl)phenyl]propyl 2-propanesulphonamide (LY404187), N-2-(4-(N-benzamido)phenyl) fluoro-1-methyl-2-(propane-2-sulphonylamino)-ethyl]-biphenyl- 4 carboxylic acid methylamide (LY503430) (Miu, Jarvie et al. 2001; Zarrinmayeh, Bleakman et al. 2001; Quirk and Nisenbaum 2002; Murray, Whalley et al. 2003), (R)-4′-[1-, Cyclothiazide, 7-chloro-3- methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide (IDRA-21), ((4-[2-(phenylsulphonylamino)ethylthio] -2,6-difluorophenoxyacetamide (PEPA) (Ito, Tanabe et al. 1990; Yamada and Tang 1993; Arai, Kessler et al. 2000; Danysz 2002; Lynch 2004) are the most common AMPA receptors positive allosteric modulators.

Significance of AMPA receptors

AMPA-selective ionotropic glutamate receptors (iGluRs) are the class of neurotransmitter receptors which are involved in a large number of CNS disorders such as Fragile-X mental retardation (O'Donnell and Warren 2002; Bear, Huber et al. 2004; Bear 2005; Dolen and Bear 2005; Marenco and Weinberger 2006; Koukoui and Chaudhuri 2007), schizophrenia (O'Neill,

Bleakman et al. 2004; Black 2005; Marenco and Weinberger 2006), neurodegenerative conditions-, such as Alzheimer's disease, motor neuron disease or Amyotrophic Lateral Sclerosis (ALS) (Shaw and Eggett 2000; Heath and Shaw 2002; Kawahara and Kwak 2005; Kwak and Kawahara 2005; Kwak and Weiss 2006; Van Den Bosch, Van Damme et al. 2006), stroke (Kullmann, Asztely et al. 2000; Ohtani, Tanaka et al. 2002; Weiser 2002; Soundarapandian, Tu et al. 2005; Weiser 2005; Peng, Zhong et al. 2006), parkinsonism (Johnston and Brotchie 2004; Brotchie 2005; O'Neill, Murray et al. 2005; Wu and Frucht 2005; O'Neill and Witkin 2007), epilepsy (Kullmann, Asztely et al. 2000; Madsen, Stensbol et al. 2001; De Sarro, Gitto et al. 2005; Rogawski 2006; Howes and Bell 2007) and Rasmussen's syndrome (Granata 2003; Lang, Dale et al. 2003). Excessive activation of Ca^{2+}- permeable AMPA-receptors (CP-AMPARs) leads to cell death (Fig. 8) (Cull-Candy, Kelly et al. 2006; Kwak and Weiss 2006) in chronic neurodegenerative conditions. In neuronal cell death, cytochrome c is released which triggers cell death via a caspase dependent-pathway (Kwak and Weiss 2006) by activation of Poly(ADP-ribose) polymerase 1 (PARP-1) (Hong, Dawson et al. 2004). As stated above, GluA2 is the only AMPAR subunit which forms Ca^{2+}-impermeable homomers (Hollmann, Hartley et al. 1991; Verdoorn, Burnashev et al. 1991). All other AMPAR subunits, GluA1, 3 and 4, lead to an appreciable divalent permeability. The activation of AMPA receptors may lead to proliferation and migration through a pathway mediated by the serinethreonine kinase Akt (Ishiuchi, Yoshida et al. 2007; Bowie 2008). Recently, it has been reported that AMPA receptors are regulated by G proteins (Wang, Small et al. 1997)-, although the interaction between AMPA receptors and G proteins is still unclear but it might be that an adaptor protein is involved (Dingledine, Borges et al. 1999). Signaling involved in apoptosis by the activation of glutamate receptors is summarized in Fig. 8.

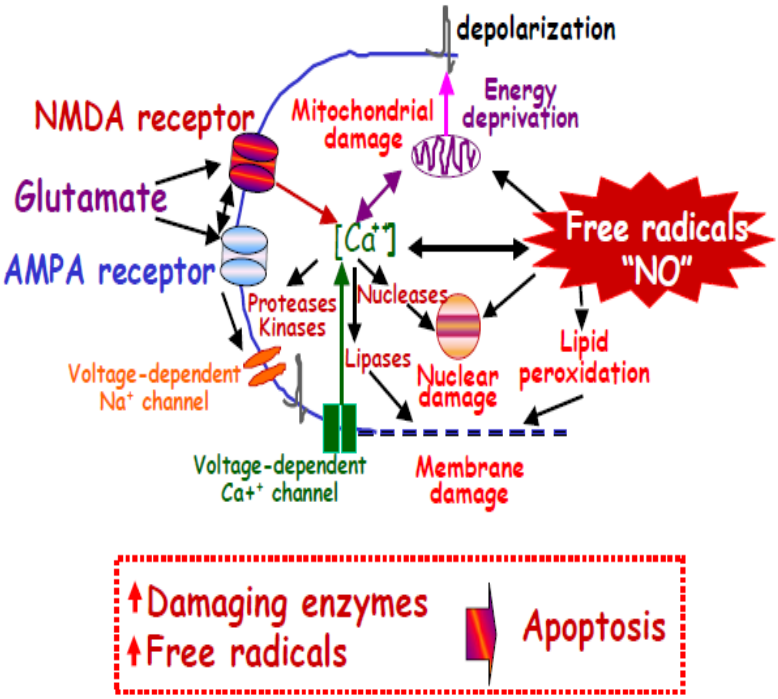

Figure 8. Mechanism of cell death by glutamate receptors, modified (Bezprozvanny and Hayden 2004; Marambaud, Dreses-Werringloer et al. 2009)

Leukotriene C4

Biosynthesis and metabolism of leukotriene

Leukotrienes were discovered in 1938 (Feldberg and Kellaway 1938) and described as slow reacting substance. They are a family of biological active molecules which are mainly formed by leukocytes, macrophages, mastcells and other tissues and cells in response to immunological and nonimmunological stimuli (Hammarstrom 1983). They are important in many diseases involving inflammatory or immediate hypersensitivity reactions (Brocklehurst 1960). Leukotrienes are generated from arachidonic acid by a specific synthesis pathway (Fig.9) whose key enzyme is 5-lipoxygenase (Hammarstrom, Samuelsson et al. 1980). Arachidonic acid is initially metabolized to hydroperoxyeicosatetraenoic acids (HPETE). The 5- lipoxygenase pathway leads to form 5-HPETE which is spontaneously hydrolysed to 5-HETE or further converted by 5-lipoxygenase into leukotriene A4 (LTA4). LTA4 can be

transformed to LTB4 or LTAC4 by a zinc metallohydrolase (LTA4 hydrolase) or by a transferase (LTC4 synthase).

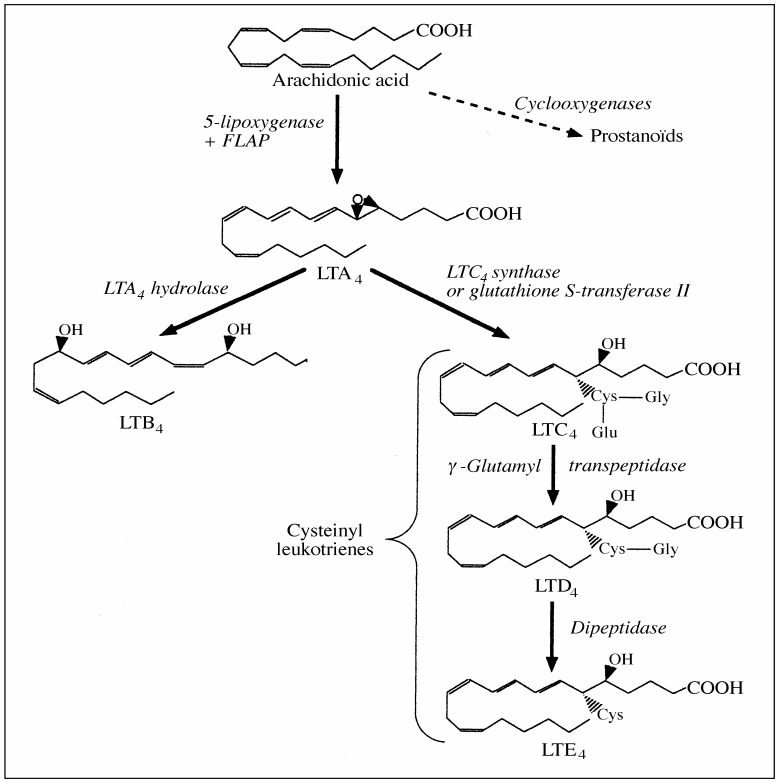

Figure 9 . The leukotrienes formation (Devillier, Baccard et al. 1999)

Furthermore, LTC4 is converted to LTD4 by a g-glutamyl-transpeptidase and then LTD4 into LTE4 by a dipeptidase (Borgeat and Samuelsson 1979; Samuelsson, Borgeat et al. 1979; Woods, Evans et al. 1993). Except for LTB4; LTC4, LTD4 and LTE4 are called cysteinyl leukotrines because they possess a common structural feature, i.e. a peptide chain with a cysteine. The key enzyme 5-lipoxygenase exists in the cytoplasm of inflammatory cells in an active form and can only act on arachidonic acid after binding to a specific protein, the 5-lipoxygenase activating protein (FLAP). FLAP expressed in the nuclear envelope (Ford-Hutchinson 1991; Woods, Evans et al. 1993) and the binding of 5-lipoxygenase to FLAP occurs during cell activation in a calcium-dependent manner (Brock, McNish et al. 1997; Peters-Golden 1998).

Leukotriene receptors

There are three types of leukotriene receptors, BLT receptors stimulated by LTB4 and *CysLT1* and *CysLT2* receptors are stimulated by cysteinyl leukotrienes LTC4, LTD4 and LTE4 (Fig.10).

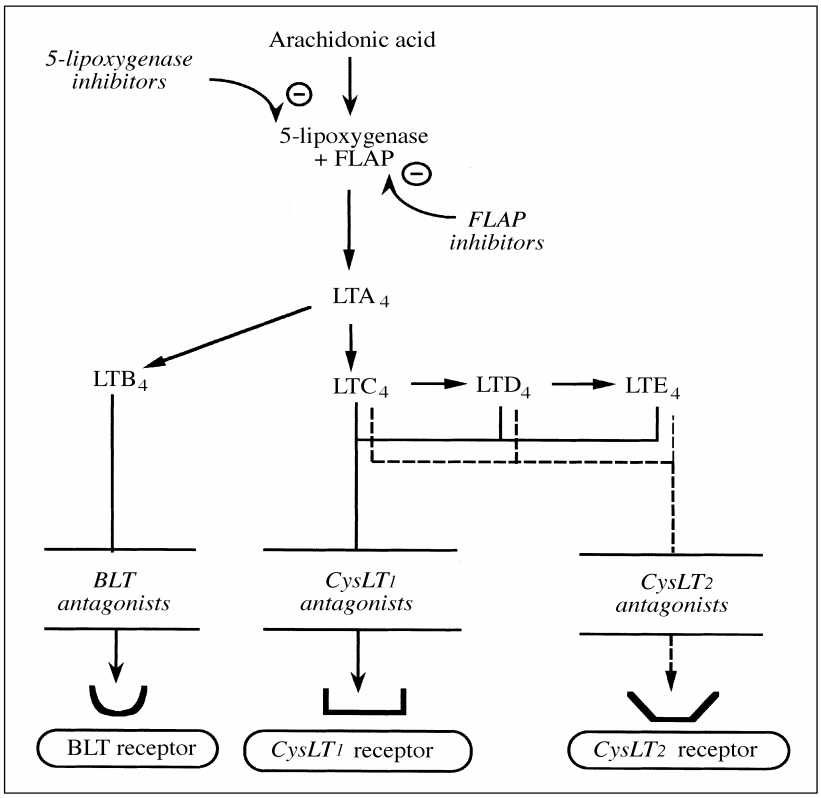

Figure 10 Leukotrienes receptors (Devillier, Baccard et al. 1999)

The BLT receptor is identical to the chemoattractant-receptor-like-1 (CMKRL1) and belongs to the seven transmembrane spanning family of G-protein coupled receptors (Owman, Blay et al. 1996). The three cysteinyl leukotrienes have high and similar affinities for the *CysLT1* receptor. However, LTC4 and LTD4 have a ten fold higher affinity to the *CysLT2* receptor than LTE4 (Labat, Ortiz et al. 1992). 3-Fluorophenylboronic acid is commonly used as potent leukotriene B4 receptor agonist (Sofia, Floreancig et al. 1993). A large number of selective antagonists for cys-LTs have been developed:- BAYu9773, LY293111, montelukast, pranlukast, pobilukast, iralukast and SB209247 are most commonly used (Fleisch, Rinkema et al. 1982; Labat, Ortiz et al. 1992). MK-886 is the potent and specific inhibitor of the 5-lipoxygenase (Dittmann, Mayer et al. 1998). LTB4 also acts as a ligand for the peroxisome proliferator-activated receptor (PPARα) leading to the expression of genes which control lipid

metabolism through the α and β-oxidation pathways (Krey, Braissant et al. 1997). In addition to LTB4, several *CysLT1* antagonists also induce peroxisome proliferation (Bendele, Hoover et al. 1990; Foxworthy, Perry et al. 1990).

