Studying mechanisms of eryptosis

Thomas Wieder¹, Philipp A. Lang¹, Karl S. Lang¹, Daniela S. Kempe¹,
Olivier M. Niemoeller¹, Christophe Duranton¹, Erich Gulbins²,
Stephan M. Huber¹ and Florian Lang^{1,3,*}

¹Department of Physiology, University of Tübingen, Germany; ²Institute of Molecular Biology, University of Duisburg-Essen, Essen, Germany; ³Physiologisches Institut, Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen, Germany; *Author for correspondence (e-mail: florian.lang@uni-tuebingen.de; phone: +49-7071-29-72194; fax: +49-7071-29-5618)

Received 7 December 2005; accepted in revised form 2 January 2006

Key words: Annexin, Apoptosis, Cell volume, Phosphatidylserine, Signaling

Abstract

Cellular stress leads to activation of erythrocyte cation channels with subsequent Ca^{2+} entry and stimulates a sphingomyelinase with subsequent formation of ceramide. Both signaling molecules then activate the death program of erythrocytes (eryptosis) which is characterized by phosphatidylserine exposure, cellular shrinkage, membrane blebbing and activation of death-inducing proteases. Some of the mediators accounting for activation of the erythrocyte death machinery, i. e. prostaglandin E_2 (PGE_2) and platelet activating factor (PAF), have been described and the respective signaling cascades disclosed. The present article outlines and discusses the methods which have been used to analyze erythrocyte death pathways. Furthermore, some of the pathophysiological implications of eryptosis signaling are delineated and the methods to screen for eryptosis defects in those conditions are presented. Needless to say that further research will be required to fully understand the mechanisms leading to suicidal red blood cell death and to elucidate the role of eryptosis during anemic complications.

Introduction

Cell injury of erythrocytes, such as osmotic shock, glucose depletion and oxidative stress, activates an amiloride-sensitive, Ca^{2+} -permeable cation channel (Duranton et al. 2002; K.S. Lang et al. 2003a). The subsequent elevation of intracellular Ca^{2+} activity triggers membrane blebbing, cellular shrinkage, activation of the Ca^{2+} -dependent protease μ -calpain and scrambling of the cell membrane with breakdown of phosphatidylserine asymmetry (Berg et al. 2001; Bratosin et al. 2001; K.S. Lang et al. 2003a; P.A. Lang et al. 2005b). Since the sequence of events resembles apoptosis of

nucleated cells (Green and Reed 1998; Gulbins et al. 2000), the term eryptosis was introduced to describe this particular kind of cell death (K.S. Lang et al. 2005). In contrast to nucleated cells (Essmann et al. 2000; Prokop et al. 2000; Wieder et al. 2001a, b; von Haefen et al. 2003), however, erythrocyte programmed cell death after most triggers of cell injury does not require caspase activation (Weil et al. 1998; Berg et al. 2001; K.S. Lang et al. 2004a), even though caspases are expressed in erythrocytes and activated by oxidative stress (Mandal et al. 2003, 2002). Thus, erythrocytes could be utilized as a 'stripped down' cellular system to investigate caspase-independent suicide mechanisms.

Ca^{2+} entry into erythrocytes is mainly regulated by three distinct mechanisms: (i) by protein kinase C-dependent pathways leading to activation of Ca^{2+} fluxes across the membrane (Andrews and Low 1999; Yang et al. 2000), (ii) by phospholipase A_2 -mediated PGE_2 signaling leading to activation of the erythrocyte cation channels (Kaestner et al. 2004; P.A. Lang et al. 2005b) and (iii) by erythropoietin-mediated inhibition of the cation channels (Myssina et al. 2003).

In addition to the Ca^{2+} -dependent signaling cascades, eryptosis is regulated by ceramide (K.S. Lang et al. 2004a), shown to be involved in apoptosis of several cell types (Frago et al. 1998; Goldkorn et al. 1998; Raisova et al. 2000; von Haefen et al. 2002; Kolesnick and Fuks 2003; Ogretmen and Hannun 2004). Ceramide is generated from membrane phospholipids by a shrinkage-dependent sphingomyelinase and sensitizes the erythrocytes for cytosolic Ca^{2+} thereby triggering scrambling of the cell membrane even in the absence of Ca^{2+} entry (K.S. Lang et al. 2004a). Further detailed investigations revealed that erythrocyte ceramide formation and cell membrane scrambling is governed by platelet activating factor (PAF) via a PAF receptor-dependent mechanism (P.A. Lang et al. 2005c). Along those lines, PAF, a phospholipid mediator involved in the regulation of inflammation, thrombosis, atherogenesis and cardiovascular function (Bussolino et al. 1989; Montrucchio et al. 1993, 2000; Subbanagounder et al. 1999; Haynes and Obiako 2002; Zimmerman et al. 2002; Goggel et al. 2004) is released from erythrocyte progenitor cells upon increase of cytosolic Ca^{2+} activity (Dupuis et al. 1997). PAF further activates Ca^{2+} -sensitive K^+ channels (Gardos channels) in the erythrocyte cell membrane (Garay and Braquet 1986) by sensitising them for the stimulating effects of cytosolic Ca^{2+} (Rivera et al. 2002). Thus, PAF is involved in the regulation of two hallmarks of eryptosis, namely K^+ -mediated shrinkage and ceramide-mediated phospholipid scrambling.

The scrambling of the cell membrane following increase of cytosolic Ca^{2+} -activity (Woon et al. 1999) or ceramide formation (K.S. Lang et al. 2004a) leads to phosphatidylserine exposure at the erythrocyte surface which may either facilitate cell-cell interactions and the formation of cell aggregates (Eda and Sherman 2002; K.S. Lang et al. 2004b) or lead to accelerated clearance of

damaged erythrocytes by macrophages which are equipped with receptors specific for phosphatidylserine (Fadok et al. 2000).

Erythrocytes can be easily obtained at high purity and in large amounts by standard purification procedures. In contrast to previous assumptions, erythrocytes still contain the molecular machinery to regulate different intracellular signaling pathways on a posttranslational level, e.g. phospholipase-mediated, receptor-triggered and kinase-dependent pathways (Barvitenko et al. 2005; K.S. Lang et al. 2005). Here, we present and discuss the experimental procedures that shed light on the suicide machinery of this organelle-free cell type. Due to their good reproducibility and high throughput, we focus on flow cytometric measurements to screen for effects of different stressors on erythrocyte membrane asymmetry (annexin binding assay), cell volume (forward scatter), Ca^{2+} concentration (Fluo-3-dependent fluorescence) and cellular ceramide levels (antibody-based determination). The methods are complemented by more sophisticated biochemical analyses, e. g. enzymatic determination of ceramide, breakdown of metabolically labeled sphingomyelin, Western blotting, and patch clamp techniques for electrophysiological analysis.

Materials

Equipments

Automated blood cell counter CellDyn3000: Abbott GmbH¹
 Coulter Epics XL: Beckman Coulter²
 FACS-Calibur: Becton Dickinson³
 MDM 905 electronic hematology particle counter: Medical Diagnostics Marx⁴
 VAPRO 5520 vapor pressure osmometer: Wescor⁵
 Polyacrylamide gelelectrophoresis (PAGE) unit: Biorad⁶

¹Abbott GmbH: Wiesbaden, Germany

²Beckman Coulter: Krefeld, Germany

³Becton Dickinson: Heidelberg, Germany

⁴Medical Diagnostics Marx: Butzbach, Germany

⁵Wescor: Logan, Utah, USA

⁶Biorad: München, Germany

β-Scintillation counter: Wallac⁷
 Microplate reader Sunrise: Tecan⁸
 Microprocessor-driven DMZ puller: Zeitz⁹
 MS314 electrical micromanipulator: MW, Märzhäuser¹⁰
 EPC-9 amplifier: Heka¹¹
 ITC-16 Interface: Instrutech¹²

Labware

OptiPure RC quadruple blood pack set (#HGR8484B): Baxter¹³
 Fetal calf serum (FCS; #S0115 (500 ml)): Biochrom AG¹⁴
 Protran BA83 nitrocellulose membranes (#10402426): Schleicher und Schuell¹⁵
 Silica G60 thin layer chromatography plates (#034.5721 (20×20 cm)): Merck Biosciences GmbH¹⁶
 Borosilicate glass pipettes (GC 150 TF-10; #30-0068): Clark Medical Instruments¹⁷

