Regulation of Phosphatidylserine Exposure in Red Blood Cells

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Key Words

Red blood cells • Lysophosphatidic acid • Scramblase • Flippase • Calcium concentration • Phosphatidylserine • Fluorescence imaging

lipid flop caused by LPA. In sheep RBCs, only the latter mechanism occurs suggesting absence of scramblase activity.

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Abstract

The exposure of phosphatidylserine (PS) on the outer membrane leaflet of red blood cells (RBCs) serves as a signal for eryptosis, a mechanism for the RBC clearance from blood circulation. The process of PS exposure was investigated as function of the intracellular Ca^{2+} content and the activation of PKC α in human and sheep RBCs. Cells were treated with lysophosphatidic acid (LPA), 4-bromo-A23187, or phorbol-12 myristate-13 acetate (PMA) and analysed by flow cytometry, single cell fluorescence video imaging, or confocal microscopy. For human RBCs, no clear correlation existed between the number of cells with an elevated Ca^{2+} content and PS exposure. Results are explained by three different mechanisms responsible for the PS exposure in human RBCs: (i) Ca^{2+} -stimulated scramblase activation (and flippase inhibition) by LPA, 4-bromo-A23187, and PMA; (ii) PKC activation by LPA and PMA; and (iii) enhanced

Introduction

Phospholipids are asymmetrically distributed in the plasma membrane of most, if not all, biological cells. Sphingomyelin (SM) and phosphatidylcholine (PC) are found predominantly in the outer leaflet of the membrane bilayer, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located mostly in the inner leaflet [1]. The distribution of the membrane phospholipids is regulated by three proteins: flippase [2], floppase, and scramblase [3-5]. PS exposure on the outer leaflet of the membrane has been described as a marker for apoptosis in nucleated cells [5]. Although the apoptosis of RBCs is still under discussion, they undergo suicidal death with signs of apoptosis such as PS exposure, membrane blebbing and vesicle formation [6]. This process was termed eryptosis by Lang et al. [7].

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Based on a correlation between decreased haematocrit and longer bleeding times [8] and experiments of Andrews and Low [9], an active role of RBCs in thrombus formation has been proposed [9]. Kaestner et al. published a more detailed signalling cascade based on Ca^{2+} uptake via a non-selective cation channel that could be activated by prostaglandin E_2 (PGE_2) [10, 11]. This channel was believed to be a voltage-activated non-selective cation channel [12, 13]. Recent considerations [14] propose the PGE_2 receptor-activated non-selective cation channel [10, 11] and the voltage activated cation channel [12, 13] are of different identity. Ca^{2+} entry can also be caused by a mechanical deformation of RBCs as described by Dyrda et al. [15].

PGE_2 and LPA are local mediators released by platelets after their activation within the coagulation cascade and, in case of PGE_2 , are released by RBCs under mechanical stress [16]. Moreover, in recent reports, we showed that PS exposure induced by LPA was associated with cell-cell adhesion of human RBCs [17, 18]. PS exposure at the outer leaflet of the RBC membrane is of importance for the adhesion of RBCs to endothelium in some diseases such as sickle cell anaemia, malaria, and diabetes [19].

Although it is known that in RBCs LPA induces PS exposure at the outer membrane leaflet, there are conflicting reports about its mechanism. While Chung et al. [20] claim that it is a totally Ca^{2+} -independent process, we showed that Ca^{2+} alone is sufficient to induce PS exposure in human RBCs [17]. Woon et al. found that an increase of the intracellular Ca^{2+} level in RBCs results in the exposure of PS to the outer membrane leaflet due to activation of the scramblase and inhibition of the flippase [21]. Protein kinase $\text{C}\alpha$ ($\text{PKC}\alpha$) has been also described to be involved in the PS exposure on RBCs [20, 22, 23]. In human RBCs, in addition to $\text{PKC}\alpha$, only a limited number of PKC isoforms (ζ , λ/ι , and μ) have been detected [24]. The molecular mechanisms for the participation of these proteins in the Ca^{2+} uptake and/or in the PS exposure remain to be elucidated.

Here, we investigated the parameters regulating PS exposure at the outer membrane leaflet of human RBCs by addressing Ca^{2+} content manipulated by an ionophore and PKC activation by phorbol-12 myristate-13 acetate (PMA). For comparison, sheep RBCs were studied because these cells have a completely different membrane phospholipid distribution. Like in human RBCs, PS and PE are present at the inner membrane leaflet, however, sheep RBC membranes lack PC and the outer layer consists exclusively of SM [25, 26].

Materials and Methods

Blood and solutions

Human venous blood from healthy donors was obtained from the Institute of Clinical Haematology and Transfusion Medicine of Saarland University Hospital. Sheep blood samples were obtained from the sheep farm Ernst in Blieskastel, Germany. Citrate or EDTA (for human blood) or heparin (for sheep blood) were used as anticoagulants. Freshly drawn blood samples were stored at 4 °C and used within one day.