Biological effects of leukotrienes

The significance of leukotrienes in biology is very important because of their pharmacological and physiological properties. They are the biological regulatory molecules that are important in many diseases. The leukotrienes are mediators which alter the respiratory function and therefore have implication in asthma. Especially LTC4, LTD4, and LTE4 are potent stimulators of airway smooth muscles (Drazen, Austen et al. 1980; Hedqvist 1980; Krell, Osborn et al. 1981). It is also known that LTC4 and D4 also impair human respiratory function in vivo (Holroyde, Altounyan et al. 1981). Several other effects of LTC, D, and E have already been reported such as effects on blood pressure (Sirois, Roy et al. 1981), stimulation of prostaglandin synthesis in macrophages (Feuerstein, Bash et al. 1981; Feuerstein, Foegh et al. 1981) and lung (Omini, Folco et al. 1981; Peck, Piper et al. 1981; Piper and Samhoun 1981), constriction of coronary arteries and a negative ionotropic effect on the heart (Burke, Levi et al. 1982; Levi, Burke et al. 1982), excitation of cerebellar Purkinje neurons (Palmer, Mathews et al. 1980; Palmer, Mathews et al. 1981), and inhibition of mitogen-induced lymphocyte transformation (Webb, Nowowiejski et al. 1982). Leukotrienes also increase the vascular permeability (Orange and Austen 1969), although LTB4 has no direct effect on vascular permeability but LTB4 induces the adhesion of leukocytes to the endothelial cells (Dahlen, Bjork et al. 1981; Bjork, Hedqvist et al. 1982). Because of the adhesion of leukocytes to endothelial cells (Dahlen, Bjork et al. 1981; Bjork, Hedqvist et al. 1982), LTB4 stimulates directional (chemotaxis) and random movements (chemokinesis) of polymorphonuclear leukocytes (Ford-Hutchinson 1991). LTB4 can also induce degranulation and release of lysosomal enzymes from human and rabbit polymorphonuclear leukocytes (Bokoch and Reed 1981). Leukotrienes may act as a calcium ionophore and induce calcium mobilization in rabbit neutrophils (Molski, Naccache et al. 1981; Sha'afi, Molski et al. 1981) and in liposomes (Serhan, Anderson et al. 1981). LTC4, D4, E4, and F4 also induce contractions of stomach tissue (Orning, Hammarstrom et al. 1980; Hiscott, Murphy et al. 1981).

5. AIM OF THE STUDY

The objective of this study was to elucidate the regulation of suicidal erythrocytes death by membrane receptors and host pathogen interactions.

In a first series of experiments, the functional significance of host pathogen interactions for suicidal erythrocyte death was investigated. This study aimed to explore whether the synthetic lipopetide Pam3CSK4 mimicking a constituent of the bacterial cell wall triggers eryptosis. Using flowcytometry, it could be shown that Pam3CSK4 increased the cytosolic Ca^{2+} activity, moderately stimulated ceramide formation and significantly stimulated phosphatidylserine exposure of human erythrocytes.

In further series of experiments it was investigated, whether the AMPA receptor GluA1 is expressed in human erythrocytes and whether the AMPA receptor antagonist NBQX modifies Ca^{2+} entry and suicidal death of human erythrocytes. Confocal microscopy, flow cytometry and patch clamp recordings were used to illustrate the expression and participation of GluA1 in the regulation of suicidal erythrocytes death.

The objective of third study was to investigate the participation of cysteinyl-leukotrienes in the signaling of eryptosis. Western blotting was employed to detect the cysteinyl-leukotriene receptor cysLT1, competitive immune assay to determine leukotriene release from erythrocytes and flow cytometry to estimate cytosolic the Ca^{2+} concentration, cell volume, phosphatidylserine and activated caspases.

6. MATERIALS AND METHODS

Lipopeptides in the triggering of erythrocyte cell membrane scrambling

Erythrocytes, solutions, and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers. The volunteers provided informed consent. The study has been approved by the ethics committee of the University of Tübingen (184/2003V). For this study, 11 blood samples were studied. Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4. In Ca^{2+}-free Ringer, 1 mM $CaCl_2$ was substituted for 1 mM ethylene glycol tetraacetic acid (EGTA). The Ca^{2+} ionophore ionomycin (Sigma, Schnelldorf, Germany; dissolved in dimethyl sulfoxide [DMSO]) was used at a concentration of 1 μM. The final concentration of the solvent DMSO was 0.1%. The synthetic tripalmitoylated lipopeptide Pam3CSK4 (N-Palmitoyl- S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl- [S]- seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 HCl) that mimics the acylated amino terminus of bacterial lipoproteins was purchased from Invivogen (San Diego, USA) and has an endotoxin level of <0.125 EU/mg according to the manufacturer. In some experiments Pam3Cys from EMC Microcollections GmbH (Sindelfinger Str.3, 72070 Tübingen) was used yielding similar results. Pam3CSK4 and Pam3Cys are the same compounds sold under different names by different manufacturers.

FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described (Andree, Reutelingsperger et al. 1990). After incubation, cells were washed in Ringer solution containing 5 mM $CaCl_2$. Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} measurements were performed as described previously (Lang, Kempe et al. 2005). Briefly, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3-AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1.

Determination of ceramide formation

To determine formation of ceramide, which is exposed at the cell surface, a monoclonal antibody-based assay (Grassme, Jendrossek et al. 2002; Bieberich, MacKinnon et al. 2003) was used in FACS analysis. After incubation, cells were stained for 1 hour at 37°C with 1 μg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analysed by flow cytometric analysis on a FACS-Calibur in FL-1.

Statistics

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by unpaired t-test or ANOVA using Tukey's test as post hoc test, as appropriate.

Modulation of erythrocyte survival by AMPA

Erythrocyte, solution and chemicals

Four to six different leukocyte-depleted erythrocyte concentrates provided by the blood bank of the University of Tübingen were studied. For retrieval of the concentrates, citrate was used as anticoagulant. Prior to our experiments, the concentrates were stored at 4°C in the commonly-used SAG mannitol solution (0.41 – 0.26 ml/ml concentrate) with CPD stabilisator solution (0.015 – 0.007 ml/ml concentrate). 100 ml SAG mannitol solution contained 0.877 g NaCl, 0.9 g glucose, 0.0169 g adenosine, 0.525 g mannitol. 100 ml CPD stabilisator solution contained 0.327 g citric acid monohydrate, 2.63 g sodium citrate, 2.55 g glucose monohydrate, 0.251 g sodium dihydrogenphosphate. The erythrocyte concentrates were 7 – 20 days old when starting the experiment. Furthermore, erythrocytes from EDTA blood freshly retrieved from volunteers were investigated. The study is approved by the Ethical Commission of the University of Tübingen.

The experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$. Where indicated, Cl^- was substituted for gluconate or glucose was removed. The AMPA-receptor inhibitor NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) were obtained from Sigma (Schnelldorf, Germany).

FACS analysis of PS exposure and forward scatter

After incubation, erythrocytes were washed once in Ringer solution containing 5 mM $CaCl_2$ (Bentzen, Lang et al. 2007). The cells were then stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution (Nicolay, Gatz et al. 2007). After 15 min**,** samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3/AM (Foller, Shumilina et al. 2007). The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis (Attanasio, Shumilina et al. 2007).

Patch clamp recordings

Patch pipettes made of borosilicate glass (150 TF-10, Clark Medical Instruments) were pulled using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 8 to 12 MOhm were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA) as already described (Duranton, Huber et al. 2002). For current measurements (room temperature), erythrocytes were held at a holding potential (V_h) of -30 mV, and 200 ms pulses from -100 to +100 mV were applied in increments of 20 mV. The original whole-cell current traces are depicted without filtering (acquisition frequency of 5 kHz). The currents were analysed by averaging the current values measured between 90 and 190 ms of each square pulse (I-V relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solution and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch (Barry and Lynch 1991). Data were corrected for liquid junction potentials. The pipette solution contained (in mM): 125 Na-gluconate, 10 NaCl, 1 MgATP, 1 EGTA, and 10 HEPES/NaOH (pH 7.4). The recorded cells were superfused with standard NaCl bath solution containing (in mM): 145 NaCl, 5 KCl, 10 HEPES/NaOH, 5 D-glucose, 2 $MgCl_2$, 1 $CaCl_2$ (pH 7.4). In Na-gluconate bath solution Cl^- was substituted for gluconate. NMDG-gluconate bath solution contained (in mM): 180 N-methyl-D-glucamine (NMDG)-gluconate, 10 HEPES/NMDG, 1 $MgCl_2$, 1 $CaCl_2$ (pH 7.4).

Preparation of erythrocyte membrane and ghost

10 µl banked erythrocytes were lysed at 4°C in 2 ml hypotonic buffer containing 10 mM HEPES/NaOH (pH 7.4) and a protease inhibitor cocktail tablet (Roche, Mannheim, Germany). Ghost membranes were pelleted at 17,000 g for 15 min at 4°C and resupsended in 10 µl Ringer solutions. The ghost membranes were then subjected to confocal microscopy.

Confocal microscopy

Fresh EDTA whole blood or erythrocyte ghosts prepared from banked erythrocyte concentrates were taken and suspended in PBS (EDTA blood) or Ringer (ghosts) at a cell density of 5×10^7 cells/ml. Ten to twenty µl of the suspension were smeared onto a glass slide which was air dried for 30 min and then fixed with methanol for 2 min. After four washing steps with PBS for 10 min, the specimen was blocked by incubation with 10% goat serum. Following three washing steps with PBS for 5 min, the specimen was incubated with rabbit GluR1 antibody (1:200; Millipore, Billerica, MA, USA) at 4°C overnight. The slide was washed again three times for 5 min and then incubated with Cy3-conjugated Affinipure goat anti-rabbit antibody (Jackson Immuno Research, Hamburg, Germany) at room temperature for 1.5 h. Then, the specimen was mounted using Prolong® Gold antifade reagent (Invitrogen, Karlsruhe, Germany). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40 or 63_/1.3 NA DIC. As a control for the specificity of the primary antibody, erythrocytes from mice lacking GluA1 (*GluA1*$^{-/-}$) and from their wild type littermates (*GluA1*$^{+/+}$) were retrieved (EDTA blood) and similarly analysed in confocal microscopy.

Statistics

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by ANOVA using Tukey's test as post-hoc test or by two-tailed t-test, as appropriate. $p<0.05$ was considered as statistically significant.

Participation of leukotriene C4 in eryptosis

Volunteers and solution

Experiments were performed at 37°C with banked erythrocyte concentrates provided by the blood bank of the University of Tübingen. According to legislative standards in Germany, 1 µl of the erythrocyte concentrate could have contained at most 150 leukocytes compared to 10^7 erythrocytes. In erythrocyte concentrates leukocytes are, therefore, depleted by a factor of at least 2000 in respect of whole blood. The study was approved by the ethics committee of the University of Tübingen (184/2003V). Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4.

Leukotriene B4 and C4, the CysLT1 antagonist cinalukast, and the 5-lipoxygenase inhibitor BW B70C were purchased from Sigma (Schnelldorf, Germany). Leukotriene C4 was stored under argon gas to prevent degradation. Leukotriene B4 was dissolved in ethanol, leukotriene C4 in methanol. The effect of the solvent methanol was investigated as follows: In the experiments shown in Fig. 23A,B, 24A,B, and 25A,B,C,D,E,F the control samples contained the same amount of methanol as the samples treated with the highest concentration of Leuktriene C4 shown in the respective figures.

FACS analysis of PS exposure and forward scatter

FACS analysis was performed as described (Nicolay, Gatz et al. 2007). After incubation, cells were washed in Ringer solution containing 5 mM $CaCl_2$. Erythrocytes were stained with Annexin V-Fluos (Roche, Mannheim, Germany) at a 1:500 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and annexin V fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured 48 hours after incubation as described previously (Foller, Shumilina et al. 2007). Briefly, erythrocytes were washed in Ringer solution and then loaded

with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo-3-AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 µl Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1. To study Leukotriene C4-induced Ca^{2+} uptake of erythrocytes at physiologically low concentrations, erythrocytes were stained with Fluo-3/AM in Ringer containing 5 mM Ca^{2+} as decribed above. Then, erythrocytes were further handled in a box filled with argon gas to prevent Leukotriene C4 degradation. The samples were purged by argon gas for 2 minutes to remove oxygen. Subsequently, the samples were kept under argon gas for different time periods in the absence or presence of 10 nM Leukotriene C4. Then, Fluo3-dependent fluorescence was determined as a measure of the cytosolic Ca^{2+} concentration as described above.

Determination of leukotriene formation

5×10^8 erythrocytes taken from erythrocyte concentrates were incubated in Ringer solution either with or without 5 mM glucose for 24 hours. After incubation, cells were pelleted by centrifugation at 4°C, 450 *g* for 5 min. The supernatants were removed and stored at -80°C. Leukotriene C_4,D_4,E_4 concentrations in the supernatant were determined using the Cysteinyl Leukotriene Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI, USA) according to the manufacturer`s instructions. Leukotriene release from erythrocytes is expressed as pg cysteinyl leukotriene determined in the supernatant per 10^9 erythrocytes. Despite the high depletion factor leukocyte contaminations could in theory account for leukotriene formation. To rule out this possibility, 50 µl fresh whole blood and erythrocytes were similarly exposed to 37°C, and leukotriene formation was detected. As a result, leukotriene formation in whole blood was at the most 10 times higher than in erythrocyte concentrates (data not shown). Thus, the contribution of the residual leukocytes in the erythrocyte concentrates was too low to significantly bias the result.

Erythrocyte membrane preparation

100 µl whole blood, buffy coat or concentrates of banked erythrocytes were washed in Ringer solution and then hypotonically lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors composed of 2.5 mM EDTA, 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) from Roche

(Mannheim, Germany) at 4°C. Membranes were pelleted (15000 rpm for 20 min at 4°C). Then, membranes were solubilized in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton-X-100, 0.4% β-mercaptoethanol.

Immunoblotting

The protein concentration of the samples was determined with the Bradford method (Biorad, München, Germany) with bovine serum albumin (BSA; Sigma) as standard. Equal amounts of lysate protein (40 µg per lane) were separated by 10% SDS-PAGE, and proteins were transferred to a PVDF membrane. After blocking with 5% nonfat milk in TBS-0.1% Tween 20 at room temperature for 1 h, the blot was probed at 4°C overnight with a commercial rabbit CysLT1antibody (Gene Tex, Hiddenhausen, Germany; 1:500 dilution in TBS-0.1% Tween 20- 5% nonfat milk) After washing in TBS-0.1% Tween 20, the blot was incubated with a secondary anti-rabbit antibody (1:2000 in TBS-0.1% Tween 20- 5% nonfat milk) conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) for 1 h at room temperature. Antibody binding was detected with the enhanced chemoluminescence ECL kit (Amersham).