Chemicals

Percoll (#L6135): Biochrom AG¹⁴
 TruCount kit (#340334), Retic-COUNT (Thiazole orange) reagent (#349204): Becton Dickinson³
 2-Deoxyglucose (#D6134), ionomycin (#I0634), arachidonic acid (#A9673), amiloride (#A7410), ethylisopropylamiloride (EIPA; #A3085), quina-crine (#22530), acetylsalicylic acid (#A5376), diclofenac (#D6899), calpain inhibitor I (#A6185), calpain inhibitor II (#A6060), prostaglandin E₂ (PGE₂; #P6532), platelet activating factor (PAF18;

#P6537), thromboxane B₂ (#T0516), purified sphingomyelinase from *Staphylococcus aureus* (#S8633), bovine serum albumin (BSA; #A7906), *n*-octyl glucopyranoside (#O0630), cardiolipin (#21979), hemolysin Kanagawa from *Vibrio parahaemolyticus* (no longer available), clotrimazole (#C6019), inosine (#I4125), sodium orthovanadate (#S6508): Sigma¹⁸

PACOCF3 (#506274) and AACOCF3 (#100109): Merck Biosciences GmbH¹⁶

Platelet activating factor (PAF16; #511075), Fluo-3/AM (#343242): Calbiochem¹⁹

Annexin-V-Fluos (#11988549001), cocktail of protease inhibitors composed of 2.5 mM ethylenediamine tetraacetic acid (EDTA), 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (#04693124001): Roche Diagnostics²⁰

Anti-ceramide antibody, clone MID 15B4 (#ALX-804-196-T050): Alexis²¹

Isotype-matched pure mouse IgM antibody, clone TEPC 183 (#290-010): Ancell²²

Polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM specific antibody (#555988): Pharmingen²³

Bradford protein assay kit (#500-0201): Biorad⁶

Polyclonal goat anti-PAF receptor antibody directed against the N-terminus of the human PAF receptor (PAF-R (N-17); #sc-8741), secondary peroxidase-conjugated donkey anti-goat antibody (#sc-2020): Santa Cruz Biotechnology²⁴

Enhanced chemi-luminescence ECL kit (#RPN 2109), sheep anti-mouse antibody conjugated with horseradish peroxidase (#RPN4201), PAF [³H] Scintillation Proximity Assay (SPA) System (#TRK990), diacylglycerol (DAG)-kinase (no longer available), [³²P]γATP (#PB10168-250UCI (250 μCi)), [*methyl*-³H]choline chloride (#TRK593-1MCI (1 mCi)): Amersham Biosciences²⁵

Monoclonal mouse anti-human CD61 antibody, clone PM6/13 (#MCA728GA): Serotec²⁶

⁷Wallac: Freiburg, Germany

⁸Tecan: Crailsheim, Germany

⁹Zeitz: Augsburg, Germany

¹⁰MW, Märzhäuser: Wetzlar, Germany

¹¹Heka: Lambrecht, Germany

¹²Instrutech: Port Washington, NY, USA

¹³Baxter: Unterschleißheim, Germany

¹⁴Biochrom AG: Berlin, Germany

¹⁵Schleicher und Schuell: Dassel, Germany

¹⁶Merck Biosciences GmbH: Bad Soden, Germany

¹⁷Clark Medical Instruments: Pangbourne, UK

¹⁸Sigma: Taufkirchen, Germany

¹⁹Calbiochem: Schwalbach, Germany

²⁰Roche Diagnostics: Mannheim, Germany

²¹Alexis: Grünberg, Germany

²²Ancell: Bayport, MN, USA

²³Pharmingen: Hamburg, Germany

²⁴Santa Cruz Biotechnology: Santa Cruz, CA, USA

²⁵Amersham Biosciences: Freiburg, Germany

²⁶Serotec: Oxford, United Kingdom

Correlate-EIATM Prostaglandin E₂ Enzyme Immunoassay Kit (#901-001): Assay Designs, Inc.²⁷

C₁₆-ceramide (#CAY-62530-M005): Biozol²⁸
⁴⁵Ca²⁺ as CaCl₂ in aqueous solution (specific activity: 0.185–1.11 TBq/g Ca; #016200501): ICN Biomedicals GmbH²⁹

Procedures

Blood cells, purification of erythrocytes and determination of blood cell numbers

For erythrocyte signaling experiments, highly purified erythrocyte concentrates should be used. Human whole blood is drawn from healthy volunteers and erythrocyte concentrates are obtained by filtration (P.A. Lang et al. 2005c). In any case, the purity of the erythrocyte concentrates has to be measured by different automated procedures as outlined below.

Human whole blood is drawn from healthy volunteers and erythrocyte concentrates are obtained using the OptiPure RC quadruple blood pack set equipped with a soft housing red cell filter. About 500 ml of whole blood are automatically mixed with 70 ml CPD buffer containing 3.27 mg/ml citric acid, 26.30 mg/ml sodium citrate, 2.50 mg/ml sodium dihydrogenphosphate dihydrate and 25.50 mg/ml dextrose monohydrate. Blood components are separated by centrifugation at 4795 g for 10 min at 22 °C. Plasma, buffy coat and erythrocytes are then pressed into the respective blood bags. During this process SAG-M stabilizer solution containing 8.77 mg/ml NaCl, 9.00 mg/ml dextrose monohydrate, 0.17 mg/ml adenine and 5.25 mg/ml mannitol is added to the erythrocytes and the erythrocytes are passed through the integrated leukocyte depletion filter at room temperature. Aliquots of the erythrocyte concentrates are stored at 4 °C until usage.

Alternatively (e.g. for Western blot analyses or in animal experiments), erythrocytes are purified by centrifugation of whole blood for 25 min,

2000 g over Percoll, washed 3 times in phosphate buffered saline (PBS) and centrifuged at 450 g for 5 min. Purified erythrocytes should be immediately used for experimental purposes.

Platelet numbers in whole blood and in erythrocyte concentrates as well as leukocytes in whole blood are measured using an automated blood cell counter (CellDyn3000). The cells are identified by a combination of their optical and electrical properties in the appropriate counter medium. Representative erythrocyte concentrates should not contain more than 3% of the original platelet number of the respective whole blood samples. Furthermore, platelet numbers in whole blood and in erythrocyte concentrates are determined by flow cytometric analysis of thiazole orange-stained cells using the Retic-COUNT (Thiazole orange) reagent according to the manufacturer's instructions. Measurements are performed on a FACS-Calibur and the number of cells in the thrombocyte gate of the respective forward scatter (FSC) versus thiazole orange-fluorescence intensity (FL-1H) dot plots is determined using the CellQuestTM software.

Leukocyte numbers in erythrocyte concentrates are quantified by flow cytometric analysis on a Coulter Epics XL using the internally normalized TrueCount kit. The kit is based on the detection of nucleated cells by the fluorescent DNA-intercalator propidium iodide. Erythrocyte concentrates should contain less than 0.1% of the original leukocyte number of the respective whole blood samples.

Erythrocytes, platelets and leukocytes of whole blood and erythrocyte concentrates are further quantified by the use of a MDM 905 electronic hematology particle counter.

For determination of relative reticulocyte numbers, 5 µl of whole blood are added to 1 ml Retic-COUNT (Thiazole orange) reagent. Samples are stained for 30 min at room temperature in the dark. Then, FACS analysis of stained samples is performed according to the manufacturer's instructions. Forward scatter (FSC), side scatter (SSC) and thiazole orange-fluorescence intensity (in the fluorescence channel FL-1) of the blood cells are measured and the percentage of Retic-COUNT positive, gated erythrocytes is calculated. Gating of erythrocytes is achieved by analysis of FSC vs. SSC dot plots using the CellQuestTM software.

²⁷ Assay Designs, Inc.: Ann Arbor, MI, USA

²⁸ Biozol: Eching, Germany

²⁹ ICN Biomedicals GmbH: Eschwege, Germany

Eryptosis in mice: the use of knockout models and dietary manipulation

Erythrocytes are devoid of nuclei and therefore direct genetic manipulation of signaling pathways is not possible. However, as we have shown previously, mouse erythrocytes likewise undergo Ca^{2+} -dependent programmed cell death (Birka et al. 2004; P.A. Lang et al. 2003b) and may serve as a model system for human eryptosis. Thus, the use of specific genetic knockout models (e.g. PAF receptor knockout (PAF-R $-/-$) mice and the corresponding PAF receptor wild type (PAF-R $+/+$ littermates) enables us to specifically target distinct parts of the death signaling pathway (P.A. Lang et al. 2005c). Furthermore, animals (mice or rats) can be fed special diets to investigate the influence of different dietary deficiencies (Birka et al. 2004). Animal experiments have to be performed according to international and local regulations and need to be approved by the local authorities. Murine erythrocytes are drawn by retroorbital venopuncture and used after purification over Percoll.