Blood was centrifuged at 2,000 g for 5 min at room temperature (RT) and the plasma and buffy coat was removed by aspiration. Subsequently, RBCs were washed 3 times in HEPES-buffered physiological solution (HPS) containing (mM): NaCl 145, KCl 7.5, glucose 10, HEPES 10, pH 7.4, under the same conditions. Finally, RBCs were re-suspended in HPS and kept at 4 °C. To assess the effect of cell volume, the last wash was done in a high- K^+ solution (mM): NaCl 2.5, KCl 150, glucose 10, HEPES 10, pH 7.4.

Intracellular Ca^{2+} content

The application of fluo-4 for Ca^{2+} measurements in intact RBCs has been reported elsewhere [27]. It has been also shown that fura-2 or indo-1 cannot be applied for those measurements due to quenching by haemoglobin [27].

RBCs were loaded with 5 μM fluo-4 AM from a 1 mM stock solution in dimethyl sulfoxide (DMSO) with 20% Pluronic F-127 in 1 ml HPS at a haematocrit of 1% for 45 min at 37 °C in the dark. Cells were washed 3 times by centrifugation (20 s, 12,000 g) in HPS and finally equilibrated for de-esterification for 15 min at a haematocrit of 0.5% at RT.

Intracellular free Ca^{2+} levels of single RBCs were monitored using an inverted fluorescence microscope (Eclipse TE2000-E, Nikon, Tokyo, Japan). A diluted RBC suspension (approximately 0.025% haematocrit), prepared in HPS with 2 mM CaCl_2 , was placed on a cover slip coated with poly-L-lysine 5 min before the start of the imaging procedure. Ca^{2+} ionophore 4-bromo-A23187 (the non-fluorescent variant of A23187, thereafter named A23187), LPA, and PMA were added immediately before the start of the imaging procedure. The experiments were performed at RT in a dark room. Control conditions consisted of the same procedure but cells were re-suspended in the presence of respective amounts of reagent solvents (ethanol, water, or DMSO).

Our experimental approach was designed to provide a quantitative assessment of fluorescence intensity while maintaining identical imaging parameters under all conditions. This was accomplished by taking images with an electron multiplication CCD camera (CCD97, Photometrics, Tucson, USA) using a 100 $\times$ 1.4 (NA) oil immersion lens with infinity corrected optics. An image was taken every 20 s (exposure time 500 ms) for a 30 min experiment using the imaging software Metavue (Universal Imaging Corp., Marlow, UK).

Fluo-4 was excited with a xenon lamp-based monochromator (Visitron Systems, Puchheim, Germany) at a centre wavelength of 488 nm. Emission was recorded at 520/15 nm. The background corrected fluorescence signals were normalized to the initial fluorescence (F_0). For each experiment 20 - 30 cells

were analysed from each sample and at least three different blood samples were used.

Additionally, the intracellular free Ca^{2+} content of RBCs was measured by flow cytometry (FACS Calibur and Cell Quest Pro software, Becton Dickinson Biosciences, Franklin Lakes, USA). Preparation of cells, solutions and experimental conditions were the same as described above (except that samples were analysed after 12 min). Ca^{2+} content was measured in the FL-1 channel and analysed using the mean value of the relative fluorescence of 30,000 cells from each blood sample. For each experiment at least three different blood samples were used.

PS exposure

PS exposure on the outer membrane leaflet of RBCs was evaluated by annexin V-FITC binding to this phospholipid. RBCs prepared as described above were treated with A23187, LPA, or PMA or with ethanol, water, or DMSO (for controls) in HPS with 2 mM CaCl_2 at 37 °C for 30 min (0.1% haematocrit). Cells were washed in the ice-cold HPS with 2 mM CaCl_2 by centrifugation (20 s, 12,000 g). Approximately 10^6 cells were incubated with 5 μl of annexin V-FITC in 500 μl of annexin binding buffer containing (mM): NaCl 145, HEPES 10, CaCl_2 2.5, pH 7.4, for 15 min at RT. Annexin V-FITC was delivered in unit size of 500 μl containing 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, and 0.1% bovine serum albumin. After incubation, samples were kept on ice and analysed immediately by flow cytometry (FACS Calibur and Cell Quest Pro software). PS exposure was measured in the FL-1 channel excited by an argon laser at 488 nm. Control conditions for the negative fluorescent gate were obtained in the absence of A23187, LPA, or PMA. Annexin V positive RBCs were determined as percentage by relating positive and negative signal events to the control condition. For each experiment at least three different blood samples were used and 30,000 events for each sample were counted.