Confocal microscopy

Fresh EDTA whole blood was taken and suspended in PBS at a cell density of 5×10^7 cells/ml. 20 µl of the suspension were smeared onto a glass slide, air dried for 30 min, and then fixed with methanol for 2 min. After four washing steps with PBS for 10 min, the specimen was blocked by incubation with 10% goat serum. Following three washing steps with PBS for 5 min, the specimen was incubated with rabbit CysLT1antibody (Gene Tex, Hiddenhausen, Germany; 1:200) at 4°C overnight. The slide was washed again three times for 5 min and then incubated with Cy3-conjugated affinipure goat anti-rabbit antibody (Jackson Immuno Research, Hamburg, Germany) at room temperature for 1.5 h. Then, the specimen was mounted using Prolong® Gold antifade reagent (Invitrogen, Karlsruhe, Germany). Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Germany) with a water immersion Plan-Neofluar 40_/1.3 NA DIC.

Caspase 3 and 8 assays

After incubation in the presence of LTC_4 or of vehicle alone, the activities of caspase 3 and 8 were determined independently using the CaspGlow Fluorescein Active Caspase-3 or -8 Staining kits from BioVision (Mountain View, CA, USA) according to the provided protocol.

Statistics

Data are expressed as arithmetic means ± SEM, and statistical analysis was made by paired or unpaired t-test or ANOVA using Tukey's test as post hoc test, as appropriate.

7. RESULTS

Lipopeptides in the triggering of erythrocyte cell membrane scrambling

Annexin V-binding was determined to identify phosphatidylserine (PS)-exposing erythrocytes. The percentage of erythrocytes exposing annexin V at their surface was low after incubation in plain Ringer solution (Fig. 11B). A 48 hours treatment with Pam3CSK4 (5 µg/ ml) at 37°C significantly increased annexin V-binding (Fig. 11B).

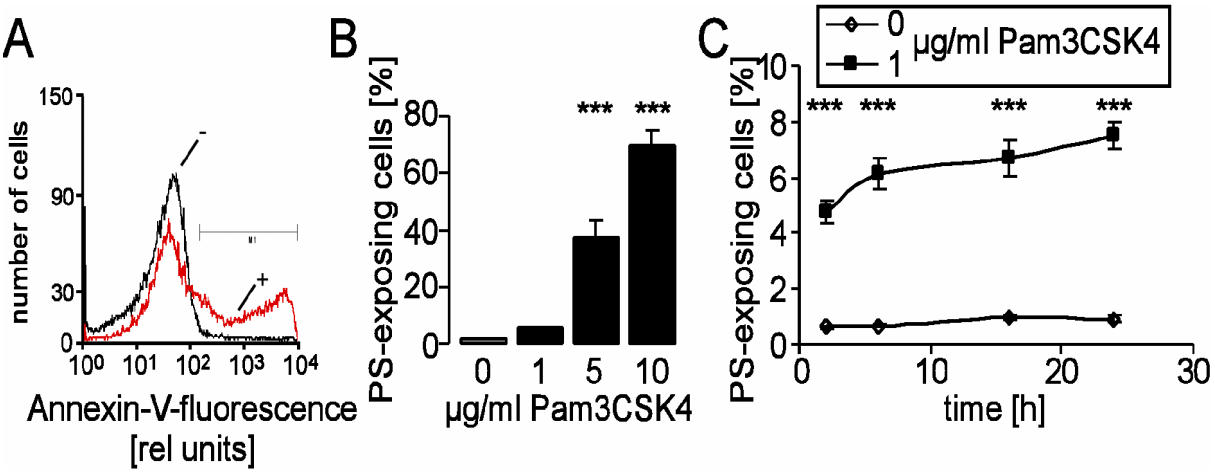

Figure 11. Stimulation of phosphatidylserine exposure of erythrocytes by Pam3CSK4.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 5 µg/ml Pam3CSK4 (indicated as ,+).

B. Effect of Pam3CSK4 (1, 5, 10 µg/ml) on phosphatidylserine exposure. Arithmetic means ± SEM (n = 4-20) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars). *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA).

C. Time dependence of the effect of 1 µg/ml Pam3CSK4 on phosphatidylserine exposure. Arithmetic means ± SEM (n = 8) of the percentage of annexin V-binding erythrocytes exposed for 2, 6, 16, and 24 hours to plain Ringer solution (open symbols) or to 1 µg/ml Pam3CSK4 (closed symbols). *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (t-test).

At 1 µg/ml Pam3CSK4, annnexin V binding similarly tended to be increased, but in the series displayed in 11B, the effect escaped statistical significance due to large scatter of the data. To further explore whether Pam3CSK4 could be effective at this low concentration, an additional series was performed analysing the effect of 1µg/ml at different exposure times. As shown in Fig. 11 C, Pam3CSK4 increased PS exposure of erythrocytes significantly in this series.

Forward scatter in FACS analysis was employed to detect alterations of cell volume. As illustrated in Fig. 12A, B, a 48 hours treatment with Pam3CSK4 (5 µg/ml), was followed by an increase in cell volume.

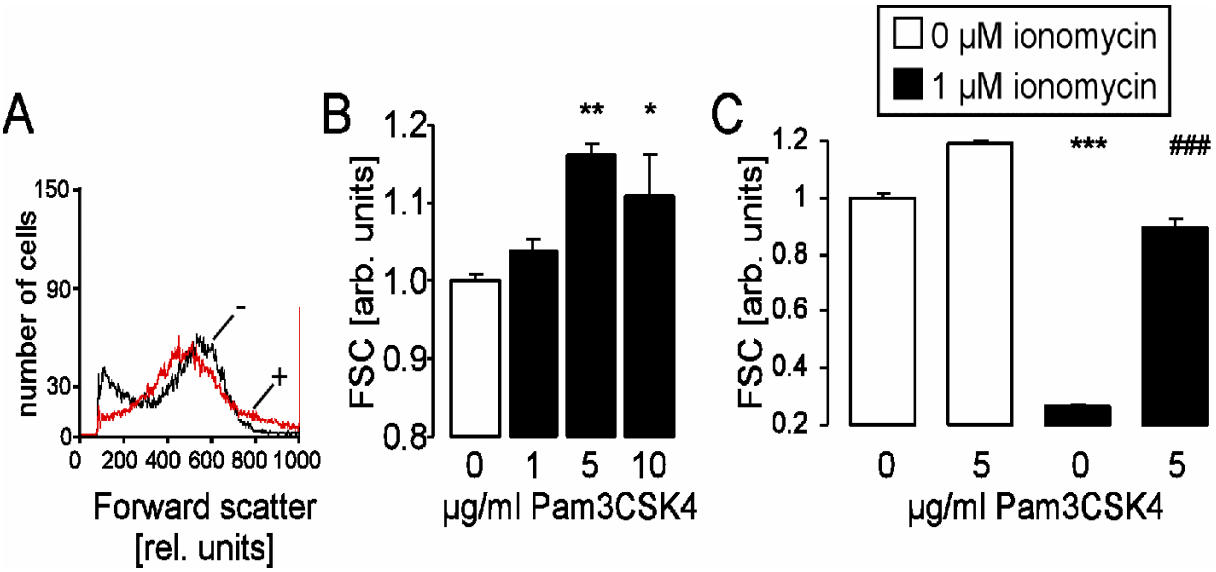

Fig. 12. Effects of Pam3CSK4 on erythrocyte forward scatter.

A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 5 µg/ml Pam3CSK4 (indicated as, +).

B. Effect of Pam3CSK4 (1, 5, 10 µg/ml) on forward scatter. Arithmetic means ± SEM (n = 4-16) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars). *, ** ($P < 0.05$, $P < 0.01$) indicate significant difference from values in control Ringer solution (ANOVA).

C. Arithmetic means ± SEM (n = 6) of forward scatter of erythrocytes exposed for 120 min to plain Ringer solution or to 5 µg/ml Pam3CSK4 and further 30 min in the absence (left bars) or presence (right bars) of 1 µM ionomycin. *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA). ### ($P < 0.001$) indicates significant difference between absence and presence of Pam3CSK4.

At least in Cell Physiology theory, Pam3CSK4 might inhibit the erythrocyte Ca^{2+}-dependent K^+ (Gardos) channels which, upon increase in intracellular Ca^{2+}, leads to an efflux of K^+, Cl^- and osmotically obliged water resulting in cell shrinkage. To check this possibility, erythrocytes were exposed to the Ca^{2+} ionophore ionomycin, which within 30 min led to the expected decrease of cell volume (Fig. 12C).

Additional experiments were performed to elucidate the underlying mechanisms for the observed phosphatidylserine scrambling following exposure to Pam3CSK4. Fluo3

fluorescence was employed to determine the cytosolic Ca^{2+} activity. As illustrated in Fig. 13, a 48 hours exposure to Pam3CSK4 (5 µg/ml) significantly increased Fluo3 fluorescence.

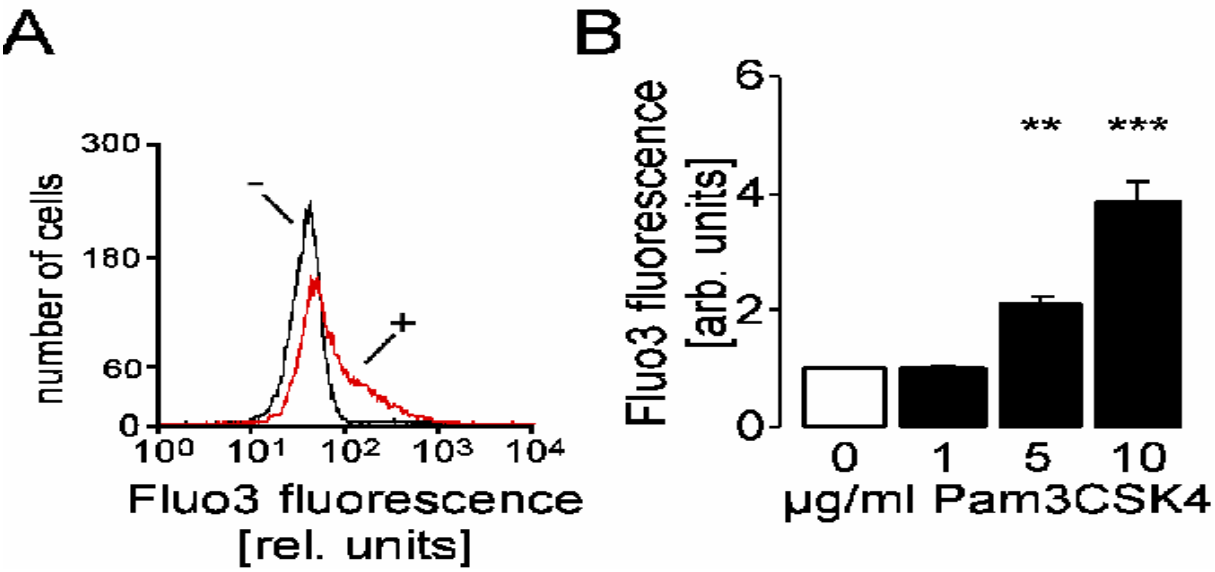

Figure 13. Effects of Pam3CSK4 on cytosolic Ca^{2+} activity.

A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 5 µg/ml Pam3CSK4 (indicated as, +).

B. Dose dependence of the effect of Pam3CSK4 on Fluo3 fluorescence. Arithmetic means ± SEM (n = 4-13) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Pam3CSK4 (black bars) at the indicated concentrations. **, *** ($P < 0.01$, $P < 0.001$) indicate significant difference from values in control Ringer solution (ANOVA).

To determine, whether the increase in cytosolic Ca^{2+} indeed contributes to or even accounts for the increase in phospholipid scrambling, experiments were performed in the presence and absence of extracellular Ca^{2+}. As shown in Fig. 14, 1 µg/ml led to a statistically significant increase in annexin V binding. Removal of extracellular Ca^{2+} indeed significantly blunted the stimulation of annexin V-binding following a 48 hours exposure to Pam3CSK4.

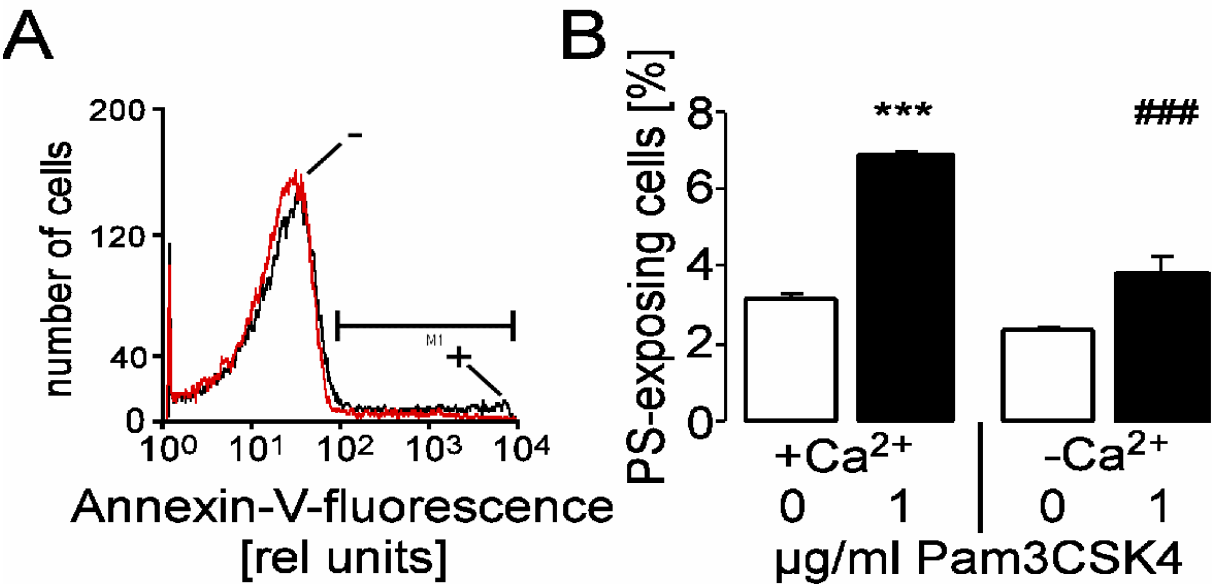

Figure 14. Role of Ca^{2+} in the stimulation of phosphatidylserine exposure by Pam3CSK4.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to 1 µg/ml Pam3CSK4 in the absence (4.1% annexin V-binding cells, indicated as,-) and presence (6.9% annexin V-binding cells, indicated as ,+) of 1 mM Ca^{2+}.