Solutions

In vitro experiments are performed at 37 °C in standard Ringer solution in the presence or absence of specific effector molecules, such as PAF (P.A. Lang et al. 2005c), PGE_2 (P.A. Lang et al. 2005b), erythropoietin (Myssina et al. 2003) phorbol ester (Andrews et al. 2002) etc. In addition, erythrocytes are stressed by increasing or decreasing osmolarity of the standard Ringer solution (K.S. Lang et al. 2003a), by removal of glucose from the standard Ringer solution (K.S. Lang et al. 2003a) or by replacing Cl^- ions by gluconate (P.A. Lang et al. 2005b). Eryptosis can also be induced by treatment of erythrocytes with low concentrations of heavy metals, i.e. lead (Kempe et al. 2005) or mercury ions (Eisele et al. 2005). To investigate the role of eryptosis in the development of anemic conditions, erythrocytes from patients suffering from genetic disorders, e.g. sickle cell anemia, thalassemia or glucose-6-phosphate dehydrogenase deficiency (K.S. Lang et al. 2002), are tested *in vitro* in the experimental set ups described above and compared with erythrocytes from healthy volunteers.

The Ringer solution contains (in mM): 125 NaCl, 5 KCl, 1 MgSO_4 , 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)/NaOH, 5 glucose, 1 CaCl_2 ; pH 7.4. In some experiments, osmolarity is increased to 850 mOsm by adding sucrose. Osmolarity is measured by the use of a VAPRO 5520 vapor pressure osmometer. Furthermore, glucose is removed from the standard Ringer solution to achieve energy depletion. The effect of glucose removal is enhanced by supplementing glucose-free Ringer solution with 5 mM 2-deoxyglucose which efficiently blocks glycolytic ATP synthesis. Cl^- -free Ringer solutions are composed of (in mM): 125 Na-D-glucuronate, 5 K-D-gluconate, 1 MgSO_4 , 32 HEPES/NaOH, 5 glucose, 1 Ca-gluconate₂; pH 7.4.

The Ca^{2+} ionophore ionomycin (1 μM), the cation channel inhibitor EIPA (10 μM), phospholipase A_2 inhibitors quinacrine (25 μM), palmitoyltrifluoromethyl ketone (PACOCF₃; 1–4 μM) or arachidonyltrifluoromethyl ketone (AACOCF₃; 8 μM), cyclooxygenase inhibitors acetylsalicylic acid (50 μM) or diclophenac (10 μM), prostaglandin PGE_2 (0.1 nM–50 μM), 1-*O*-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF16; 100 nM–4 μM), 1-*O*-octadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF18; 100 nM–4 μM), calpain inhibitor I (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; 177 μM) and calpain inhibitor II (*N*-acetyl-L-leucyl-L-leucyl-L-methioninal; 70 μM), arachidonic acid (1 μM) and thromboxane B_2 (5–50 μM) could be added to modify eryptosis. The substances are dissolved in final concentrations of 0.1% dimethyl sulfoxide (DMSO) (ionomycin, EIPA, quinacrine, acetylsalicylic acid, diclophenac, PACOCF₃, AACOCF₃, PAF16, PAF18) or of 0.5% ethanol (PGE_2 , thromboxane B_2 , calpain inhibitors I and II). For appropriate controls, it is mandatory to incubate erythrocytes from the same donor in Ringer solution containing the same concentration of solvent.

Determination of phosphatidylserine exposure, cell volume, cellular ceramide levels and intracellular Ca^{2+} concentration by flow cytometry

Fluorescence activated cell sorting (FACS) analysis (Andree et al. 1990) is performed with some modifications to adapt the method to erythrocyte

research (K.S. Lang et al. 2002, 2003a). Erythrocyte concentrates (diluted to 0.3% hematocrit) are treated with different agonists or antagonists. For determination of phosphatidylserine exposure, the cells are washed in annexin-binding buffer containing (in mM): 125 NaCl, 10 HEPES; pH 7.4 and 5 CaCl₂. Erythrocytes are stained with AnnexinV-Fluos at a 1:100 dilution. After 15 min, samples are diluted 1:5, thoroughly mixed on a vortex mixer to achieve single cell suspensions, and measured by flow cytometric analysis on a FACS-Calibur (see Figure 1a). AnnexinV-fluorescence intensity is measured in the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

To determine the cell volume of stressed erythrocytes, the forward scatter of unstained cells is measured and analyzed on a FACS-Calibur (see Figure 1b) (K.S. Lang et al. 2003a). In case that the cells have been incubated in standard Ringer solution in the presence or absence of different effector molecules, forward scatter analysis can also be accomplished with Annexin V-labelled erythrocytes.

For determination of ceramide, a monoclonal antibody-based assay is used which has been validated earlier (Grassme et al. 2002; Bieberich et al. 2003). After incubation, cells are stained for 1 hour at 4 °C with 1 µg/ml anti-ceramide antibody or 1 µg/ml isotype-matched pure mouse IgM antibody in PBS containing 1% fetal calf serum (FCS) at a dilution of 1:5 (K.S. Lang et al. 2004a). After three washes with PBS/1% FCS, cells are stained with polyclonal fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG + IgM specific antibody in PBS/1% FCS at a dilution of 1:50 for 30 min. Unbound secondary antibody is removed by repeated washing with PBS/1% FCS. The samples are analyzed by flow cytometric analysis on a FACS-Calibur. Mean values of FITC-fluorescence intensity in the fluorescence channel FL-1 are determined using the CellQuestTM software. Isotype matched pure mouse antibody should not display increased fluorescence intensity in FL-1 and 1 U/ml purified sphingomyelinase from *Streptomyces* sp. for 5 min should be used as a positive control.

Intracellular Ca²⁺ measurements are performed using the Ca²⁺-dependent fluorescence dye Fluo-3/AM (Andrews et al. 2002). For this, 10 ml erythrocyte suspension (0.16% hematocrit) are

loaded with Fluo-3/AM by addition of 10 µl of a Fluo-3/AM stock solution (2.0 mM in DMSO). After incubation at 37 °C for 15 min under vigorous shaking and protection from light, an additional 10 µl of Fluo-3/AM is added, with incubation carried out for 25 min. Fluo-3/AM-loaded erythrocytes are centrifuged at 1000 *g* for 3 min at 22 °C and washed twice with Ringer solution containing 0.5% bovine serum albumin (BSA) and once with Ringer. Fluo-3/AM-loaded erythrocytes (0.32% hematocrit) are treated with the respective agonist or with 1 µM ionomycin as a positive control to increase intracellular Ca²⁺ activity. After incubation, cells are analyzed by flow cytometric analysis on a FACS-Calibur and Ca²⁺-dependent fluorescence intensity is measured in the fluorescence channel FL-1 (see Figure 1c).

Human red blood cell (RBC) membrane preparation and Western blotting

Expression of different receptors, protein kinases or proteases is determined by Western blot analysis (Wieder et al. 2001a). Before analysis, erythrocyte preparations should be checked for platelet and white blood cell contaminations, for example by the use of an electronic hematology particle counter (MDM 905). Purified erythrocyte preparations should contain less than 3–5% contaminating cells as compared with the platelet counts and white blood cell counts of the respective, original whole blood samples. To remove the hemoglobin, 200 µl of the erythrocyte pellet are hemolyzed in 50 ml of 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors composed of 2.5 mM ethylenediamine tetraacetic acid (EDTA), 10 µg/ml pepstatin A, 10 µg/ml leupeptin, 5 µg/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Ghost membranes are pelleted (15,000 rpm for 20 min at 4 °C) and solubilized in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.3), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholic acid, 1% Triton-X-100, and 10 µl β-mercaptoethanol. The protein concentration of the samples is determined with the Bradford method with BSA as standard. Lysates are separated by 10% SDS-PAGE (50 µg protein per lane), and transferred to Protan BA83 nitrocellulose membranes. Protein transfer is controlled

Figure 1. Flow cytometric analysis of phosphatidylserine exposure (a), cell volume (b), and intracellular Ca^{2+} concentration (c) in ionomycin-treated, human erythrocytes. (a) Original histograms of Annexin-V binding of erythrocytes treated for 1 h with vehicle-containing Ringer (left histogram) or ionomycin-containing Ringer (1 μ M; right histogram). The numbers depict the percentage of annexin-V-positive cells. (b) Original histograms of forward scatter of erythrocytes treated for 1 h with vehicle-containing Ringer (left histogram) or ionomycin-containing Ringer (1 μ M; right histogram). The numbers depict the geo mean of the forward scatter of the respective erythrocyte population. (c) Original histogram overlay of Ca^{2+} -dependent fluorescence of Fluo3-labelled erythrocytes treated for 1 h with vehicle-containing Ringer (black line) or ionomycin-containing Ringer (1 μ M; red line). (d) Means $\pm$ SEM (n = 4) of annexin-V positive cells in % (left columns), of the forward scatter geo mean (middle columns) or of Fluo3-dependent fluorescence (right columns) after treatment of human erythrocytes for 1 h with Ringer solution (R; open columns) or 1 μ M ionomycin (I; closed columns).