The kinetics of PS exposure was recorded using a fluorescence microscope (the same as described above for Ca^{2+} measurements). A diluted RBC suspension (approximately 0.025% haematocrit) was prepared in the HPS with 2 mM CaCl_2 and 5 μl annexin V-FITC and transferred to a cover slip (coated with poly-L-lysine) 5 min before the start of the imaging procedure. A23187, LPA, and PMA (or the solvents ethanol, water, or DMSO, for controls) were added immediately before the start of the imaging procedure. Experiments were performed at RT in a dark room. An image was taken every 30 s (exposure time 500 ms).

Double labelling experiments

To evaluate the relationship between the intracellular Ca^{2+} content and the PS exposure, RBCs were labelled with both fluo-4 AM and annexin V-alexa 568. Briefly, cells loaded with fluo-4 AM as described before were induced for PS exposure using A23187, LPA, or PMA. After washing in the HPS at RT, cells were incubated with annexin V-alexa 568 for 15 min at RT. The double labelling experiments were carried out using the confocal laser scanning fluorescence microscope LSM 510 META (Carl Zeiss AG, Jena, Germany). Samples were scanned with an argon laser (488 nm) for fluo-4 and

Fig. 1. Fluo-4 AM fluorescence intensity (arbitrary units) of human RBC for Ca^{2+} content after treatment with 2.5 μM LPA, 2 μM A23187 or 6 μM PMA in the presence of 2 mM extracellular Ca^{2+} measured with single cell fluorescence microscopy over a time interval of 30 min. Curves show the mean values of at least 60 RBCs from 3 different blood samples. Error bars represent 25% of SD.

HeNe laser (543 nm) for annexin V-alexa 568.

Reagents

All chemicals used (except fluorescent dyes and Pluronic F-127) were purchased from Sigma-Aldrich (Munich, Germany). Fluo-4 AM, annexin V-FITC, and Pluronic F-127 were obtained from Molecular Probes (Eugene, USA) and annexin V-alexa from Roche Diagnostics GmbH (Mannheim, Germany). Stock solutions for A23187 (1 mM), LPA (1 mM), and PMA (10 mM) were dissolved in ethanol, water, and DMSO, respectively.

Statistical significance

Data are presented as mean values $\pm$ SD of at least 3 independent experiments. The significance of differences was tested by Student's *t*-test. Statistical significance of the data was defined as follows: $p > 0.05$ (n.s.), $p = 0.05$ (*); $p = 0.01$ (**), $p = 0.001$ (***)

Results

An increased intracellular Ca^{2+} content of RBCs results in the activation of several processes, important for PS exposure: (i) activation of the scramblase and inhibition of flippase [4], (ii) activation of the Ca^{2+} -activated K^+ channel leading to loss of KCl and water, causing cell shrinkage [28–30], (iii) activation of PKC α [22], and (iv) activation of calpain resulting in cytoskeleton destruction, membrane blebbing, and micro-vesiculation [31, 32]. In addition, elevated intracellular Ca^{2+} activates the Ca^{2+} pump.

In order to correlate between increased intracellular Ca^{2+} content and PS exposure of human RBCs, we focussed on LPA treatment shown to increase the intra-

Fig. 2. Flow cytometric analysis of human RBC Ca^{2+} content after treatment with 2.5 μM LPA, 2 μM A23187 or 6 μM PMA in the presence of 2 mM extracellular Ca^{2+} after 12 min of incubation. Mean values of 3 different blood samples (with 30,000 cells per sample) are shown. Error bars represent SD, $p = 0.05$ (*); $p = 0.01$ (**), $p = 0.001$ (***)). Control measurements were done in the presence of solvents (ethanol, water, or DMSO, see Material and Methods). All data measured in the presence of the reagents

are significantly different from control, $p = 0.001$. A: Percentage of RBCs showing a significant higher Ca^{2+} content compared to control after treatment with LPA, A23187 or PMA. B: Increase of Ca^{2+} content of RBCs in response to LPA, A23187 or PMA treatment shown as arbitrary units (a.u.) of fluorescence intensity analysed in cells with higher Ca^{2+} content.

Fig. 3. PS exposure evaluation of human RBCs treated with 2 μM A23187 or 2.5 μM LPA in the presence of 2 mM extracellular Ca^{2+} up to 120 min and labelled with annexin V-FITC using fluorescence microscopy. A: Representative images out of 3 independent experiments. Note that blood samples from different donors are presented. B: Kinetics of fluorescence intensity shown as arbitrary units (a.u.); curves show the mean values of at least 30 RBCs from 3 different blood samples. Error bars represent SD.

cellular Ca^{2+} content [17, 18, 20, 22, 27]. For comparison, we used the Ca^{2+} ionophore A23187. In addition, we treated the RBCs with PMA, a known activator of the Ca^{2+} -dependent conventional PKC (cPKC) isoforms (α , βI , βII , γ) and the Ca^{2+} -independent novel PKC (nPKC) isoforms (δ , ϵ , η , θ) but not the Ca^{2+} -independent, non phorbol ester-binding atypical PKC (aPKC) isoforms (ζ , $\lambda/1$, μ) [33, 34]. As mentioned before, PKC α [22] as well as PKC ζ [24], and PKC $\lambda/1$ [24] are present in human RBCs and may play a role in PS exposure. So far no nPKC isoforms were detected in RBCs.