B. Arithmetic means ± SEM (n = 8) of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bars) or to Pam3CSK4 (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. *** ($P < 0.001$) indicates significant difference from values in control Ringer solution (ANOVA). ### ($P < 0.001$) indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

Similar to Pam3CSK4, Pam3Cys stimulated annexin V-binding, increased forward scatter, and enhanced Fluo3 fluorescence (data not shown).

The second major stimulus of eryptosis is ceramide. Therefore, further experiments were performed to investigate whether Pam3CSK4 also affects ceramide formation. As illustrated in Fig. 15A, B, 1 µg/ml Pam3CSK4 within 48 hours significantly stimulated the generation of erythrocytic ceramide.

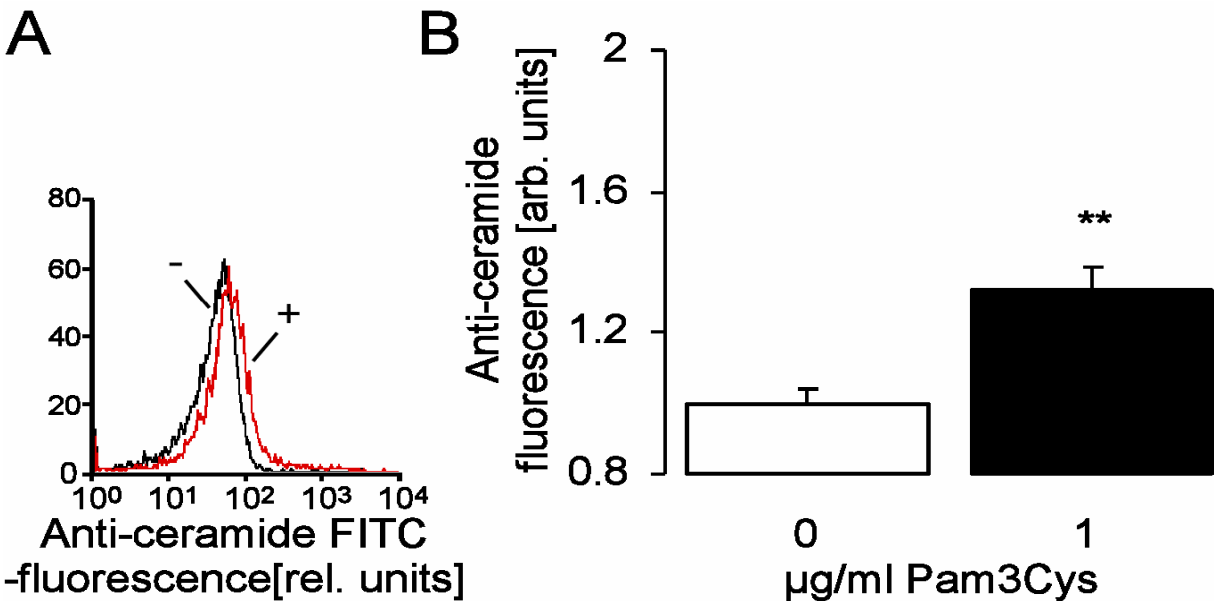

Figure 15. Effect of Pam3CSK4 on ceramide formation.

A. Histogram of ceramide abundance in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (indicated as, -) or with (indicated as, +) 1 µg/ml Pam3CSK4.

B. Arithmetic means ± SEM (n = 6-8) of ceramide abundance in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) 1 µg/ml Pam3CSK4. ** ($P < 0.01$) indicates significant difference from values in control Ringer solution (t-test).

Modulation of erythrocyte survival by AMPA

Confocal microcopy was utilized to explore whether GluA1 is expressed in human erythrocytes. As illustrated in Fig. 16, upper panels, a preparation of human whole blood indeed revealed the expression of GluA1 in the cell membrane of human erythrocytes. Similarly, GluA1 could be detected in the membranes of erythrocyte ghosts (Fig. 16 middle panel). To check for the specificity of the antibody against GluA1, the antibody was probed against erythrocytes from GluA1-deficient mice (*gluA1*$^{-/-}$) and from their wild type littermates (*gluA1*$^{+/+}$). As shown in Fig. 16, lower left panel, GluA1 could be readily detected in *gluA1*$^{+/+}$ erythrocytes while no signal was observed in *gluA1*$^{-/-}$ erythrocytes (Fig. 16, lower right panel).

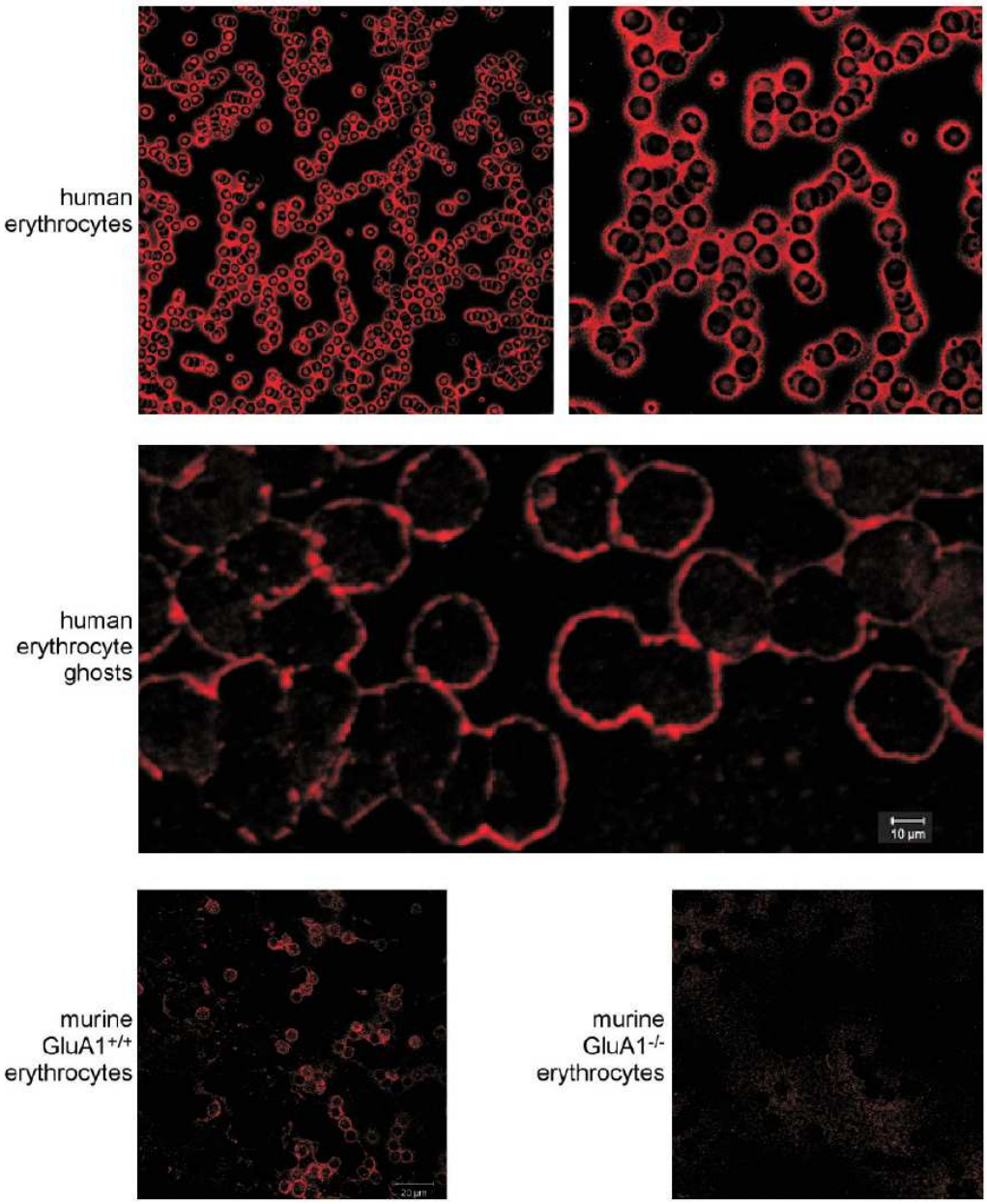

Figure 16 Expression of GluA1 in erythrocytes.

Examination of GluA1 expression in different erythrocyte preparations. The two upper panels show GluA1-dependent fluorescence in human erythrocytes. The middle panel depicts GluA1-dependent fluorescence in human erythrocyte ghosts. The lower panels illustrate GluA1-dependent fluorescence in murine *gluA1*$^{+/+}$ (left panel) and *gluA1*$^{-/-}$ (right panel) erythrocytes.

Whole cell patch clamp recordings were performed to elucidate the sensitivity of the Ca^{2+}-permeable cation channels of human erythrocytes to the AMPA receptor blocker NBQX. In confirmation of earlier observations, a cation channel could be observed in the absence of Cl^- (Fig. 17). NBQX added to the bath solution at a concentration of 10 µM significantly decreased the cation current (Fig. 17).

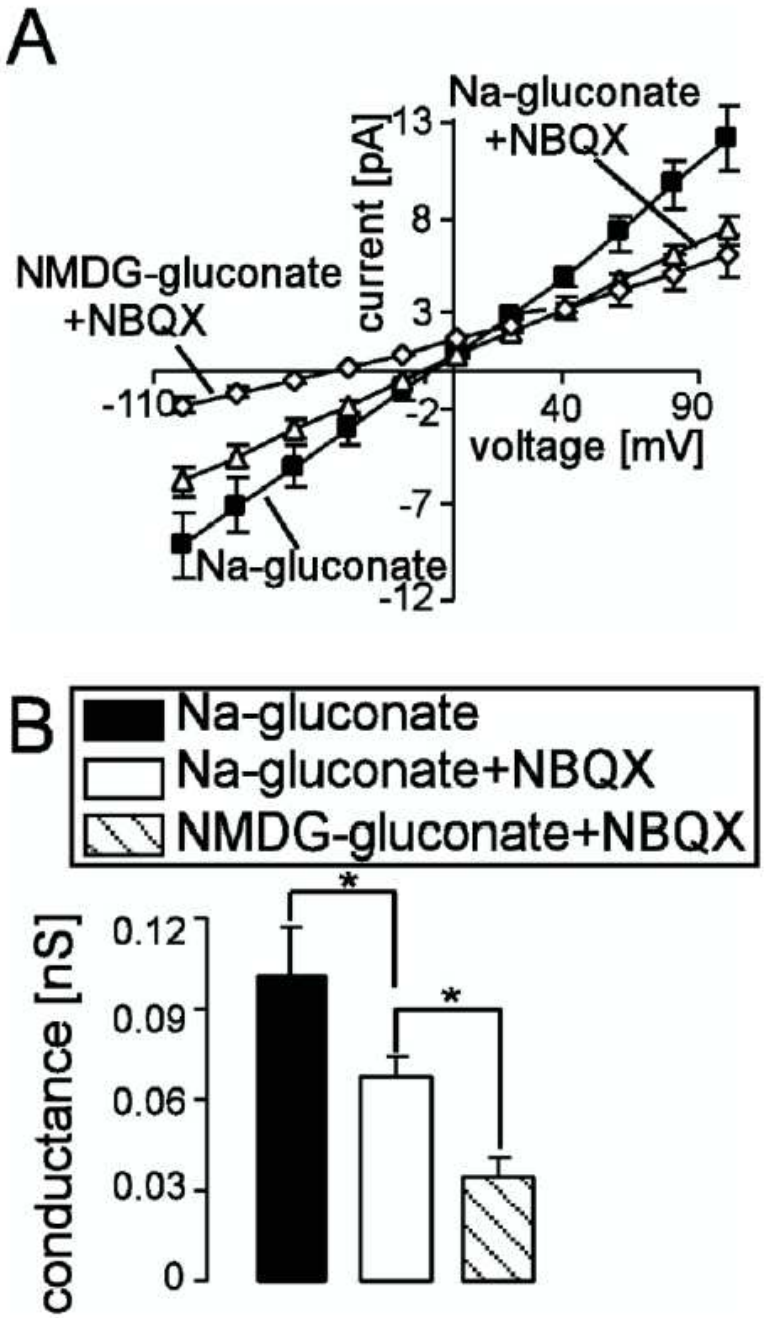

Figure 17 Inhibition of the non-selective cation channels by NBQX in human erythrocytes

A. Mean current voltage (I-V) relationships (± SEM, n=6) of human erythrocytes recorded in Na^+-gluconate (closed squares), then in Na-gluconate + 10 µM NBQX (open triangles) and then in NMDG-gluconate + 10 µM NBQX (open diamonds) bath solutions.

B. Mean conductance of the inward currents (± SEM, n=6) recorded as in (A) calculated by linear regression between -100 mV and -40 mV) in Na^+-gluconate (closed bar), Na-gluconate + 10 µM NBQX (open bar) and NMDG-gluconate + 10 µM NBQX (striped bar). * ($p<0.05$) indicates significant difference (one-way ANOVA).