by Ponceau red staining. After blocking with 5% nonfat-dried milk at room temperature for 1 h, the blots are probed overnight at 4 $^{\circ}$ C with a specific primary antibody against the protein in question (e.g. polyclonal goat anti-PAF receptor antibody directed against the N-terminus of the human PAF receptor (PAF-R (N-17)) at 1:200 dilution. The blots are incubated overnight in the presence or absence of 10 μ g/ml specific blocking peptides. After washing, the blots are incubated with a secondary peroxidase-conjugated antibody, e.g. a donkey anti-goat antibody (at 1:2000 dilution) for 1 h at room temperature. Antibody binding is detected with the enhanced chemiluminescence

ECL kit. To test for contamination of the erythrocyte membrane preparations by platelets, Western blot analysis is performed in parallel using a monoclonal mouse anti-human CD61 antibody at 1:200 dilution combined with a sheep anti-mouse antibody conjugated with horseradish peroxidase at 1:1000 dilution.

Determination of phospholipase A_2 (PLA_2)-derived signaling molecules after cellular stress

Membrane as well as cytosolic PLA_2 isoenzymes are expressed in mature erythrocytes (MacDonald

et al. 2004; Zahler and Kramer 1981). Thus, PLA₂-mediated signaling pathways are good candidates to regulate eryptosis. To unravel PLA₂ signaling in erythrocytes, cells are treated with hypertonic Ringer solution (850 mOsm) or other stressors, or isotonic Ringer solution (300 mOsm) as control. After incubation, lipids are extracted by a modified method of Bligh and Dyer (K.S. Lang et al. 2004a). Lipids are transferred to scintillation vials and dried under nitrogen. Then, the lipid-derived signaling molecule PAF is determined using the PAF [³H] Scintillation Proximity Assay (SPA) system based on the competition of radioactive [³H]PAF with non-radioactive PAF for binding to a specific anti-PAF antibody coupled to fluomicrospheres (P.A. Lang et al. 2005c). In detail, one hundred million erythrocytes are treated with 90 μ l hypertonic Ringer solution (850 mOsm) or isotonic Ringer solution (300 mOsm) as control. After incubation, lipids are extracted by addition of 250 μ l methanol and 125 μ l chloroform to the erythrocyte suspension. Samples are stirred on a vortex mixer and centrifuged at 13,000 g for 2 min. Phase separation is accomplished by the addition of 125 μ l chloroform and 125 μ l water. The suspension and centrifugation steps are repeated, and 100 μ l of the chloroform phases are transferred to scintillation vials and dried under nitrogen. Then, PAF is determined using the PAF [³H] Scintillation Proximity Assay (SPA) system according to the manufacturer's instructions. [³H]PAF bound to the fluomicrospheres is counted in a β -scintillation counter and the concentration of PAF in the samples is calculated from a PAF calibration curve (20–1280 pg/tube) which has been run in parallel.

For determination of PGE₂ release following osmotic shock, erythrocytes are incubated in hypertonic Ringer solution (850 mOsm) or in the presence of other stressors and subsequently pelleted. PGE₂ is determined in the supernatant. In detail, one billion erythrocytes are treated with 0.9 ml hypertonic NaCl Ringer solution (850 mOsm) in the absence or presence of Ca²⁺ (1 mM), with hypertonic NaCl Ringer solution (850 mOsm) in the absence or presence of 25 μ M PLA₂ inhibitor quinaquine, with isotonic Na-glucuronate solution (0 mM Cl⁻), with ionomycin (1 μ M) or with isotonic NaCl Ringer solution (300 mOsm) as control. After incubation, cells are

pelleted by centrifugation at 4 °C, 450 g for 5 min. The supernatant is removed and stored at -20 °C until usage. PGE₂ concentrations in the supernatant are then determined using the Correlate-EIATM Prostaglandin E₂ Enzyme Immunoassay Kit according to the manufacturer's instructions. The samples are diluted 1:2.5 with assay buffer. Then, 100 μ l sample, 50 μ l alkaline phosphatase PGE₂ conjugate and 50 μ l monoclonal anti-PGE₂ EIA antibody are applied to goat anti-mouse IgG microtiter plates and incubated at room temperature for 2 h. After washing, 200 μ l *p*-nitrophenyl phosphate substrate solution is added and incubated at room temperature for 45 min. Finally, the optical density at 405 nm is measured in a microplate reader. PGE₂ concentrations in the samples are calculated from a PGE₂ standard curve (39.1–5000 pg/ml) which is run in parallel. PGE₂ levels in the supernatants of control (Ringer-treated) erythrocytes should be in the range of 100 pg per 10⁹ cells and are set as 100%. PGE₂ levels in the samples of erythrocytes exposed to hyperosmotic Ringer, Cl⁻-free extracellular fluid or ionomycin are calculated as % of the appropriate controls.

Biochemical determination of cellular ceramide using diacylglycerol (DAG)-kinase

In some cases (e.g. in mouse erythrocytes or when human erythrocytes are stimulated with antibodies), it is not possible to determine ceramide levels by the antibody-based flow cytometric method. Furthermore, absolute ceramide levels cannot be measured by this technique. We therefore established a biochemical method to confirm the data from FACS analysis. After stimulation of erythrocytes, the samples are extracted in chloroform:methanol:1N HCl (100:100:1), the lower phase is collected and dried. Diacylglycerol is then degraded by alkaline hydrolysis of the samples in 100 μ l of 0.1 N methanolic KOH at 37 °C for 60 min. The samples are re-extracted, dried and resuspended in 20 μ l of detergent solution (7.5% (w/v) *n*-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DETAPAC)). The samples are then sonicated for 10 min in a bath sonicator and the kinase reaction is initiated by addition of 70 μ l reaction mix consisting of 10 μ l DAG-kinase (in 5 mM potassium phosphate buffer (pH 7.0), 10% glycerol, 1 mM

2-mercaptoethanol, 0.005 M imidazole/HCl, 0.5 mM DETAPAC (pH 6.6), 50 μ l assay buffer (0.1 M imidazole/HCl (pH 6.6), 0.1 M NaCl, 25 mM MgCl₂ and 2 mM EGTA), 2.8 mM dithiothreitol (DTT), 5 μ M ATP and 10 μ Ci [³²P] γ -ATP. The kinase reaction is performed for 30 min at room temperature. The reaction is terminated by addition of 1 ml of chloroform: methanol: 1 N HCl (100:100:1). About 170 μ l of a buffered saline solution (135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES pH 7.2) and 30 μ l of a 100 mM EDTA-solution are added. The samples are extracted, the lower phases collected, dried, dissolved in 20 μ l of chloroform: methanol (1:1) and separated on Silica G60 thin layer chromatography plates with chloroform: methanol: acetic acid (65:15:5). The plate is exposed, ceramide identified by co-migration with an identical standard, scraped from the plate and quantified by liquid scintillation counting. The amount of ceramide is determined by comparison with a ceramide standard curve using C₁₆-ceramide.

Determination of the sphingomyelin cycle

To determine the origin of elevated ceramide levels, erythrocytes are metabolically labelled using [*methyl*-³H]choline chloride (K.S. Lang et al. 2004a). After labelling, the cells are washed and lipids are extracted. Then, sphingomyelin is quantified using bacterial sphingomyelinase to release [³H]phosphocholine (Jayadev et al. 1994). In detail, cells are labelled by incubation of erythrocyte concentrates (10% hematocrit) for 72 h in the presence of 7.4×10^4 Bq/ml [*methyl*-³H]choline chloride. After labelling, cells are washed twice with Ringer and reseeded at 2×10^8 cells/ml in control Ringer solution or in Ringer solution containing different cellular stressors. After incubation, cells are washed twice using 1 ml of Ringer solution. Lipids are extracted by the modified method of Bligh and Dyer as described above. About 20 μ l of the chloroform phases are taken for scintillation counting, and 50 μ l of the chloroform phases are dried under nitrogen and used for sphingomyelin measurements. To this end, cellular lipids are resuspended in 100 μ l assay buffer (100 mM Tris-HCl, pH 7.4, 6 mM MgCl₂, 0.1% Triton X-100). Samples are sonicated and 1 U/ml

sphingomyelinase from *streptomyces* sp. is added. Reaction mixtures are incubated for 2 h at 37 °C. Reactions are stopped by addition of 1.0 ml chloroform/methanol (2:1, v/v). Phase separation is completed by addition of 100 μ l water. Sphingomyelin is quantified by counting the upper, aqueous phase, containing the liberated [³H]phosphocholine, and phosphatidylcholine is quantified by drying and counting the lower, organic phase in a β -scintillation counter. Where appropriate, sphingomyelin is normalized using phosphatidylcholine measurements. Blank reactions contain no sphingomyelinase. The radioactivity of control samples normally reaches approx. 1800 dpm/10⁹ cells and is set as 100%. Subsequently, the sphingomyelin concentration in the samples of stressed cells is calculated as % of control. Optimization studies have illustrated that the above conditions yielded maximal sphingomyelin hydrolysis (100%).