Fig. 1 shows the kinetics of Ca^{2+} uptake after treating RBCs with LPA, A23187, or PMA measured by fluorescence video imaging. Addition of 2.5 μM LPA immediately led to an increase of the intracellular Ca^{2+} content that remains relatively constant up to 30 min. At higher LPA concentrations (3.5 - 10 μM) the rate of haemolysis continuously increased (data not shown). Treatment with 2 μM A23187 significantly increased the Ca^{2+} content but only after a delay of about 5 min. The final fluorescence intensity, i.e. the intracellular Ca^{2+} content, was the same under both conditions after 30 min. At higher concentrations (up to 10 μM) the lag phase was reduced without affecting the maximal fluorescence intensity (data not shown). Similar to the treatment with LPA, exposure to 6 μM PMA, a recommended concentration to activate cPKCs and nPKCs [22], increased the Ca^{2+} content with-

Fig. 4. Flow cytometry analysis of human RBCs showing a significant higher percentage PS exposure compared to control after a 30 min treatment with 2.5 μ M LPA, 2 μ M A23187 or 6 μ M PMA in the presence of 2 mM extracellular Ca²⁺ [(+) Ca²⁺]. For comparison cells were treated in the absence of extracellular Ca²⁺ (presence of 1 mM EGTA) [(-) Ca²⁺]. Bars show mean value of 3 different blood samples (30,000 cells of each blood sample were analysed). Error bars represent SD, $p \leq 0.05$ (*); $p \leq 0.01$ (**). Control measurements were done in the presence of solvents (ethanol, water, or DMSO) and extracellular Ca²⁺ (see Material and Methods). LPA or A23187 treatments in the absence of extracellular Ca²⁺ were not significantly different from control. All data measured in the presence of reagents were significantly different from control, $p \leq 0.001$.

out a lagphase, but failed to reach comparably high fluorescence intensities.

Comparable experiments were carried out using flow cytometry analysis and results are represented in Fig. 2. Fig. 2A depicts the percentage of cells with an elevated Ca²⁺ content after LPA, A23187, or PMA treatment in comparison to control conditions after 12 min when the fluorescence intensity after A23187 treatment had reached maximal values (see Fig. 1). Fig. 2B shows the corresponding fluorescence intensities of the reacting cells, i.e. a significantly enhanced Ca²⁺ content after stimulation by the three substances. Fig. 2A shows that in 99.2% of the cells there was a significant increase of Ca²⁺ content in response to A23187. In the presence of LPA about 80% of the cells raised their Ca²⁺ content, comparable to the Ca²⁺ ionophore treatment. With PMA only about 40% of the cells increased their Ca²⁺ content reaching only half the fluorescence intensity as compared to A23187 or LPA (Fig. 2B). With 10 μ M PMA, the number of cells reacting by exposing PS increased significantly, reaching a value of about 70%, although the Ca²⁺ content was not enhanced under these conditions (not shown in Fig. 2, but cf. with fluorescence microscopy results presented in Fig. 1). It should be mentioned that on average only 1.3% of control cells had about 5-fold

Fig. 5. Human RBCs double labelled with fluo-4 and annexin V-alexa 568 (Ca²⁺/PS double scan, green and red, right column) after stimulated to increase Ca²⁺ content (green, left column) and PS exposure (red, middle column), respectively. Upper panels - 2.5 μ M LPA, middle panels - 2 μ M A23187, lower panels - 6 μ M PMA. White arrows indicate cells with both, elevated Ca²⁺ content and an increased PS exposure, green arrows cells with increased Ca²⁺ content only, and red arrows cells with externalised PS only. These are representative images out of 3 independent experiments.

Fig. 6. Sheep RBCs double labelled with fluo-4 and annexin V-alexa 568 (Ca²⁺/PS double scan, green and red, right column) after stimulation to increase Ca²⁺ content (green, left column) and PS exposure (red, middle column), respectively. Upper panels - 2.5 μ M LPA, lower panels - 2 μ M A23187. There are two cells with elevated Ca²⁺ content and increased PS exposure. Representative images out of 3 independent experiments.

higher fluo-4 fluorescence intensity compared to the non-reacting control cells (for non-reacting cells data not shown).