However, a residual cation conductance was observed even in the presence of NBQX. Consequently, substitution of Na^+ by impermeable $NMDG^+$ in the bath solution led to a prominent decrease of the rest inward current and to the shift of the reversal potential (Fig.17). The patch clamp experiments thus reveal that NBQX- sensitive cation channels contribute to the cation conductance of human erythrocytes.

Further experiments were performed to elucidate whether the channel modifies the intracellular Ca^{2+} concentration. According to Fluo3 fluorescence, Cl^- deficiency (replacement of Cl^- by gluconate) markedly increased the cytosolic Ca^{2+} activity in human erythrocytes (Fig. 18). Similarly, energy depletion (incubation of erythroytes in glucose-free solution) was followed by a significant increase in the intracellular Ca^{2+} concentration (Fig. 18). Exposure to NBQX (10 or 50 µM) significantly blunted the increase in the intracellular Ca^{2+} concentration in erythrocytes following Cl^- removal and glucose depletion. The effect of 50 µM NBQX was more pronounced than the effect of 10 µM NBQX (Fig. 18).

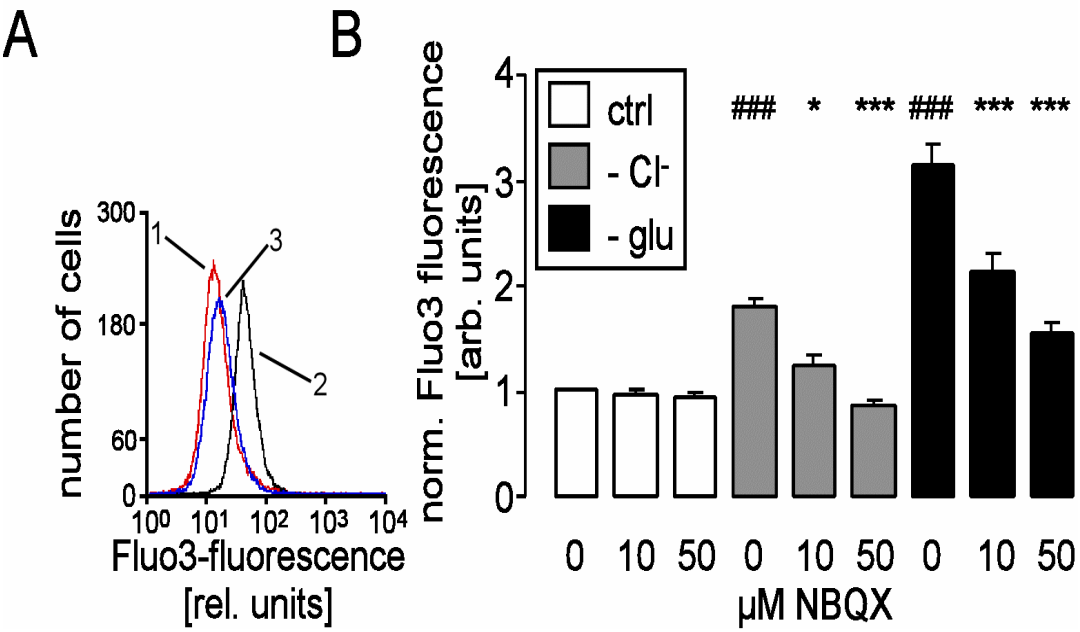

Figure 18 Cytosolic Ca^{2+} concentration in human erythrocytes

A. Histogram of Fluo3 fluorescence in a representative experiment of human erythrocytes exposed for 48 hours to plain Ringer (indicated as, 1) or to Cl^--depleted Ringer without (indicated as, 2) or with AMPA receptor blocker NBQX (50 µM, indicated as, 3).

B. Arithmetic means ± SEM (n = 16) of the normalized Fluo3 fluorescence in human erythrocytes exposed for 48 hours to plain Ringer (white bars), to Cl^--depleted Ringer (grey bars) or to glucose-free Ringer (black bars) in the presence of 0-50 µM NBQX. ### indicates significant difference from plain Ringer (ANOVA, $p<0.001$). *, *** indicate significant difference from the absence of NBQX (ANOVA, $p<0.05$, $p<0.001$).

Stimulation of Ca^{2+} entry and the resulting increase in the cytosolic Ca^{2+} activity are expected to activate Ca^{2+}-sensitive K^+ channels with following cell shrinkage (Lang, Kaiser et al. 2003). The forward scatter of human erythrocytes as a measure of cell volume was indeed decreased by both, Cl^- removal and glucose depletion (Fig. 19). Treatment with the AMPA receptor blocker NBQX (50 µM) significantly blunted the decrease of forward scatter following Cl^- removal (Fig. 19). Lower concentrations of NBQX (10 µM) tended to increase the forward scatter in the absence of Cl^- or glucose, an effect, however, not reaching statistical significance.

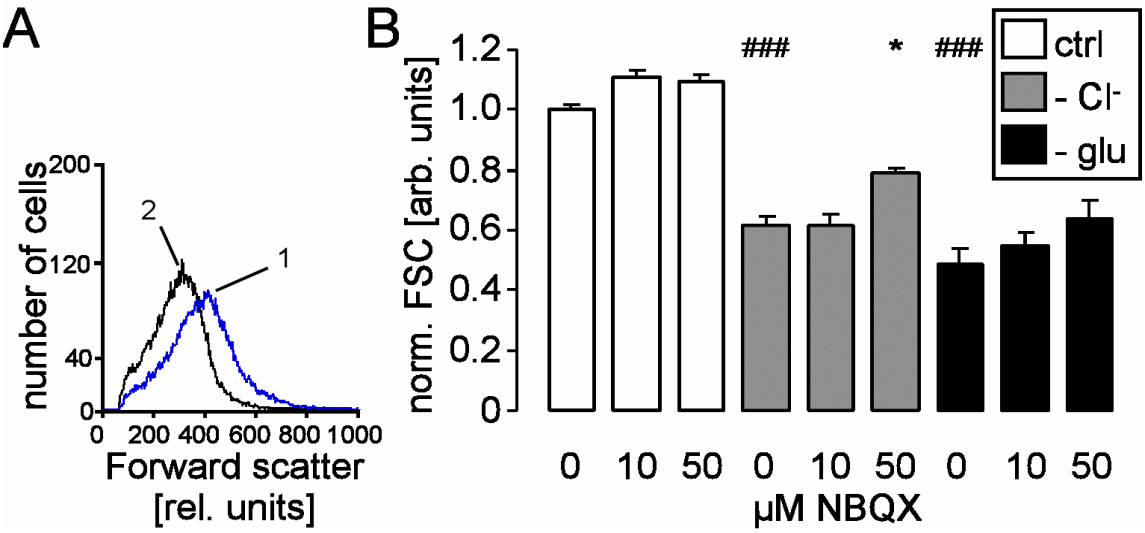

Figure 19 Forward scatter in human erythrocytes

A. Histogram of forward scatter in a representative experiment of human erythrocytes exposed for 48 hours to Cl^--depleted Ringer without (indicated as, 1) or with AMPA receptor blocker NBQX (indiated as, 2).

B. Arithmetic means ± SEM (n = 12-16) of the normalized forward scatter of human erythrocytes exposed for 48 hours to plain Ringer (white bars), to Cl^--depleted Ringer (grey bars) or to glucose-free Ringer (black bars) in the presence of 0-50 µM NBQX. ### indicates significant difference from plain Ringer (ANOVA, $p<0.001$). * indicates significant difference from the absence of NBQX (ANOVA, $p<0.05$).

Stimulation of Ca^{2+} entry further triggers cell membrane scrambling with subsequent PS exposure (Berg, Engels et al. 2001). As illustrated in Fig. 20, annexin V-binding of human erythrocytes was indeed enhanced by both, Cl^- removal and glucose depletion. The presence of NBQX (10 or 50 µM) significantly blunted the increase in annexin-V binding following Cl^- removal and glucose depletion (Fig. 20).

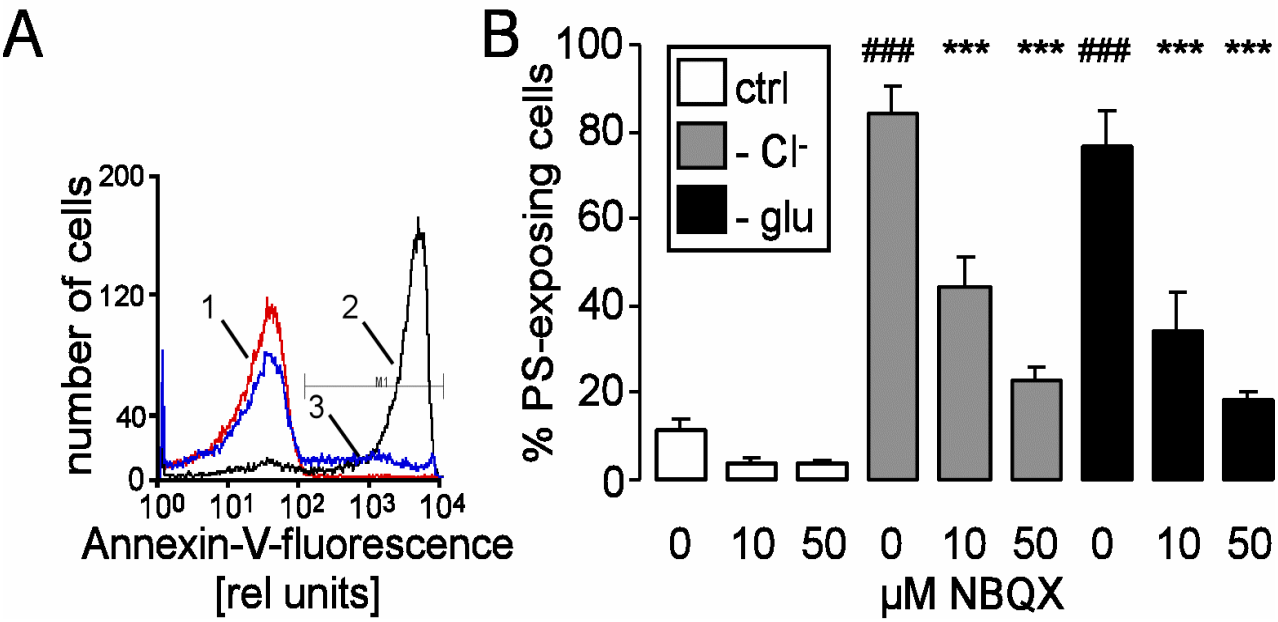

Figure 20 Annexin V-binding of human erythrocytes

A. Histogram of annexin V-binding in a representative experiment of human erythrocytes exposed for 48 hours to plain Ringer (indicated as, 1) or to Cl^- depleted Ringer without (indicated as, 2) or with AMPA receptor blocker NBQX (50 µM, indicated as, 3).

B. Arithmetic means ± SEM (n = 16) of the percentage of annexin V-binding human erythrocytes exposed for 48 hours to plain Ringer (white bars), to Cl^--depleted Ringer (grey bars) or to glucose-free Ringer (black bars) in the presence of 0-50 µM NBQX. ### indicates significant difference from plain Ringer (ANOVA, $p<0.001$). *** indicates significant difference from the absence of NBQX (ANOVA, $p<0.001$).

In a further series of experiments we compared the inhibitory potency of NBQX in freshly drawn blood with that in banked erythrocytes. As a result, following Cl^- removal for 48 hours the percentage of annexin V-binding banked erythrocytes approached 39.0 ± 5.9 % in the absence and of 16.9 ± 0.8 % in the presence of 50 µM NBQX (n=8). Exposure of freshly drawn erythrocytes to Cl^--free Ringer resulted in 36.9 ± 4.6% annexin V-binding cells in the absence and in 11.1 ± 2.2% annexin V-binding cells in the presence of 50 µM NBQX (n = 4-6). Thus, NBQX was similarly effective in freshly drawn blood and banked erythrocytes.

Additonal experiments explored whether the antieryptotic efficacy of NBQX is shared by the other AMPA receptor-blocker CNQX. As a result, in the presence of 50 µM CNQX the percentage of annexin V binding following Cl^- removal for 48 h was significantly ($p<0.05$) decreased from 31.9 ± 5.2 % (n = 16) to 14.0 ± 2.8 % (n = 16).

Participation of leukotriene C4 in eryptosis

The cysteinyl-leukotriene receptor CysLT1 is expressed in haematopoetic progenitor cells (Bautz, Denzlinger et al. 2001). Accordingly, we explored, whether the receptor is similarly expressed in mature erythrocytes. As illustrated in Fig. 21A, in erythrocyte membrane preparations a specific antibody directed against CysLT1 indeed bound to a protein band with the correct size (right lanes).