Patch clamp

To study the functional significance of cation channels during eryptosis, the patch clamp technique has been adapted to mature red blood cells (Huber et al. 2001). Patch clamp experiments are performed in voltage-clamp, fast whole-cell mode according to Hamill et al. (Hamill et al. 1981) as outlined in detail below. In human and mouse erythrocytes, several (e.g. K⁺-, nonselective cation-, and Cl⁻-selective) whole-cell conductances have been defined and their role for eryptosis has been characterized (for review see Huber et al. 2005).

The erythrocytes are sedimented on a plastic cell culture dish mounted on an inverted microscope. The cells are continuously superfused with NaCl Ringer solution through a flow system inserted into the dish. The bath is grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes manufactured by a microprocessor-driven DMZ puller are used in combination with a MS314 electrical micromanipulator. The currents are recorded by an EPC-9 amplifier using Pulse software (Heka) and an ITC-16 Interface. The holding potential is set to -30 mV which induces and stabilizes the seal resistances in the range of 10 gigohms. Voltage-dependent sealing may take minutes. Seal formation may be improved by bath

solutions containing high concentrations (5–10 mM) of Ca^{2+} and Mg^{2+} (for instance a solution designed by Desai et al. (2000)). Electrical access to the whole plasma membrane is accomplished by short negative pressure pulses applied to the pipette lumen optionally combined with 500 mV/150 μs voltage pulses. Whole cell recording mode is indicated by a minute increase of the capacitive current transients and simultaneous bleaching of the recorded erythrocyte. Whole cell currents are determined during successive square pulses to voltages between –100 mV and +80 mV (+100 mV).

In erythrocytes, whole-cell recording seal resistances may be highly voltage-dependent. As a consequence, leak currents which exhibit a slight cation-selectivity may become rectifying and time-dependent activating/inactivating and, thus, be misinterpreted as biological currents. To distinguish leak currents from currents through nonselective cation channels, inhibitors such as amiloride (Gamper et al. 2000), GdCl_3 (Duranton et al. 2002), or ethylisopropylamiloride (K.S. Lang et al. 2003b; P.A. Lang et al. 2005b) may be used. In addition, substitution of Na^+ by the impermeant cation N-methyl-D-glucamine $^+$ (NMDG $^+$) results in an about 50 mV shift of the reversal potential of cation channel-generated currents which is paralleled by a dramatic decrease of inward current (P.A. Lang et al. 2005b). Leak currents, in contrast, shift only by some 10 mV and exhibit only small current declines upon bath cation substitution.

The nonselective cation whole-cell conductance of erythrocytes is Ca^{2+} -permeable, exhibits a permselectivity for monovalent cations of $\text{Cs}^+ > \text{K}^+ > \text{Na}^+ = \text{Li}^+ \gg \text{NMDG}^+$ (Duranton et al. 2002) and is activated by removal of intracellular or extracellular Cl^- (Huber et al. 2001; Duranton et al. 2002; Duranton et al. 2003), oxidative stress (Duranton et al. 2002), hyperosmotic cell shrinkage (Huber et al. 2001; K.S. Lang et al. 2003a) and PGE_2 (P.A. Lang et al. 2005b) (see also Figure 2). The nonselective cation currents are inhibited by hypoosmotic cell swelling (Huber et al. 2001), high intra- and extracellular Cl^- concentrations (IC_{50} of about 27 mM extracellular Cl^-) (Huber et al. 2001; Duranton et al. 2002), erythropoietin (Myssina et al. 2003) and catecholamines (P.A. Lang et al. 2005a).

To measure the nonselective cation currents, a Na^+ -containing pipette solution with reduced Cl^- concentration is used in our laboratory (115 mM Na-D-gluconate, 5 mM MgCl_2 , 5 mM HEPES/NaOH, 1 mM Mg-ATP, 1 mM EGTA; pH 7.4). In combination with NaCl Ringer solution the Cl^- equilibrium potential is at high negative voltages, which facilitates the discrimination between cation and Cl^- currents. The latter can be elicited in erythrocytes by *Plasmodium* infection (Desai et al. 2000), oxidative stress (Huber et al. 2002), cell swelling (Huber et al. 2004), and protein kinase A (Egee et al. 2002; Verlooy et al. 2004).

To measure Gardos K^+ channel activity (Egee et al. 2002; P.A. Lang et al. 2003a) erythrocytes are recorded with K-gluconate/KCl pipette solu-

Figure 2. The non selective cation channels of human erythrocytes as analyzed by whole-cell patch-clamp recording. Current traces recorded with KCl/K-gluconate pipette and Standard NaCl Ringer bath solution (as indicated) before (control), during (PGE_2), and after (wash-out) bath application of prostaglandin E_2 (PGE_2 ; 100 nM). The currents are evoked by 10 successive square-pulses to voltages between –100 mV and +80 mV (delivered in 20 mV increments). In the depicted traces the currents of the individual square pulses are superimposed and the zero current indicated by the grey line. The inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face (or the opposite movement of anions), are negative currents and depicted as downward deflections of the current traces. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space.

tion (60 mM K-D-gluconate, 60 mM KCl, 1 mM EGTA, 1 mM MgCl_2 , 1 mM Mg-ATP, and 10 mM HEPES/KOH; pH 7.4) in combination with K^+ -containing standard NaCl Ringer solution. K^+ channel activity can be stimulated in Ca^{2+} -containing bath solution by the Ca^{2+} ionophore ionomycin or by hemolysins (P.A. Lang et al. 2004), lead (Kempe et al. 2005) or mercury ions (Eisele et al. 2005). Clotrimazole is well suited to inhibit Gardos channels.

Calcium measurements

Intracellular calcium plays a crucial role in the signaling pathways leading to eryptosis (Bratosin et al. 2001; Daugas et al. 2001; Barvitenko et al. 2005; K.S. Lang et al. 2005). On the other hand, Ca^{2+} entry is more rapid in old erythrocytes than in young red blood cells (Romero and Romero 1999). To study Ca^{2+} entry during eryptosis, Ca^{2+} flux is measured using the radioactive tracer ion $^{45}\text{Ca}^{2+}$ (Staines et al. 1999; Tiffert and Lew 1997), with some modifications to adapt the experimental conditions to cellular stress (K.S. Lang et al. 2003a, 2004a). For this, erythrocytes are washed four times by centrifugation (2000 g for 5 min) and resuspension in five volumes of solution A containing in mM: 80 KCl, 70 NaCl, 10 HEPES, 0.2 MgCl_2 , 0.1 ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA); pH 7.5 to remove extracellular Ca^{2+} . The cell pellet is then washed twice in solution B to remove EGTA from the medium. Solution B has the same composition as solution A but does not contain EGTA. The cells are suspended at 10% haematocrit and preincubated for 20 min at 37 °C in the final incubation solution B supplemented with 10 mM inosine and 1 mM sodium orthovanadate. Then, $^{45}\text{Ca}^{2+}$ is added from a 100 mM CaCl_2 stock solution with a specific activity of about 10^7 c.p.m. per μmol to reach a final concentration of 150 μM . After different time periods, 100 μl aliquots are delivered into 1.2 ml of ice cold solution B with 0.2 mM CoCl_2 and 1 mM amiloride. The cells are collected by centrifugation in an Eppendorf centrifuge (14,000 rpm for 0.5 min, 4 °C) and the cell pellet is washed twice using 1 ml of the same medium. The supernatant is discarded, the cells are lysed,

and the proteins are precipitated by addition of 0.6 ml 6% trichloroacetic acid. After a further spin (14,000 rpm for 2 min, 4 °C), 0.5 ml of the clear supernatants are used for measuring $^{45}\text{Ca}^{2+}$ radioactivity by scintillation counting in a β -scintillation counter. $^{45}\text{Ca}^{2+}$ specific activity is determined in parallel by addition of 0.6 ml 6% trichloroacetic acid to 100 μl of erythrocyte suspension and centrifugation as described above. Then, 100 μl of the clear supernatants are taken for scintillation counting. The total calcium content of the cells $[\text{Ca}_T]_i$ is calculated by dividing the activity of the samples by the specific activity of $^{45}\text{Ca}^{2+}$ and by the number of cells. Different concentrations of agonists are added to the cell suspensions together with $^{45}\text{Ca}^{2+}$. Exposure of erythrocytes to hyperosmotic shock (950 mosmol/l) is achieved by addition of sucrose to solution B during the 20 min of preincubation and the 10 min of $^{45}\text{Ca}^{2+}$ uptake. Note that the delivery medium for washing the cells after radioactive labelling should be adjusted to the same osmolarity as the experimental solution.