The PS exposure was studied in LPA- and A23187-treated cells over a time period of 120 min using fluorescence microscopy (Fig. 3). At times longer than 120 min in LPA treated RBCs haemolysis occurred. In contrast, no significant haemolysis was seen with A23187-treated cells at longer incubation times (up to 24 h). PMA treatment also did not result in a significant haemolysis after long time incubation (up to 6 h, data not shown). The images of treatments with LPA and A23187 (Fig. 3A) as well as PMA (not shown) reveal that vesiculation occurred. The kinetic of PS exposure of human RBCs treated with LPA or A23187 is presented in Fig. 3B. It can be seen that the process of PS exposure is faster and more pronounced in case of LPA compared to A23187. Although the kinetics is a convolution of the annexin V binding to PS and the PS exposure rate, we can deduct from the comparison of Fig. 1 and Fig. 3B that the PS exposure is a slower process than the Ca^{2+} entry.

PS exposure was additionally measured by flow cytometry 30 min after incubation of RBCs with LPA, A23187, or PMA and results are presented in Fig. 4. The number of annexin V-positive cells after LPA treatment in the presence of Ca^{2+} was about 35% and significantly higher as compared to the A23187 treatment (20%). Also, PMA induced the highest PS exposure in human RBCs. It should be stated that these measurements using fluorescence microscopy (Fig. 3B) represent integrated values of the fluorescence intensity whereas the data obtained from the flow cytometry experiments depict the numbers of PS exposing cells (in %).

Based on the data obtained from flow cytometry experiments (quadrant analysis), we estimated the number of vesicles detached from the RBC membrane after 30 min. In case of LPA, A23187, or PMA treatment the number of vesicles per 30,000 RBCs was estimated as $10,698 \pm 2,363$ ($n = 3$), $6,626 \pm 2,898$ ($n = 4$), and $15,509 \pm 6,382$ ($n = 4$), respectively.

Experiments were also carried out after treating human RBCs with LPA, A23187, or PMA in the absence of extracellular Ca^{2+} (presence of 1 mM EGTA). In case of LPA and A23187, there were no significant differences in the PS exposure between treatment and control conditions (Fig. 4, (-) Ca^{2+}). Only in the case of PMA treatment, about 50% of RBCs showed PS exposure (compared to about 80% in the presence of Ca^{2+} , see Fig. 4, (+) Ca^{2+}). The data obtained for control conditions in the presence of Ca^{2+} shown in Fig. 4 were not significantly different from the control conditions in the absence of Ca^{2+} (data not shown).

Table 1. FACS analysis of RBCs showing a significant higher PS exposure compared to control conditions (in %) after treatment with 2.5 μM LPA, 2 μM A23187 or 6 μM PMA in the presence of 2 mM extracellular Ca^{2+} in HPS (7.5 mM KCl) or in a high K^+ solution (150 mM KCl). Mean values $\pm$ SD of 3 different blood samples (30,000 cells of each blood sample were analysed).

	7.5 mM KCl [%]	150 mM KCl [%]
LPA	36.3 ± 7.3	23.1 ± 2.9
4-bromo-A23187	27.7 ± 7.7	10.8 ± 5.5
PMA	78.5 ± 6.0	76.9 ± 8.8

Fig. 7. Flow cytometry analysis of sheep RBCs showing a significant higher PS exposure compared to control (in %) after treatment with 2.5 μM LPA, 2 μM A23187 or 6 μM PMA in the presence of 2 mM extracellular Ca^{2+} (incubation time 30 min). Columns show mean value of 3 different blood samples (30,000 cells of each blood sample were analysed). Error bars represent SD, $p \leq 0.05$ (*); $p \leq 0.01$ (**). Control measurements were done in the presence of solvents only (ethanol, water, or DMSO) and extracellular Ca^{2+} (see Material and Methods). Data for treatments with A23187 or PMA are statistically not significantly different from control.

Comparing the results of Figs. 2 and 4 it is evident that there is no correlation between the number of cells showing an elevated Ca^{2+} content and increased PS exposure. It should be noted that the threshold for the forward scatter in the flow cytometry measurements was set at a value so that only events based on the size of intact cells were counted, since micro-vesicle formation occurred after treatment with all 3 substances (see Fig. 3).

A lack of correlation between Ca^{2+} content and PS exposure was further supported by confocal fluorescence microscopy with double labelling experiments (for both, intracellular Ca^{2+} and PS in the outer membrane leaflet). The upper panels of Fig. 5 show the effect of LPA treat-

ment. Most of the cells exhibited both, an elevated Ca^{2+} content and external PS exposure (Fig. 5, upper panels, white arrows). However, some cells displayed an increased Ca^{2+} level, but no PS exposure (Fig. 5, upper panels, green arrows), whereas other cells externalise PS without a higher Ca^{2+} content (Fig. 5, upper panels, red arrows). The same observation was found for A23187-treated cells (middle panels of Fig. 5) although it should be noted that PS exposure without high Ca^{2+} content is relatively rare in both cases. The lower panels of Fig. 5 show that after PMA treatment PS exposure was more prevalent at low Ca^{2+} content in agreement with the findings presented in Figs. 2 and 4.