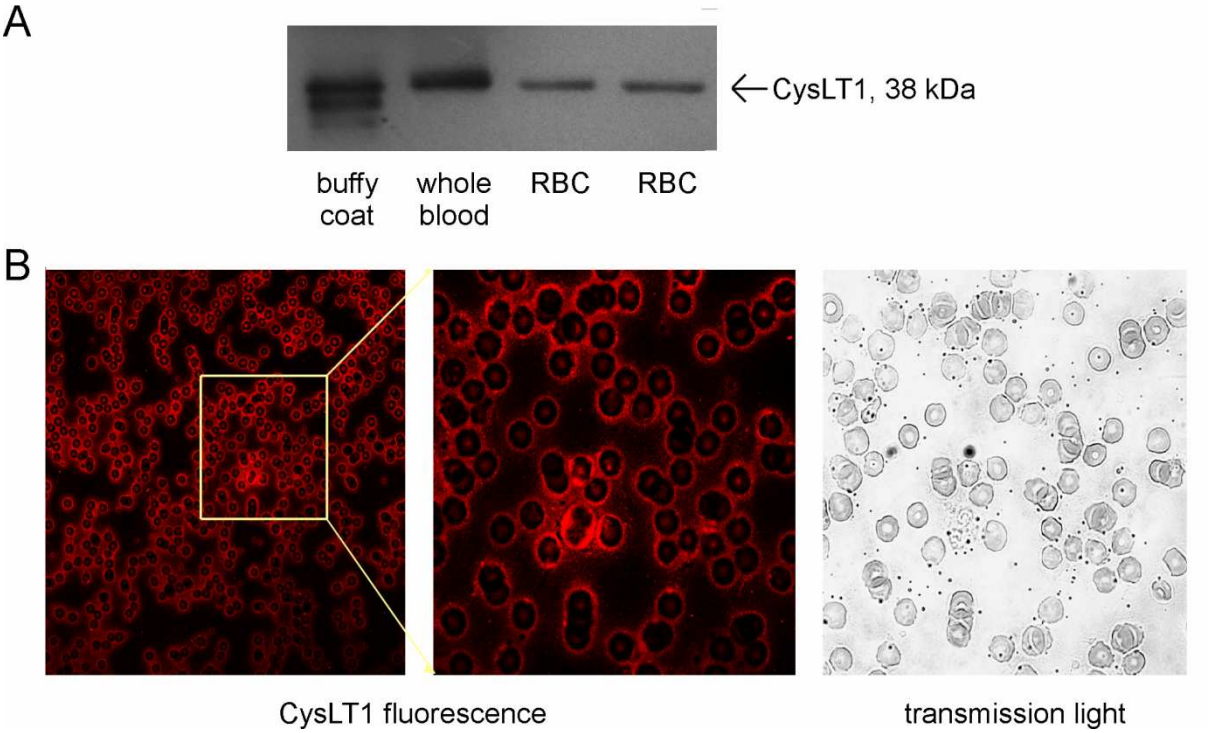

Figure 21 Expression of the leukotriene receptor CysLT1 in human erythrocytes.

A. Original Western Blot demonstrating the expression of CysLT1 in membrane preparations of enriched leukocytes (buffy coat), whole blood cells (whole blood) and human erythrocytes (RBC).

B. Examination of CysLT1 expression in erythrocytes from a whole blood preparation by confocal microscopy. The left panels show CysLT1-dependent fluorescence in human erythrocytes. For comparison, the right panel shows the corresponding transmission light photograph.

The same band was readily detected in membrane preparations of whole blood cells containing leukocytes as well as in the membrane preparation of a buffy coat enriched in leukocytes. Further experiments were performed to determine CysLT1 expression in human erythrocytes using confocal microscopy. As shown in Fig 21B, confocal microscopy of a

preparation of human whole blood indeed revealed the expression of CysLT1 on human erythrocytes.

In a next step, we explored the possibility that erythrocytes synthesize cysteinyl-leukotrienes. As shown in Fig.22, a competitive immune assay indeed detected cysteinyl-leukotrienes in the medium (Ringer solution) of incubated erythrocytes. Moreover, the assay revealed that glucose depletion significantly increased the formation of cysteinyl-leukotrienes. Due to extreme instability of the different cysteinyl leukotrienes we could not discriminate between LTC_4, LTD_4 and LTE_4. All three leukotrienes are known to activate cysLT1 (Xue, Cai et al. 2007).

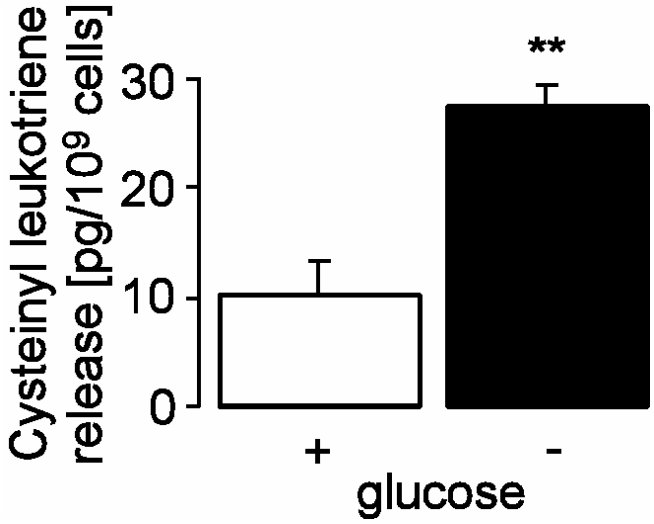

Figure 22 Effect of glucose depletion on leukotriene formation in human erythrocytes.

Arithmetic means ± SEM (n = 4-5) of leukotriene $C_4/D_4/E_4$ abundance determined by competitive immunoassay in the supernatant following a 24 hours incubation of human erythrocytes at 37°C in glucose-containing (open bar) and glucose-deficient (closed bar) Ringer. * indicates significant difference from presence of glucose ($P < 0.05$, t-test).

Erythrocytes express cation channels permeable to Ca^{2+} (Duranton, Huber et al. 2002). Accordingly, activation of the channels is expected to increase the cytosolic Ca^{2+} concentration. Therefore, Fluo3 fluorescence was employed to determine cytosolic Ca^{2+} activity in erythrocytes prior to and following treatment with different concentrations of cysteinyl-leukotriene LTC_4. As demonstrated in Fig. 23A,B, LTC_4-treatment was indeed followed by a significant increase in the cytosolic Ca^{2+} concentration. In contrast, leukotriene

LTB4 did not significantly modify the cytosolic Ca^{2+} concentration (data not shown). LTC4 is known to be extremely instable. This might be the reason for the high concentrations of leukotriene required to induce Ca^{2+} influx into erythrocytes within 48 hours of incubation. To check this possibility, erythrocytes were exposed to 10 nM LTC4 in argon gas to prevent LTC4 degradation for different time periods, and Ca^{2+}-dependent Fluo3 fluorescence was monitored. As shown in Fig. 23C,D, 10 nM LTC4 induced a significant increase in the cytosolic Ca^{2+} concentration of erythrocytes within 5 min at room temperature.

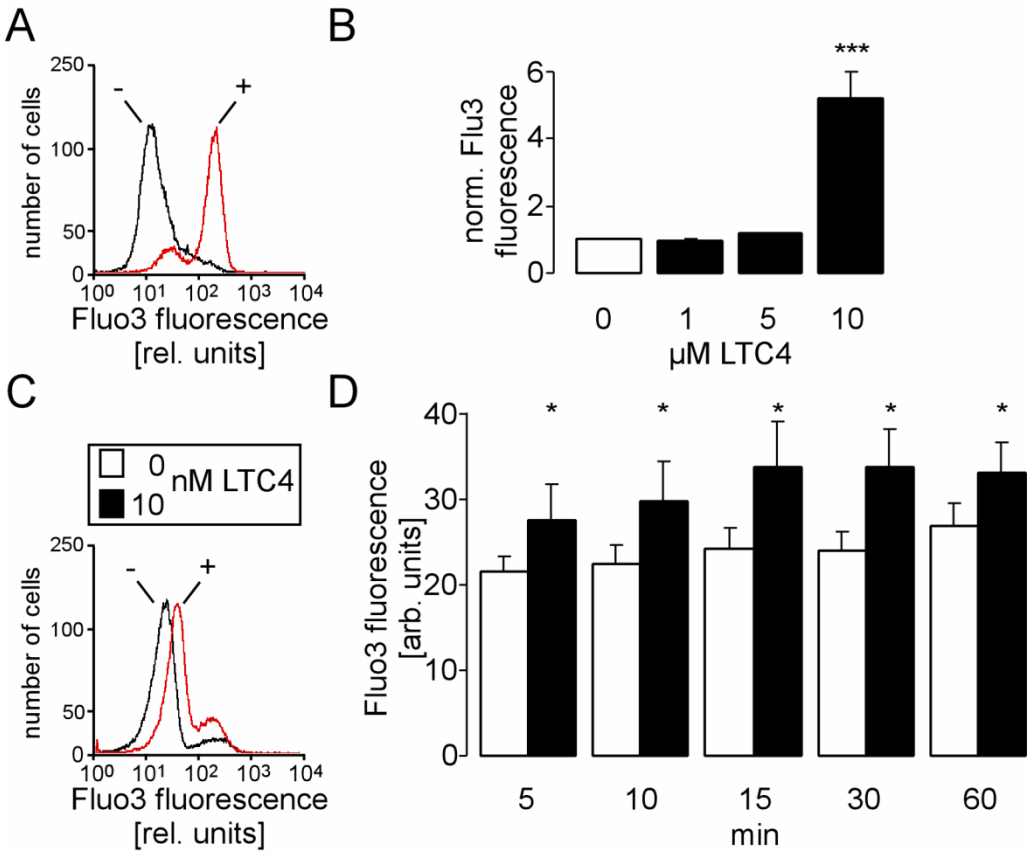

Figure 23 Effects of Leukotriene C_4 on cytosolic Ca^{2+} concentration.

A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours at 37°C to Ringer solution without (indicated as, -) or with 10 µM leukotriene C_4 (indicated as, +).

B. Dose dependence of the effect of leukotriene C_4 on Fluo3 fluorescence. Arithmetic means ± SEM (n = 12-20) of the normalized geo means of Fluo3 fluorescence of erythrocytes exposed for 48 hours at 37°C to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations. *** ($P < 0.001$, ANOVA) indicates significant difference from values in the absence of leukotriene.

C. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 15 minutes in argon gas to Ringer solution without (-, black line) or with 10 nM leukotriene C_4 (+, red line).

D. Arithmetic means ± SEM (n = 8-10) of Ca^{2+}-dependent Fluo3 fluorescence of erythrocytes incubated in argon gas to prevent leukotriene degradation at room temperature for the indicated time periods in the absence (open bars) or presence (closed bars) of 10 nM leukotriene C_4. * ($P < 0.05$, paired t-test) indicates significant difference from the absence of leukotriene C_4.

An increase in the cytosolic Ca^{2+} concentration is expected to activate Ca^{2+}-sensitive K^+ channels with subsequent exit of KCl and osmotically obliged water and thus to shrink the

cells (Lang, Kaiser et al. 2003). Accordingly, the forward scatter was determined to depict alterations of cell volume. As shown in Fig. 24, LTC_4-treatment was indeed followed by a decrease of forward scatter.

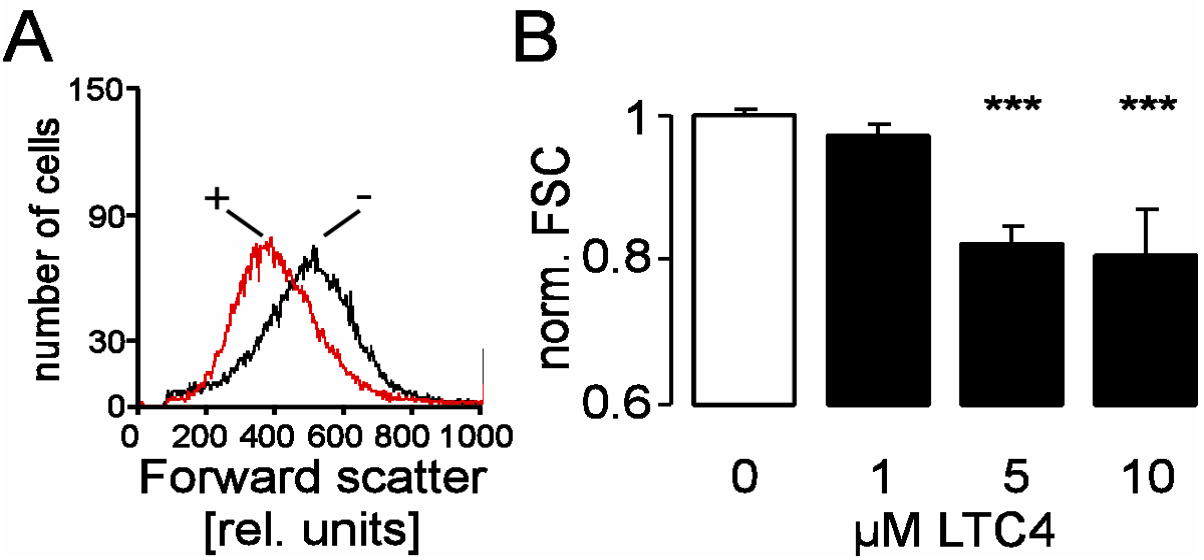

Figure 24 Effects of Leukotriene C_4 on erythrocyte forward scatter.

A. Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (-, black line) or with 5 µM leukotriene C_4 (+, red line).

B. Dose dependence of the effect of leukotriene C_4 on forward scatter. Arithmetic means ± SEM (n = 12-16) of the normalized forward scatter of erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA).

An increase in the cytosolic Ca^{2+} concentration is further expected to trigger scrambling of the cell membrane with phosphatidylserine exposure at the cell surface (Berg, Engels et al. 2001; Bratosin, Estaquier et al. 2001). Annexin V-binding to phosphatidylserine at the cell surface was utilized to detect cell membrane scrambling. As displayed in Fig. 25A,B, the treatment of erythrocytes with LTC_4 indeed significantly enhanced the percentage of annexin V-binding erythrocytes. LTB_4, applied at the same concentrations, did not induce appreciable annexin V-binding of erythrocytes (data not shown). Further experiments revealed that LTC_4-induced eryptosis is associated with activation of caspases.