Statistics

Erythrocytes from different donors show a relatively high degree of variability. Thus, all experiments using the described methods have to be repeated with several blood samples from different individuals (4 to 10 donors). If the researchers follow the instructions described above a good reproducibility of the results can be expected with a SEM below 10% of the mean value (see also Figure 1d). Data are always expressed as arithmetic means $\pm$ SEM and statistical analyses have to be made by paired or unpaired *t*-test, or ANOVA using Dunnett's or Tukey's test as *post hoc* test, where appropriate.

Results and discussion

The induction of phosphatidylserine exposure and erythrocyte shrinkage after ionomycin treatment was originally described *in vitro* with purified erythrocyte preparations from healthy donors. In these reports, purification of erythrocytes was achieved by centrifugation procedures (Berg et al.

2001; Bratosin et al. 2001) and it was shown in a later study that purified and non-purified erythrocytes from the same donor showed similar susceptibility towards cellular stress (K.S. Lang et al. 2003a). However, for analysis of eryptosis signaling, particular care should be placed on the purification and analysis of the cell population. In a study dealing with PAF signaling, we thus used highly purified erythrocyte concentrates which had been prepared by filtration over leukocyte depletion filters (P.A. Lang et al. 2005c).

To demonstrate the significance of eryptosis signaling for anemic diseases, it is necessary to analyze red blood cells from patients suffering from anemia (K.S. Lang et al. 2002). Since erythrocytes from healthy patients show differences in their susceptibility towards stress-induced eryptosis, it is mandatory to always investigate diseased erythrocytes side by side with normal controls. This approach has already been successfully used in patients suffering from chronic renal failure and receiving erythropoietin therapy (NeoRecormon). In these experiments, we could demonstrate that erythropoietin treatment significantly decreases phosphatidylserine exposure of circulating erythrocytes *in vivo* (Myssina et al. 2003).

Genetic manipulation of mature erythrocytes is not possible. Thus, classical genetic approaches to interfere with the human erythrocyte signaling machinery, i.e. antisense technology or RNA interference, can be carried out only in erythrocyte progenitor cells or cell lines with similarities to erythrocytes (e.g. the K-562 cell line). Using the antisense technique, we were able to characterize the role of Ca^{2+} -activated K^+ channels in human erythrocyte 'apoptosis' (P.A. Lang et al. 2003a).

Over the last decade, knockout mice have been widely used to study the role of effector proteins in apoptosis (for review see Los et al. (1999)). In the case of erythrocyte research, the knockout technique represents an elegant method to specifically target eryptosis signaling pathways. Using PAF receptor knockout mice, experimental evidence has been provided that PAF induces erythrocyte phosphatidylserine exposure via a PAF receptor-dependent mechanism (P.A. Lang et al. 2005c). In another study, erythrocytes from taurin transporter-deficient mice showed blunted eryptosis sensitivity (P.A. Lang et al. 2003b). It should be kept in mind, though, that there may be differences between human and murine erythrocytes. For

instance, mouse erythrocytes apparently lack shrinkage-dependent sphingomyelinase activity (unpublished observation).

Flow cytometry is a very convenient and fast method to measure large cell populations. This method has been used to screen for the effects of diverse regulator molecules of apoptosis and to analyze the pathways involved. Thus, different assays using this technique have been applied to erythrocyte suspensions. These assays comprise determination of membrane lipid asymmetry (phosphatidylserine exposure), measurement of cell volume (forward scatter analysis), detection of elevated ceramide levels and determination of intracellular Ca^{2+} concentration. Nevertheless, one has to keep in mind that the assays depend on the fluorescence properties of the erythrocytes. Therefore positive as well as negative controls have to be included. In the case of the ceramide assay, treatment of erythrocytes with purified sphingomyelinase may serve as a positive control and the use of an unspecific, isotype matched control antibody as a negative control (Grassme et al. 2002; Bieberich et al. 2003; K.S. Lang et al. 2004a). The same is true for the flow cytometric determination of intracellular Ca^{2+} activity where treatment with ionomycin or other Ca^{2+} ionophores should be included as positive control into each series of experiments (Andrews et al. 2002; P.A. Lang et al. 2005b).

The flow cytometric measurements of cell volume, cellular ceramide levels and intracellular Ca^{2+} concentrations should be confirmed by independent (biochemical) methods. In detail, cell volume can be measured conventionally using micro hematocrit tubes in combination with a microhematocrit centrifuge (Myssina et al. 2004), cellular ceramide can be determined enzymatically by the use of DAG kinase (P.A. Lang et al. 2005c) and Ca^{2+} entry can be measured by $^{45}\text{Ca}^{2+}$ uptake assays (K.S. Lang et al. 2003a).

The application of novel experimental procedures and tools allowed to redefine some features of erythrocyte physiology. For example, in binding studies using radioactive PAF it was demonstrated that mature erythrocytes do not contain high affinity binding sites for PAF. The authors concluded from those data that erythrocytes do not contain PAF receptors (Hwang et al. 1983) although specific antibodies against the human PAF receptor were not available at this time. In a recent

study, however, it was shown by Western blot that highly purified erythrocyte concentrates contain significant amounts of PAF receptor and undergo PAF-mediated eryptosis (P.A. Lang et al. 2005c). Interestingly, these data can serve as an additional explanation for the PAF-mediated adherence of sickle erythrocytes to endothelium (Kaul et al. 2000, 2001).

Another example for new concepts in erythrocyte biology is the assumption that mature erythrocytes still contain erythropoietin receptors and that erythropoietin is able to influence eryptosis at the level of the nonspecific cation channels. This concept was confirmed by patch clamp measurements in the presence or absence of erythropoietin. Additionally, high affinity erythropoietin binding sites on mature erythrocytes were determined by the use of [125 I]iodotyrosylerythropoietin (molecular weight: 34,000 D) (Myssina et al. 2003).

In summary, human and murine erythrocytes represent well characterized cellular systems which will enable future research on cell death signaling pathways which are independent of intracellular organelles, i.e. nuclei or mitochondria.

Acknowledgements

The authors acknowledge the technical assistance of E. Faber, K. Bauer, S. Heuser, and M. Halter and the meticulous preparation of the manuscript by T. Loch and L. Subasic. The work of the authors is supported by the Deutsche Forschungsgemeinschaft, Nr. La315/6-1, La315/11-1, and La315/13-1, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Center for Interdisciplinary Clinical Research) 01KS9602, P.A.L. was a stipend of the 17KF, the Else-Übelmeyer Stiftung and the Biomed program of the EU (BMH4-CT96-0602).

References

Andree H.A., Reutelingsperger C.P., Hauptmann R., Hemker H.C., Hermens W.T. and Willems G.M. 1990. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J. Biol. Chem.* 265: 4923–4928.
 Andrews D.A. and Low P.S. 1999. Role of red blood cells in thrombosis. *Curr. Opin. Hematol.* 6: 76–82.