Experiments after PMA treatment, but in the complete absence of external Ca^{2+} (presence of 1 mM EGTA), were carried out using confocal fluorescence microscopy and double labelling of the cells (for Ca^{2+} and PS). In agreement with the flow cytometry measurements, many cells revealed PS exposure but none increased Ca^{2+} (data not shown). In addition, the presence of 1 mM *o*-vanadate to inhibit the Ca^{2+} pump failed to produce further significant changes (data not shown).

To consider a possible effect of cell volume alteration on PS exposure, experiments were carried out with the HPS replaced by a solution containing 150 mM KCl plus 2.5 mM NaCl instead of 145 mM NaCl plus 7.5 mM KCl. Under these conditions no net K^+ efflux occurs through the Ca^{2+} -dependent K^+ channel and hence no KCl loss and accompanying cell shrinkage. Table 1 reveals that a substantial part of the PS exposure in the presence of LPA or A23187 was due to volume decrease which was confirmed by analysing the forward scatter (FSC) by flow cytometry recordings (data not shown). Note that the PS exposure data in HPS (7.5 mM KCl) presented in Table 1 were not identical to the data shown in Fig. 4 since different blood samples were analysed for both investigations.

In comparison, in sheep RBCs (Fig. 6) LPA (upper panels) also increased the Ca^{2+} content. However, in comparison to human RBCs only very few cells showed PS exposure (see Fig. 5, upper panels). For A23187 (Fig. 6, lower panels) and PMA treatment (data not shown) there was no PS exposure although the intracellular Ca^{2+} content was increased in both cases in comparison to control conditions. These results were confirmed by flow cytometry and are depicted in Fig. 7. Only 2% of sheep RBCs treated with LPA expose PS on the outer membrane leaflet whilst A23187 and PMA treatment failed to induce PS exposure (Fig. 7), thus differing significantly from those obtained with human RBCs (see Fig. 4).

Discussion

The main idea was to investigate the process of PS exposure as a function of the intracellular Ca^{2+} content and the activation of PKC α in human and sheep RBCs. Flow cytometry, single cell fluorescence microscopy, and confocal fluorescence microscopy were used.

In HPS, the Ca^{2+} permeability of the RBC membrane is very low [35] and the basal level of free cytoplasmic Ca^{2+} is in the range of 60 nM maintained by the activity of the Ca^{2+} pump [36]. Our data reveal a fast uptake of Ca^{2+} by LPA stimulation (Fig. 1). This effect is probably mediated by a specific pathway rather than a non-specific leak since it has been proposed that a non-selective cation channel is activated by a still unclear mechanism [17, 27]. Other Ca^{2+} permeable cation channels in the RBC membrane also may be involved [7, 32, 37] (for a recent review see [14]). Furthermore the Ca^{2+} entry showed a clear LPA dose-dependent response [18].

Ca^{2+} uptake mediated by A23187 reached the same levels as LPA treatment after 30 min, although a delay in the response of about 5 min occurred, probably because A23187 partitions into the membrane before acting as ionophore. The effect of PMA (even at 10 μM) was fast at the beginning of the experiment without ever inducing high levels of intracellular Ca^{2+} , as compared to LPA and A23187 treatments. Flow cytometry experiments (Fig. 2B) performed at 12 min (when the Ca^{2+} content reached the maximum values, cf. Fig. 1) are consistent with data obtained by fluorescence microscopy. The mechanism by which PMA increases intracellular Ca^{2+} is different from the LPA and A23187 stimulations. Andrews et al. [38] assume that PMA activates PKC isoforms that in turn opens a ω -agatoxin-TK-sensitive, Cav2.1-like (P/Q-type) Ca^{2+} channel. It has been proposed that this channel may be identical with the voltage-activated non-selective cation channel [14]. PKC activation by PMA is Ca^{2+} -independent, whereas the channel opening leads to an increase of the intracellular Ca^{2+} content. In addition, PMA may activate the Ca^{2+} pump [34] and inhibit the Ca^{2+} -activated K^+ channel [34, 39].

From the images depicting PS exposure at different time points presented in Fig. 3, one can see that the increased Ca^{2+} content also leads to a vesiculation of the cells, in agreement with findings reported in literature [31, 32]. Calpain, a calcium-dependent proteolytic enzyme, cleaves spectrin and actin, leading to cytoskeleton breakdown [40–42], and therefore may be involved in vesiculation.

PMA induced a greater PS exposure in comparison to LPA and A23187 (Fig. 4, (+) Ca^{2+}). PS exposure was not directly correlated with the Ca^{2+} content since RBCs stimulated with PMA, at the same time point (30 min), displayed two-fold lower Ca^{2+} -dependent fluorescence when compared to LPA and A23187 treatment (cf. Figs. 1 and 4).