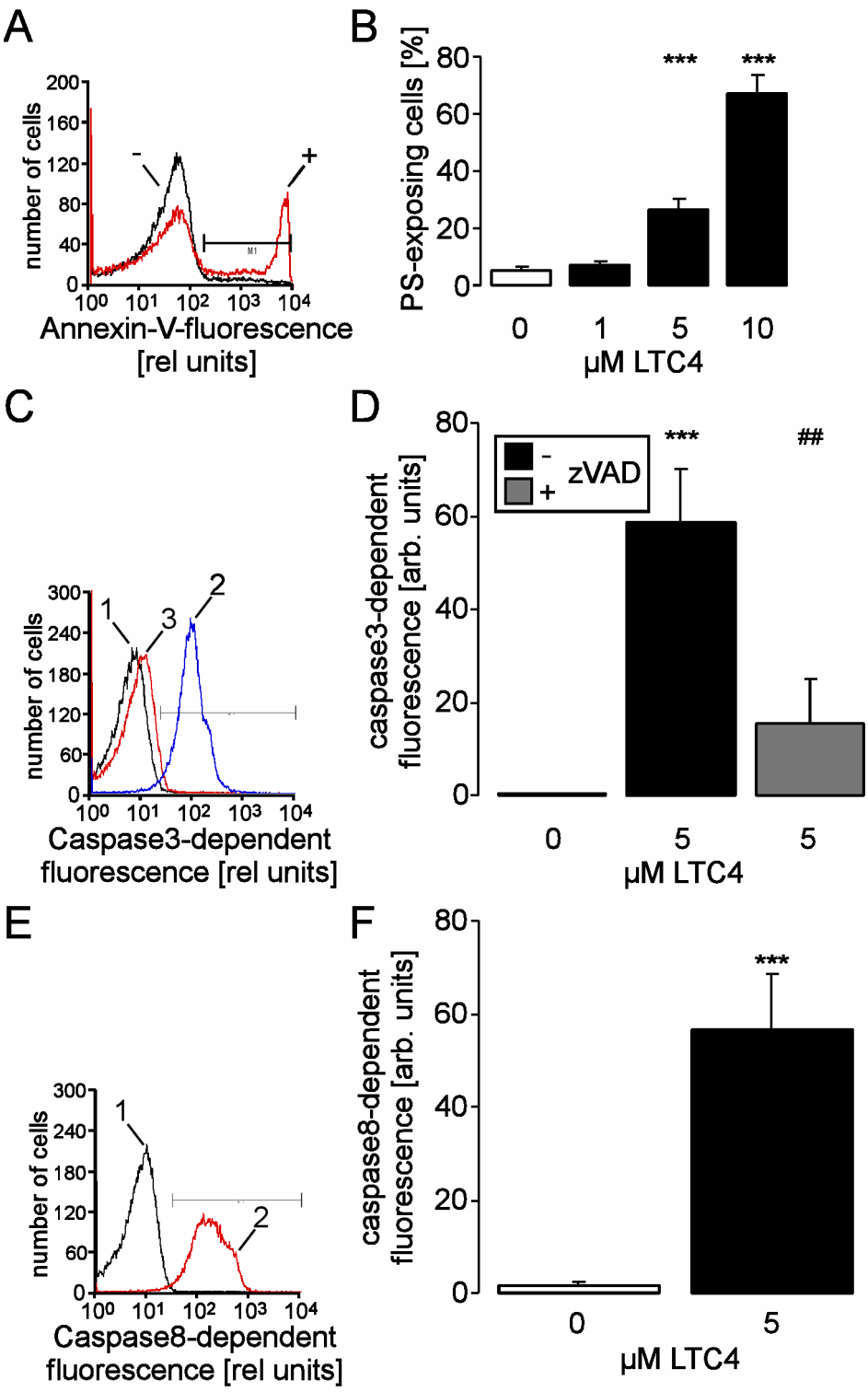

Figure 25 Stimulation of phosphatidylserine exposure and caspase activation by Leukotriene C_4.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (indicated as, -) or with 5 µM leukotriene C_4 (indicated as, +).

B. Dose dependence of the effect of leukotriene C_4 on phosphatidylserine exposure. Arithmetic means ± SEM (n = 12-20) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with leukotriene C_4 (black bars) at the indicated concentrations. *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA).

C. Histogram of caspase3-dependent fluorescence of erythrocytes exposed for 48 hours to Ringer solution without (indicated as, 1) or with 5 µM leukotriene C_4 in the absence (indicated as, 2) or presence (indicated as, 3) of pancaspase inhibitor zVAD-FMK.

D. Arithmetic means ± SEM (n = 10-12) of the percentage of erythrocytes with activated caspase 3 after exposure for 48 hours to Ringer solution without (white bar) or with 5 µM leukotriene C_4 in the absence (black bar) or presence of pancaspase inhibitor zVAD-FMK (grey bar). *** ($P < 0.001$) indicates significant difference from values in the absence of leukotriene (ANOVA). ## ($P < 0.01$) indicates significant difference from the absence of zVAD-FMK.

E. Histogram of caspase 8-dependent fluorescence of erythrocytes exposed for 48 hours to Ringer solution without (indicated as, 1) or with 5 µM leukotriene C_4 (indicated as, 2).

F. Arithmetic means ± SEM (n = 12) of the percentage of erythrocytes with activated caspase 8 after exposure for 48 hours to Ringer solution without (white bar) or with 5 µM leukotriene C_4 (black bar). *** ($P < 0.001$) indicates significant difference from values inthe absence of leukotriene (u-test).

As shown in Fig. 25C,D, LTC_4 led to activation of caspase 3, an effect, significantly blunted in the presence of the pancaspase inhibitor zVAD-FMK (Fig. 25C,D). Similarly, caspase 8 activity was significantly enhanced upon incubation in the presence of LTC_4 (Fig. 25E,F).

A further series of experiments explored, whether the activation of the cysteinyl-leukotriene receptor cysLT1 is involved in the stimulation of phosphatidylserine exposure and cell shrinkage during energy depletion as suggested from increased endogenous leukotriene formation during enery depletion (Fig. 22). As illustrated in Fig. 26A,B, the cysLT1 inhibitor cinalukast (1 µM) significantly blunted the effect of glucose withdrawal on cell membrane scrambling. Moreover, cinalukast significantly blunted erythrocyte shrinkage following glucose withdrawal as deduced from forward scatter (Fig. 26C).

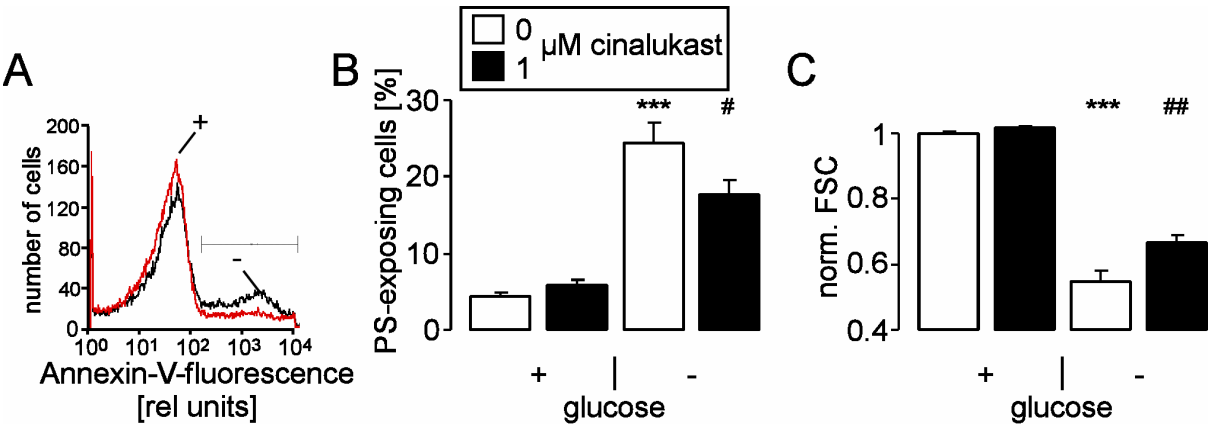

Figure 26 Inhibition of eryptosis by the CysLT1 antagonist cinalukast during energy depletion.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes incubated for 48 hours in Ringer solution free of glucose in the absence (indicated as, -) or presence (indicated as, +) of cinalukast (1 µM).
B. Arithmetic means ± SEM (n = 32) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of cinalukast (1 µM). *** indicates significant difference (ANOVA, P < 0.001) from control (presence of glucose). # indicates significant difference (ANOVA, P < 0.05) from absence of cinalukast.
C. Arithmetic means ± SEM (n = 28) of normalized forward scatter of erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of cinalukast (1 µM). *** indicates significant difference (ANOVA, p<0.001) from control (presence of glucose). ## indicates significant difference (ANOVA, P < 0.01) from absence of cinalukast.

If suicidal cell death of energy-depleted erythrocytes is indeed mediated by endogenously formed leukotrienes, inhibition of the 5-lipoxygenase should blunt phosphatdidylserine exposure and cell shrinkage following enery depletion. As shown in Fig. 27A,B, exposure of enery-depleted erythrocytes to 1 µM of the selective 5-lipoxygenase inhibitor BW B70C indeed significantly blunted phosphatidylserine exposure. Similarly, inhibition of the 5-lipoxygenase significantly reduced the cell shrinkage following enery depletion (Fig. 27C).

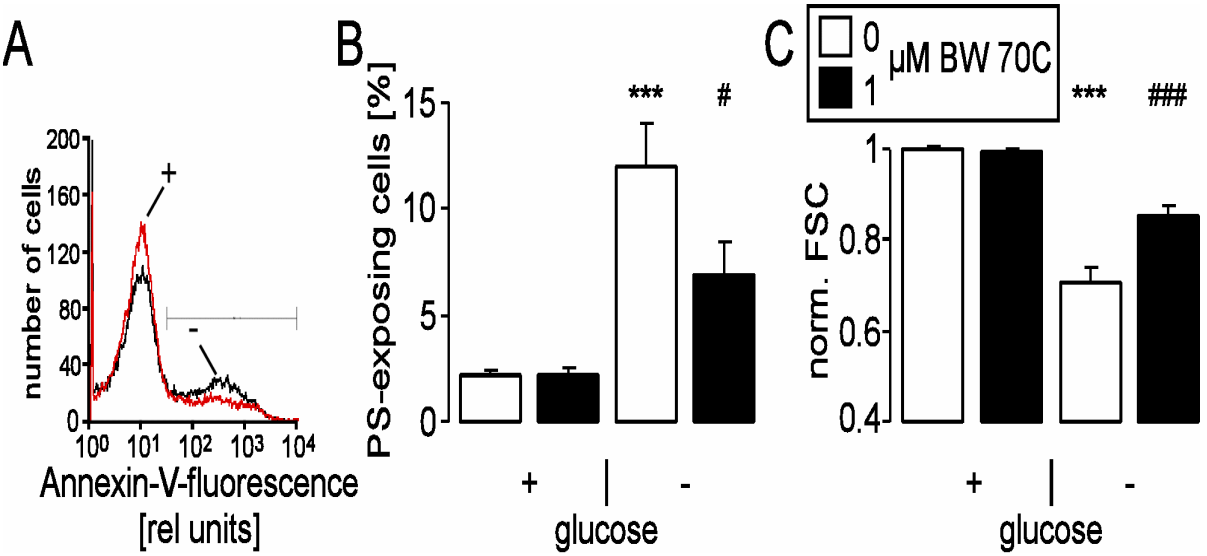

Figure 27 Inhibition of eryptosis by the 5-lipoxygenase inhibitor BW B70C during energy depletion.

A. Histogram of annexin V-binding in a representative experiment of erythrocytes incubated for 48 hours in Ringer solution free of glucose in the absence (indicated as, -) or presence (indicated as, +) of BW B70C (1 µM).
B. Arithmetic means ± SEM (n = 19-20) of the percentage of annexin V-binding erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of BW B70C (1 µM). *** indicates significant difference (ANOVA, P < 0.001)

from control (presence of glucose). # indicates significant difference (ANOVA, P < 0.05) from absence of BW B70C.

C. Arithmetic means ± SEM (n = 19-20) of normalized forward scatter of erythrocytes after a 48 hours treatment with Ringer solution in the presence (left bars) or absence (right bars) of glucose in the absence (white bars) or presence (black bars) of BW B70C (1 µM). *** indicates significant difference (ANOVA, p<0.001) from control (presence of glucose). ### indicates significant difference (ANOVA, P < 0.001) from absence of BW B70C.

8. DISCUSSION

Lipopeptides in the triggering of erythrocyte cell membrane scrambling

The present observations confirm that exposure of erythrocytes to Pam3CSK4, a known stimulator of Toll-like receptors (Bagchi, Herrup et al. 2007; Zhang, Deriaud et al. 2007), leads to scrambling of the cell membrane with subsequent phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages equipped with phosphatidylserine receptors (Boas, Forman et al. 1998) and thus eliminated from circulating blood (Kempe, Lang et al. 2006). Thus, eryptosis results in safe disposal of affected erythrocytes from the cirrculation (Lang, Lang et al. 2005). Moreover, phosphatidylserine-exposing erythrocytes adhere to the vascular wall and could thus compromise microcirculation (Lang, Kaiser et al. 2003). As a matter of fact, ischemia leads to enhanced trapping of annexin V-binding erythrocytes in the renal medulla (Lang, Kaiser et al. 2004; Curtiss and Tobias 2007). It is noteworthy that Toll-like receptor triggering could contribute to atherosclerosis (Curtiss and Tobias 2007).

As shown earlier (Lang, Kaiser et al. 2003), an increase in cytosolic Ca^{2+} activity further activates Ca^{2+}-sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, and exit of Cl^- together with osmotically obliged water, thus eventually leading to cell shrinkage (Lang, Kaiser et al. 2003). The cell shrinkage then contributes to the triggering of cell membrane scrambling (Schneider, Nicolay et al. 2007). However, surprisingly, Pam3CSK4 had no significant effect on the forward scatter. Possibly, Pam3CSK4 inhibited the Ca^{2+}-sensitive K^+ channel. Accordingly, ionomycin-induced cell shrinkage was indeed blunted in the presence of Pam3CSK4. The observations suggest that Pam3CSK4 increases Ca^{2+} entry but at the same time abrogates the stimulating effect of Ca^{2+} on the Ca^{2+} sensitive K^+ channels. On the other hand, in dendritic cells, the activation of Toll-like receptor has been shown to upregulate K^+ channels (Shumilina, Zahir et al. 2007).