Andrews D.A., Yang L. and Low P.S. 2002. Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood* 100: 3392–3399.
 Barvitenko N.N., Adragna N.C. and Weber R.E. 2005. Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cell Physiol. Biochem.* 15: 1–18.
 Berg C.P., Engels I.H., Rothbart A., Lauber K., Renz A., Schlosser S.F., Schulze-Osthoff K. and Wesselborg S. 2001. Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ.* 8: 1197–1206.
 Bieberich E., MacKinnon S., Silva J., Noggle S. and Condie B.G. 2003. Regulation of cell death in mitotic neural progenitor cells by asymmetric distribution of prostate apoptosis response 4 (PAR-4) and simultaneous elevation of endogenous ceramide. *J. Cell Biol.* 162: 469–479.
 Birka C., Lang P.A., Kempe D.S., Hoeffling L., Tanneur V., Duranton C., Nammi S., Henke G., Myssina S., Krikov M., Huber S.M., Wieder T. and Lang F. 2004. Enhanced susceptibility to erythrocyte “apoptosis” following phosphate depletion. *Pflügers Arch.* 448: 471–477.
 Bratosin D., Estaquier J., Petit F., Arnoult D., Quatannens B., Tissier J.P., Slomianny C., Sartiaux C., Alonso C., Huart J.J., Montreuil J. and Ameisen J.C. 2001. Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell Death Differ.* 8: 1143–1156.
 Bussolino F., Fischer E., Turrini F., Kazatchkine M.D. and Arese P. 1989. Platelet-activating factor enhances complement-dependent phagocytosis of diamide-treated erythrocytes by human monocytes through activation of protein kinase C and phosphorylation of complement receptor type one (CR1). *J. Biol. Chem.* 264: 21711–21719.
 Daugas E., Cande C. and Kroemer G. 2001. Erythrocytes: death of a mummy. *Cell Death Differ.* 8: 1131–1133.
 Desai S.A., Bezrukov S.M. and Zimmerberg J. 2000. A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. *Nature* 406: 1001–1005.
 Dupuis F., Levasseur S., Jean-Louis F., Dulery C., Praloran V., Denizot Y. and Michel L. 1997. Production, metabolism and effect of platelet-activating factor on the growth of the human K562 erythroid cell line. *Biochim. Biophys. Acta* 1359: 241–249.
 Duranton C., Huber S., Tanneur V., Lang K., Brand V., Sandu C. and Lang F. 2003. Electrophysiological properties of the Plasmodium Falciparum-induced cation conductance of human erythrocytes. *Cell Physiol. Biochem.* 13: 189–198.
 Duranton C., Huber S.M. and Lang F. 2002. Oxidation induces a Cl(–)-dependent cation conductance in human red blood cells. *J. Physiol.* 539: 847–855.
 Eda S. and Sherman I.W. 2002. Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell Physiol. Biochem.* 12: 373–384.
 Egee S., Lapaix F., Decherf G., Staines H.M., Ellory J.C., Doerig C. and Thomas S.L. 2002. A stretch-activated anion channel is up-regulated by the malaria parasite Plasmodium falciparum. *J. Physiol.* 542: 795–801.

- Eisele K., Lang P.A., Kempe D.S., Klarl B.A., Niemoller O., Wieder T., Huber S.M., Duranton C. and Lang F. 2006. Stimulation of erythrocyte phosphatidylserine exposure by mercury ions. *Toxicol. Appl. Pharmacol.* 210(1–2): 116–122.
- Essmann F., Wieder T., Otto A., Muller E.C., Dorken B. and Daniel P.T. 2000. GDP dissociation inhibitor D4-GDI (Rho-GDI 2), but not the homologous rho-GDI 1, is cleaved by caspase-3 during drug-induced apoptosis. *Biochem. J.* 346(Pt 3): 777–783.
- Fadok V.A., Bratton D.L., Rose D.M., Pearson A., Ezekewitz R.A. and Henson P.M. 2000. A receptor for phosphatidylserine-specific clearance of apoptotic cells. *Nature* 405: 85–90.
- Frago L.M., Leon Y., de la Rosa E.J., Gomez-Munoz A. and Varela-Nieto I. 1998. Nerve growth factor and ceramides modulate cell death in the early developing inner ear. *J. Cell Sci.* 111(Pt 5): 549–556.
- Gamper N., Huber S.M., Badawi K. and Lang F. 2000. Cell volume-sensitive sodium channels upregulated by glucocorticoids in U937 macrophages. *Pflügers Arch.* 441: 281–286.
- Garay R. and Braquet P. 1986. Involvement of K⁺ movements in the membrane signal induced by PAF-acether. *Biochem. Pharmacol.* 35: 2811–2815.
- Goggel R., Winoto-Morbach S., Vielhaber G., Imai Y., Lindner K., Brade L., Brade H., Ehlers S., Slutsky A.S., Schutze S., Gulbins E. and Uhlig S. 2004. PAF-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide. *Nat. Med.* 10: 155–160.
- Goldkorn T., Balaban N., Shannon M., Chea V., Matsukuma K., Gilchrist D., Wang H. and Chan C. 1998. H₂O₂ acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells. *J. Cell Sci.* 111(Pt 21): 3209–3220.
- Grassme H., Jendrossek V., Bock J., Riehle A. and Gulbins E. 2002. Ceramide-rich membrane rafts mediate CD40 clustering. *J. Immunol.* 168: 298–307.
- Green D.R. and Reed J.C. 1998. Mitochondria and apoptosis. *Science* 281: 1309–1312.
- Gulbins E., Jekle A., Ferlinz K., Grassme H. and Lang F. 2000. Physiology of apoptosis. *Am. J. Physiol. Renal Physiol.* 279: F605–F615.
- Hamill O.P., Marty A., Neher E., Sakmann B. and Sigworth F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391: 85–100.
- Haynes J., Jr. and Obiako B. 2002. Activated polymorphonuclear cells increase sickle red blood cell retention in lung: role of phospholipids. *Am. J. Physiol. Heart Circ. Physiol.* 282: H122–H130.
- Huber S.M., Duranton C., Henke G., Van De S.C., Heussler V., Shumilina E., Sandu C.D., Tanneur V., Brand V., Kasinathan R.S., Lang K.S., Kremsner P.G., Hubner C.A., Rust M.B., Dedek K., Jentsch T.J. and Lang F. 2004. Plasmodium induces swelling-activated ClC-2 anion channels in the host erythrocyte. *J. Biol. Chem.* 279: 41444–41452.
- Huber S.M., Duranton C. and Lang F. 2005. Patch-clamp analysis of the “new permeability pathways” in malaria-infected erythrocytes. *Int. Rev. Cytol.* 246: 59–134.
- Huber SM, Gamper N. and Lang F. 2001. Chloride conductance and volume-regulatory nonselective cation conductance in human red blood cell ghosts. *Pflügers Arch.* 441: 551–558.
- Huber S.M., Uhlemann A.C., Gamper N.L., Duranton C., Kremsner P.G. and Lang F. 2002. Plasmodium falciparum activates endogenous Cl(–) channels of human erythrocytes by membrane oxidation. *EMBO J.* 21: 22–30.
- Hwang S.B., Lee C.S., Cheah M.J. and Shen T.Y. 1983. Specific receptor sites for 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and guinea pig smooth muscle membranes. *Biochemistry* 22: 4756–4763.
- Jayadev S., Linardic C.M. and Hannun Y.A. 1994. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. *J. Biol. Chem.* 269: 5757–5763.
- Kaestner L., Tabellion W., Lipp P. and Bernhardt I. 2004. Prostaglandin E2 activates channel-mediated calcium entry in human erythrocytes: an indication for a blood clot formation supporting process. *Thromb. Haemost.* 92: 1269–1272.
- Kaul D.K., Liu X. and Nagel R.L. 2001. Ameliorating effects of fluorocarbon emulsion on sickle red blood cell-induced obstruction in an ex vivo vasculature. *Blood* 98: 3128–3131.
- Kaul D.K., Tsai H.M., Liu X.D., Nakada M.T., Nagel R.L. and Collier B.S. 2000. Monoclonal antibodies to alphaVbeta3 (7E3 and LM609) inhibit sickle red blood cell-endothelium interactions induced by platelet-activating factor. *Blood* 95: 368–374.
- Kempe D.S., Lang P.A., Eisele K., Klarl B.A., Wieder T., Huber S.M., Duranton C. and Lang F. 2005. Stimulation of erythrocyte phosphatidylserine exposure by lead ions. *Am. J. Physiol. Cell Physiol.* 288: C396–C402.
- Kolesnick R. and Fuks Z. 2003. Radiation and ceramide-induced apoptosis. *Oncogene* 22: 5897–5906.
- Lang K.S., Duranton C., Poehlmann H., Myssina S., Bauer C., Lang F., Wieder T. and Huber S.M. 2003a. Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ.* 10: 249–256.
- Lang K.S., Lang P.A., Bauer C., Duranton C., Wieder T., Huber S.M. and Lang F. 2005. Mechanisms of suicidal erythrocyte death. *Cell Physiol. Biochem.* 15: 195–202.
- Lang K.S., Myssina S., Brand V., Sandu C., Lang P.A., Berchtold S., Huber S.M., Lang F. and Wieder T. 2004a. Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell Death Differ.* 11: 231–243.
- Lang K.S., Myssina S., Lang P.A., Tanneur V., Kempe D.S., Mack A.F., Huber S.M., Wieder T., Lang F. and Duranton C. 2004b. Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl[–]. *Am. J. Physiol. Renal Physiol.* 286: F1046–F1053.
- Lang K.S., Myssina S., Tanneur V., Wieder T., Huber S.M., Lang F. and Duranton C. 2003b. Inhibition of erythrocyte cation channels and apoptosis by ethylisopropylamiloride. *Naunyn Schmiedeberg's Arch. Pharmacol.* 367: 391–396.
- Lang K.S., Roll B., Myssina S., Schittenhelm M., Scheel-Walter H.G., Kanz L., Fritz J., Lang F., Huber S.M. and Wieder T. 2002. Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cell Physiol. Biochem.* 12: 365–372.
- Lang P.A., Kaiser S., Myssina S., Birka C., Weinstock C., Northoff H., Wieder T., Lang F. and Huber S.M. 2004. Effect of *Vibrio parahaemolyticus* haemolysin on human erythrocytes. *Cell. Microbiol.* 6: 391–400.