These findings were further supported by the results of the double labelling experiments presented in Fig. 5. After treatment with LPA or A23187 many cells (in case of LPA most of the cells) showed an increased Ca^{2+} content and external PS exposure. However, in both cases some cells had an increased Ca^{2+} level but no PS exposure and, vice versa, some cells depicted externalised PS in the absence of an increased Ca^{2+} content. This situation was rare in both cases and it cannot be ruled out that, after 30 min of incubation, a reduction of the intracellular Ca^{2+} content due to the activation of the Ca^{2+} pump occurred in some cells, in agreement with the findings shown in Fig. 1 (i.e. a sub-population of cells behaved different from others). In case of the PMA treatment, some cells displayed an enhanced Ca^{2+} content and PS exposure. Interestingly, more cells as compared to LPA or A23187 treated cells, show PS exposure without having an elevated Ca^{2+} content. In a control experiment, i.e. in the absence of external Ca^{2+} (Fig. 4), some cells showed PS exposure. Thus the primary cause of PS exposure stimulated by PMA is different from the LPA or A23187 stimulation. Finally, the cell volume seems to be important for PS exposure in agreement with findings of Lang et al. [43, 44] but only in case of LPA or A23187 treatment. However, we cannot rule out the possibility that the extracellular K^{+} concentration might contribute to the observed volume effect on the PS exposure in case of LPA or A23187 treatment, which would be in agreement with the results of Wolfs et al. [45].

Surprisingly, comparable experiments with sheep RBCs did not show PS exposure after treatment with the Ca^{2+} ionophore or PMA although the intracellular Ca^{2+} content was enhanced. However, treatment with LPA resulted in a very small number of cells externalising PS. These effects can be explained by assuming absence of scramblase activity. In turn, LPA had an additional but minor effect on PS exposure (see below). The presumed absence of the scramblase activity in sheep RBCs is supported by findings of the group of A. Herrmann (Humboldt University, Berlin) applying spin-labelled lipids and investigating the PS movement across the RBC membrane of a great variety of animal species by electron spin resonance (A. Herrmann, personal communication). Indeed,

data mining using the scramblase amino acid sequences against protein databases failed to show any match with proteins from sheep. Furthermore, the external leaflet of the sheep RBC membrane contains 100% SM and no PC [25, 26]. SM is transported across the membrane very slowly (even slower than PC) [46] and therefore no need for the presence of the scramblase exists in sheep RBCs.

Based on the findings of this paper, it seems reasonable to assume at least 3 different mechanisms being responsible for the PS exposure in human RBCs:

First, a Ca^{2+} -stimulated scramblase activation (and flippase inhibition) [4, 6, 7, 47, 48] brought about by LPA, A23187, or PMA treatment inducing Ca^{2+} uptake by different pathways. LPA activates the non-selective cation channel [14] whereas PMA opens the ω -agatoxin-TK-sensitive, Cav2.1-like (P/Q-type) Ca^{2+} channel [38]. This first mechanism is a rather general one that can occur under various pathological conditions, e.g. by activation of NMDA receptors in sickle cell disease as discussed by Makhro et al. [49].

Second, PMA directly activates $\text{PKC}\alpha$ involved in PS exposure in human RBCs [22, 23]. In addition, LPA activates in human RBCs both the $\text{PKC}\alpha$ (Ca^{2+} -dependent) and the $\text{PKC}\zeta$ (Ca^{2+} -independent) [20]. Whether there is a direct activation of the scramblase in human RBCs by $\text{PKC}\alpha$ and/or $\text{PKC}\zeta$ remains to be shown. The more pronounced effect of PS exposure after treatment of RBCs with PMA as compared to LPA can be explained by assuming that PMA activates all available $\text{PKC}\alpha$ whereas LPA stimulation triggers a signalling cascade [50] resulting only in partial activation of the $\text{PKC}\alpha$ pool.

Third, an additional mechanism has to be taken into consideration in the case of sheep RBCs. LPA may insert into the membrane (more pronounced in the presence of Ca^{2+} since it becomes more hydrophobic) stimulating the flop of PS from the inner to the outer membrane leaflet. Such an event would explain the higher rate of haemolysis after LPA treatment as compared to A23187 or PMA treatment. In comparison to the first and second mechanisms, this third effect only plays a minor role in the PS exposure which may be due to the fact that the LPA concentration used was far below the micelle forming concentration [51].

How the 3 pathways are interconnected remains to be elucidated in further investigations. The situation is even more challenging since other parameters, not being addressed here, are also known to affect the PS exposure in human RBCs. For instance, it has been shown

that activation of caspases, production of ceramides, oxidative stress as well as ATP depletion increase the PS exposure [7, 31, 32, 52]. ATP depletion is a Ca^{2+} -dependent process because at higher intracellular Ca^{2+} levels the Ca^{2+} pump is activated and will consume more ATP. It has also been demonstrated that the intracellular K^{+} content and the cell shape can affect the PS exposure in RBCs [45, 53]. Furthermore, it has been demonstrated that RBCs show an increased PS exposure after long-term storage and induced by hyperosmotic shock [54].