Similar to the effect of peptidoglycans (Foller, Biswas et al. 2009), the effect of Pam3CSK4 is blunted by removal of extracellular Ca^{2+}. In polymorphonuclear leukocytes, peptidoglycans have been shown to trigger the arachidonic acid cascade with subsequent formation of prostaglandin E. In erythrocytes PGE_2 has been shown to stimulate Ca^{2+} entry by activation of the Ca^{2+}-permeable cation channels (Lang, Kempe et al. 2005). Activation of toll-like

receptors may similarly stimulate the formation of PGE_2 (Goos, Zech et al. 2007; Lenoir, Sapin et al. 2008; Noor, Goldfine et al. 2008; Xu, Xu et al. 2008). Hitherto an effect of Pam3CSK4 on prostaglandin formation has, however, not been shown.

Further mechanisms may be involved in the stimulation of Ca^{2+} entry and cell membrane scrambling following exposure to Pam3CSK4. It is noteworthy that cytosolic Ca^{2+} activity may be increased by bacterial hemolysin pneumolysin, an effect more pronounced in cells expressing a Cu/Zn SOD1 mutant and blunted by the anti-oxidant N-acetylcysteine (Goos, Zech et al. 2007), Oxidative stress is known to stimulate the erythrocyte cation channels (Duranton, Huber et al. 2002).

The present study does not address the functional consequences of Pam3CSK4 induced eryptosis for circulation in sepsis. Sepsis is known to cause severe hypotension, an effect at least partially due to nitric oxide (NO) formation (Han, Kim et al. 2006).

Accordingly, animals do not become hypotensive after an LPS exposure if they are deficient in inducible NO synthase (iNOS), an enzyme critical for the excessive production of NO (Han, Kim et al. 2006). On the other hand, Toll-like receptor activation has been shown to suppress the formation of nitric oxide (Walter, Letiembre et al. 2007), which in turn inhibits eryptosis (Nicolay, Liebig et al. 2008) at least partially through G-kinase (Foller, Feil et al. 2008).

In conclusion, the Toll-like receptor agonist Pam3CSK4 stimulates Ca^{2+} entry and leads to moderate formation of ceramide in erythrocytes, resulting in cell membrane scrambling and subsequent exposure of phosphatidylserine at the cell surface.

Modulation of erythrocyte survival by AMPA

The present research reveals a novel element in the regulation of erythrocyte survival. Both, genetic and pharmacological evidence suggest that GluA1 contributes to the Ca^{2+}-permeable cation channels, Ca^{2+} entry, cell shrinkage and phosphatidylserine exposure of mature erythrocytes. Accordingly, GluA1 or a pharmacologically similar channel presumably participates in the orchestration of suicidal death of erythrocytes.

PS-exposing erythrocytes are bound to PS receptors on macrophages (Fadok, Bratton et al. 2000), which engulf and degrade PS-exposing cells (Boas, Forman et al. 1998). Accordingly, PS-exposing erythrocytes are rapidly cleared from circulating blood (Kempe, Lang et al. 2006). Therefore, enhanced eryptosis has been observed in a variety of clinical conditions associated with anemia (Lang, Gulbins et al. 2008). Moreover, eryptosis may affect the microcirculation, as PS-exposing erythrocytes may bind to the vascular wall and participate in blood clotting (Wood, Gibson et al. 1996; Andrews and Low 1999; Closse, Dachary-Prigent et al. 1999; Gallagher, Chang et al. 2003; Zwaal, Comfurius et al. 2005; Chung, Bae et al. 2007; Pandolfi, Di Pietro et al. 2007). As a matter of fact, suicidal erythrocytes have been proposed to participate in vascular injury of metabolic syndrome (Zappulla 2008). Finally, oxidative stress may limit the life span of stored erythrocytes (Kriebardis, Antonelou et al. 2007).

According to our observations, AMPA receptor triggering does not only affect the survival of neurons (Das, Sribnick et al. 2005; Kim and Han 2005; Zou, Li et al. 2005; Segura Torres, Chaparro-Huerta et al. 2006; Beart, Lim et al. 2007; de Groot, Piao et al. 2008; Klimaviciusa, Safiulina et al. 2008; Mizuno, Zhang et al. 2008; Molz, Decker et al. 2008) and glial cells (Ishiuchi, Yoshida et al. 2007; de Groot, Piao et al. 2008) but may similarly affect erythrocyte survival. It should be kept in mind, though, that NBQX and CNQX could exert effects other than blocking AMPA receptors.

Parallel death of erythrocytes and neurons has led to the term Neuroacanthocytosis, which is characterized by nervous system abnormalities in association with acanthocytosis in the patients' blood. The disorder may be caused by a defect of the cytoskeletal protein 4.1 (Orlacchio, Calabresi et al. 2007). The protein regulates the surface expression and activity of GluA1 (Shen, Liang et al. 2000; Scott, Keating et al. 2001; Coleman, Cai et al. 2003).

Neuronal and erythrocyte survival are further affected in parallel by a mutation within GLUT-1 turning the glucose carrier into a cation channel (Weber, Storch et al. 2008). Affected

individuals of the family suffered from exertion-induced dyskinesia, epilepsy, mild developmental delay, reduced CSF glucose levels and hemolytic anemia with echinocytosis (Menard, Chartier et al. 2007).

AMPA receptors are further regulated by phospholipase A_2, which similarly participates in the regulation of erythrocyte cation channels and erythrocyte survival (Lang, Kempe et al. 2005).

Most recently, expression of AMPA receptor subunits have been discovered in platelets and shown to participate in blood coagulation. Accordingly, in mice lacking GluA1 the time required to complete thrombosis is prolonged (Morrell, Sun et al. 2008). It should be kept in mind that PS-exposing erythrocytes adhere to the vascular wall (Andrews and Low 1999; Closse, Dachary-Prigent et al. 1999; Gallagher, Chang et al. 2003; Zappulla 2008) and thus may contribute to haemostasis (Andrews and Low 1999).

In conclusion, Ca^{2+} entry into erythrocytes is blunted by an AMPA receptor antagonist. The present observations disclose a completely novel effect of AMPA-modulating drugs and unravel a novel parallelism between erythrocyte and neuronal survival.

Participation of leukotriene C4 in eryptosis

The current study discloses an innovative function of leukotrienes, i.e. the stimulation of eryptosis. The cysteinyl-leukotriene C4 presumably activates cation channels leading to influx of Ca^{2+}, cell shrinkage, and phosphatidylserine exposure at the erythrocyte surface. As glucose withdrawal enhances the formation of cysteinyl-leukotrienes, the effects participate in the signaling of eryptosis during energy depletion. Accordingly, antagonizing the cysteinyl-leukotriene receptor CysLT1 by cinalukast or inhibition of the 5-lipoxygenase by BW B70C blunt the eryptosis following glucose withdrawal and energy depletion.

The increase in phosphatidylserine exposure is the result of Ca^{2+}-sensitive scrambling of the cell membrane (Berg, Engels et al. 2001; Bratosin, Estaquier et al. 2001) and of caspase activation, the cell shrinkage due to activation of Ca^{2+}-sensitive K^+ channels with subsequent K^+ exit, hyperpolarization, and exit of Cl^- together with osmotically obliged water, thus eventually leading to cell shrinkage (Lang, Kaiser et al. 2003). The cell shrinkage then contributes to the triggering of scrambling of the cell membrane (Schneider, Nicolay et al. 2007). In addition to cell membrane scrambling and cell shrinkage, increased cytosolic Ca^{2+} activity affects the architecture of the cytoskeleton (Takakuwa and Mohandas 1988; Nunomura, Takakuwa et al. 1997) and activates several enzymes such as transglutaminase (Anderson, Davis et al. 1977), phospholipases (Allan, Billah et al. 1976) , calpain (Anderson, Davis et al. 1977), protein kinases, and phosphatises (Cohen and Gascard 1992; Minetti, Piccinini et al. 1996). Calpain-dependent degradation of membrane proteins leads to membrane blebbing, a further hallmark of eryptosis (Berg, Engels et al. 2001; Bratosin, Estaquier et al. 2001).

Eryptosis eventually results in disposal of affected erythrocytes (Lang, Lang et al. 2006), as phosphatidylserine-exposing erythrocytes are engulfed by macrophages equipped with phosphatidylserine receptors (Boas, Forman et al. 1998) and thus eliminated from circulating blood (Kempe, Akel et al. 2007). Phosphatidylserine-exposing erythrocytes could further adhere to the vascular wall and thus compromise microcirculation (Closse, Dachary-Prigent et al. 1999). Suicidal erythrocytes have thus been proposed to participate in vascular injury of metabolic syndrome (Zappulla 2008). Leukotrienes are known to interfere with microcirculation, an effect mainly attributed to their effect on vascular smooth muscle cells (Hedqvist 1980; Haeggstrom and Wetterholm 2002). Along those lines, inhibition of leukotriene formation may counteract atherosclerosis (Jawien, Gajda et al. 2007; Jawien, Gajda et al. 2008).

Leukotrienes may not only be involved in the triggering of eryptosis by energy depletion. Phosphatidylserine exposure in erythrocytes could be elicited by ligation of several surface antigens, such as glycophorin-C (Head, Lee et al. 2005), the thrombospondin-1 receptor CD47 (Head, Lee et al. 2005), and the death receptor CD95/Fas (Mandal, Mazumder et al. 2005). Moreover, phosphatidylserine exposure or eryptosis is triggered by a wide variety of chemicals and drugs (Foller, Huber et al. 2008). Cell membrane scrambling is further stimulated by sepsis (Kempe, Akel et al. 2007), iron deficiency (Kempe, Lang et al. 2006), phosphate depletion (Birka, Lang et al. 2004), Hemolytic Uremic Syndrome (Lang, Beringer et al. 2006), malaria (Koka, Lang et al. 2008), Wilson's disease (Lang, Schenck et al. 2007), glucose-phosphate dehydrogenase deficiency (Lang, Roll et al. 2002) and hemoglobinopathies (Kuypers 2007) . Future studies shall reveal, to which extent leukotrienes participate in the respective signalling of eryptosis.

Leukotrienes may similarly affect survival of nucleated cells. Apoptotic cells may release leukotrienes (Freire-de-Lima, Xiao et al. 2006). Leukotriene D4 has been shown to induce apoptosis, an effect thought to be mediated by CysLT2 (Sheng, Li et al. 2006). Over expression of CysLT1 rather attenuated apoptosis of PC12 cells (Sheng, Li et al. 2006), and CysLT1 inhibition enhanced apoptosis of intestinal cells (Paruchuri, Mezhybovska et al. 2006). On the other hand, the CysLT1 inhibitor montelukast reversed leukocyte apoptosis in chronic renal failure (Sener, Sakarcan et al. 2007). The ant apoptotic effect of leukotriene D4 has been attributed to gene transcription (Mezhybovska, Wikstrom et al. 2006), which cannot apply in erythrocytes. In nucleated cells, Ca^{2+} signalling may indeed play a dual role. While Ca^{2+} oscillations stimulate cell proliferation and confer cell survival (Berridge 2005), sustained increases in cytosolic Ca^{2+} could trigger suicidal cell death (Orrenius, Zhivotovsky et al. 2003). The stimulation of Ca^{2+} entry in erythrocytes has uniformly been shown to stimulate suicidal erythrocyte death (Lang, Lang et al. 2005).

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10. ACKNOWLEDGEMENTS

This study was carried out at the Institute of Physiology, University of Tuebingen, Germany during 2006-2009. This research work was completed by a step by step progression, accompanied with hardships, encouragement, disappointment, confident and kind help and cooperation from a number of people. Although it is not enough to express my gratitude in words but I would like to express my heartfelt thanks to all of those people.

I would like to express my deepest sense of gratitude to Prof. Dr. Florian Lang for his constant inspiration, scholastic guidance, immense encouragement, valuable suggestion, timely and solitary instruction, cordial behavior, constructive criticism and providing all facilities for successful completion of the research work as well as preparation of this thesis.

I am highly obliged to Dr. Michael Föller, for his valuable advice, creative suggestion, co-operation, encouragement and wise criticism during my study.

Furthermore, I would like to thank Prof. Dr. Friedrich Götz, Institute of Microbial Genetics, University of Tübingen, supported me as my second supervisor very kindly.

I am extremely grateful to Dr. Stephan Hubar, Dr. Ferruh Artunc, Dr. Ekaterina Shumilina, Dr. Naima Zahir, Dr. Malgorzata Sobiesiak, Dr. Raja Biswas, Dr. Lalitha Biswas, Mr. Anand Rotte, Mrs. Madhuri Bhandaru, Dr. Rexhep Rexhepaj, Dr. Omaima Nasir and Dr. Daniela Kempe for their kind cooperation and critical suggestions for my research work.

I feel much pleasure to convey the profound thanks to my colleagues Mr. Syed M. Qadri, Mr. Matthias Eberhard, Mr. Diwakar Bobballa, Mrs. Xuan Nguyen Thi, Mr. Nguyen Than, Miss. Elisa Floride, Miss. Corinna Geiger, Mr. Manual Braun, Miss Vanessa Schnor, Mr. Daneale Mauro, Mr Sargios Gratidis, Miss. Shuchen Gu and Miss. Nicole Matzner for their support, suggestions, and encouragement.

Thanks also to the all staff in this institute, especially Mrs. Elfirade Faber, Mr. Uwe Schüller, Mr. Peter Durr, Mrs. Lajla subasiak, Mrs. Tanja Loch, Mrs. Sari Rübi and Mr. Faruk Subasic for their kind cooperation. I would like to thank everyone who helped me in this study and am grateful to all of them, although it is not possible to mention everyone by name.

Finally, I express my deepest appreciation to beloved parents, brothers and relatives whose inspiration and continuous blessing opened the gate and paved the way of my higher studies.

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