- Lang P.A., Kaiser S., Myssina S., Wieder T., Lang F. and Huber S.M. 2003a. Role of Ca^{2+} -activated K^{+} channels in human erythrocyte apoptosis. *Am. J. Physiol. Cell Physiol.* 285: C1553–C1560.
- Lang P.A., Kempe D.S., Akel A., Klarl B.A., Eisele K., Podolski M., Hermle T., Niemoeller O.M., Attanasio P., Huber S.M., Wieder T., Lang F. and Duranton C. 2005a. Inhibition of erythrocyte “apoptosis” by catecholamines. *Naunyn Schmiedebergs Arch. Pharmacol.* 372: 228–235.
- Lang P.A., Kempe D.S., Myssina S., Tanneur V., Birka C., Laufer S., Lang F., Wieder T. and Huber S.M. 2005b. PGE(2) in the regulation of programmed erythrocyte death. *Cell Death Differ.* 12: 415–428.
- Lang P.A., Kempe D.S., Tanneur V., Eisele K., Klarl B.A., Myssina S., Jendrosseck V., Ishii S., Shimizu T., Waidmann M., Hessler G., Huber S.M., Lang F. and Wieder T. 2005c. Stimulation of erythrocyte ceramide formation by platelet-activating factor. *J. Cell Sci.* 118: 1233–1243.
- Lang P.A., Warskulat U., Heller-Stilb B., Huang D.Y., Grenz A., Myssina S., Duzsenko M., Lang F., Haussinger D., Vallon V. and Wieder T. 2003b. Blunted apoptosis of erythrocytes from taurine transporter deficient mice. *Cell Physiol. Biochem.* 13: 337–346.
- Los M., Wesselborg S. and Schulze-Osthoff K. 1999. The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity* 10: 629–639.
- MacDonald D.J., Boyle R.M., Glen A.C. and Horrobin D.F. 2004. Cytosolic phospholipase A2 type IVA is present in human red cells. *Blood* 103: 3562–3564.
- Mandal D., Baudin-Creuzat V., Bhattacharyya A., Pathak S., Delaunay J., Kundu M. and Basu J. 2003. Caspase 3-mediated proteolysis of the N-terminal cytoplasmic domain of the human erythroid anion exchanger 1 (band 3). *J. Biol. Chem.* 278: 52551–52558.
- Mandal D., Moitra P.K., Saha S. and Basu J. 2002. Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett.* 513: 184–188.
- Montrucchio G., Alloati G. and Camussi G. 2000. Role of platelet-activating factor in cardiovascular pathophysiology. *Physiol. Rev.* 80: 1669–1699.
- Montrucchio G., Alloati G., Mariano F., Lupia E., Lucchina P.G., Musso E., Emanuelli G. and Camussi G. 1993. Role of platelet-activating factor in hypotension and platelet activation induced by infusion of thrombolytic agents in rabbits. *Circ. Res.* 72: 658–670.
- Myssina S., Huber S.M., Birka C., Lang P.A., Lang K.S., Friedrich B., Risler T., Wieder T. and Lang F. 2003. Inhibition of erythrocyte cation channels by erythropoietin. *J. Am. Soc. Nephrol.* 14: 2750–2757.
- Myssina S., Lang P., Kempe D., Kaiser S., Huber S., Wieder T. and Lang F. 2004. Cl channel blockers NPPB and niflumic acid blunt Ca^{2+} -induced erythrocyte ‘apoptosis’. *Cell Physiol. Biochem.* 14: 241–248.
- Ogretmen B. and Hannun Y.A. 2004. active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* 4: 604–616.
- Prokop A., Wieder T., Sturm I., Essmann F., Seeger K., Wuchter C., Ludwig W.D., Henze G., Dorken B. and Daniel P.T. 2000. Relapse in childhood acute lymphoblastic leukemia is associated with a decrease of the Bax/Bcl-2 ratio and loss of spontaneous caspase-3 processing *in vivo*. *Leukemia* 14: 1606–1613.
- Raisova M., Bektas M., Wieder T., Daniel P., Eberle J., Orfanos C.E. and Geilen C.C. 2000. Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome c release. *FEBS Lett.* 473: 27–32.
- Rivera A., Jarolim P. and Brugnara C. 2002. Modulation of Gardos channel activity by cytokines in sickle erythrocytes. *Blood* 99: 357–603.
- Romero P.J. and Romero E.A. 1999. Effect of cell ageing on Ca^{2+} influx into human red cells. *Cell Calcium* 26: 131–137.
- Staines H.M., Chang W., Ellory J.C., Tiffert T., Kirk K. and Lew V.L. 1999. Passive Ca^{2+} transport and Ca^{2+} -dependent K^{+} transport in *Plasmodium falciparum*-infected red cells. *J. Membr. Biol.* 172: 13–24.
- Subbanagounder G., Leitinger N., Shih P.T., Faull K.F. and Berliner J.A. 1999. Evidence that phospholipid oxidation products and/or platelet-activating factor play an important role in early atherogenesis: *in vitro* and *in vivo* inhibition by WEB 2086. *Circ. Res.* 85: 311–318.
- Tiffert T. and Lew V.L. 1997. Cytoplasmic calcium buffers in intact human red cells. *J. Physiol.* 500(Pt 1): 139–154.
- Verloo P., Kocken C.H., Van der W.A., Tilly B.C., Hogema B.M., Sinaasappel M., Thomas A.W. and De Jonge H.R. 2004. *Plasmodium falciparum*-activated chloride channels are defective in erythrocytes from cystic fibrosis patients. *J. Biol. Chem.* 279: 10316–10322.
- von Haefen C., Wieder T., Essmann F., Schulze-Osthoff K., Dorken B. and Daniel P.T. 2003. Paclitaxel-induced apoptosis in BJAB cells proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene* 22: 2236–2247.
- von Haefen C., Wieder T., Gillissen B., Starck L., Graupner V., Dorken B. and Daniel P.T. 2002. Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent pathway in human carcinoma cells. *Oncogene* 21: 4009–4019.
- Weil M., Jacobson M.D. and Raff M.C. 1998. Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. *J. Cell Sci.* 111(Pt 18): 2707–2715.
- Wieder T., Essmann F., Prokop A., Schmelz K., Schulze-Osthoff K., Beyaert R., Dorken B. and Daniel P.T. 2001a. Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood* 97: 1378–1387.
- Wieder T., Prokop A., Bagci B., Essmann F., Bernicke D., Schulze-Osthoff K., Dorken B., Schmalz H.G., Daniel P.T. and Henze G. 2001b. Piceatannol, a hydroxylated analog of the chemopreventive agent resveratrol, is a potent inducer of apoptosis in the lymphoma cell line BJAB and in primary, leukemic lymphoblasts. *Leukemia* 15: 1735–1742.
- Woon L.A., Holland J.W., Kable E.P. and Roufogalis B.D. 1999. Ca^{2+} sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 25: 313–320.
- Yang L., Andrews D.A. and Low P.S. 2000. Lysophosphatidic acid opens a Ca^{++} channel in human erythrocytes. *Blood* 95: 2420–2425.

- Zahler P. and Kramer R. 1981. Isolation of phospholipase A2 from red cell membranes of sheep. *Methods Enzymol.* 71(Pt C): 690–698.
- Zimmerman G.A., McIntyre T.M., Prescott S.M. and Stafforini D.M. 2002. The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Crit. Care Med.* 30: S294–S301.