Conclusion

PS exposure in RBCs is a complex process involving at least 3 mechanisms. A variety of Ca^{2+} -dependent as well as Ca^{2+} -independent parameters play a substantial role for PS exposure (see e.g. [52, 55, 56]). Since the Ca^{2+} -dependent and Ca^{2+} -independent mechanisms are not additive, an interaction between those pathways can be assumed. Cell volume and probably the Ca^{2+} pump modulate the signalling cascade leading to PS exposure.

Furthermore, the majority of PS is transported to the outer membrane leaflet mediated by scramblase activity but an additional minor effect based on lipid flop has to be taken into consideration.

Externalisation of PS to the outer membrane leaflet is important for the adherence of RBCs to endothelium [19, 57, 58] as well as for the RBC-RBC adhesion [9, 17, 18]. Knowledge of the underlying mechanisms is important for clinical prevention of thrombus formation.

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References

- Haest CWM: Distribution and movement of membrane lipids; in Bernhardt I, Elorri JC (eds): Red cell membrane transport in health and disease. Berlin, Springer, 2003, pp 1-25.
- Devaux PF, Herrmann A, Ohlwein N, Kozlov MM: How lipid flippases can modulate membrane structure. *Biochim Biophys Acta* 2008;1778:1591-1600.
- Beyers EM, Comfurius P, Dekkers DW, Zwaal RF: Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta* 1999;1439:317-330.
- Daleke DL: Regulation of phospholipid asymmetry in the erythrocyte membrane. *Curr Opin Hematol* 2008;15:191-195.
- Zwaal RF, Comfurius P and Beyers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971-988.
- Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M: Eryptosis, a window to systemic disease. *Cell Physiol Biochem* 2008;22:373-380.
- Lang F, Lang KS, Lang PA, Huber SM, Wieder T: Mechanisms and significance of eryptosis. *Antioxid Redox Signal* 2006;8:1183-1192.
- Hellem AJ, Borchgrevink CF, Ames SB: The role of red cells in haemostasis: the relation between haematocrit, bleeding time and platelet adhesiveness. *Br J Haematol* 1961;7:42-50.
- Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76-82.
- Kaestner L, Bernhardt I: Ion channels in the human red blood cell membrane: their further investigation and physiological relevance. *Bioelectrochemistry* 2002;55:71-74.
- Kaestner L, Tabellion W, Lipp P, Bernhardt I: Prostaglandin E_2 activates channel-mediated calcium entry in human erythrocytes: an indication for a blood clot formation supporting process. *Thromb Haemost* 2004;92:1269-1272.
- Kaestner L, Bollensdorff C, Bernhardt I: Non-selective voltage-activated cation channel in the human red blood cell membrane. *Biochim Biophys Acta* 1999;1417:9-15.
- Kaestner L, Christophersen P, Bernhardt I, Bennekou P: The non-selective voltage-activated cation channel in the human red blood cell membrane: reconciliation between two conflicting reports and further characterisation. *Bioelectrochemistry* 2000;52:117-125.
- Kaestner L: Cation channels in erythrocytes - historical and future perspective. *Open Biol J* 2011;4:27-34.
- Dyrda A, Cytlik U, Ciuraszkiewicz A, Lipinska A, Cuffe A, Bouyer G, Egee S, Bennekou P, Lew VL, Thomas SL: Local membrane deformations activate Ca^{2+} -dependent K^{+} and anionic currents in intact human red blood cells. *PLoS One* 2010;5:e9447.
- Oonishi T, Sakashita K, Ishioka N, Suematsu N, Shio H, Uyesaka N: Production of prostaglandins E_1 and E_2 by adult human red blood cells. *Prostaglandins Other Lipid Mediat* 1998;56:89-101.
- Steffen P, Jung A, Nguyen DB, Mueller T, Bernhardt I, Kaestner L, Wagner C: Stimulation of human red blood cell leads to Ca^{2+} -mediated intercellular adhesion. *Cell Calcium* 2011;50:54-61.
- Kaestner L, Steffen P, Nguyen DB, Wang J, Wagner-Britz L, Jung A, Wagner C, Bernhardt I: Lysophosphatidic acid induced red blood cell aggregation in vitro. *Bioelectrochemistry* 2011;in press.
- Closse C, Dachary-Prigent J, Boisseau MR: Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *Br J Haematol* 1999;107:300-302.

- 20 Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH: Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *Arterioscler Thromb Vasc Biol* 2007;27:414-421.
- 21 Woon LA, Holland JW, Kable EP, Roufogalis BD: Ca^{2+} sensitivity of phospholipid scrambling in human red cell ghosts. *Cell Calcium* 1999;25:313-320.
- 22 De Jong K, Rettig MP, Low PS, Kuypers FA: Protein kinase C activation induces phosphatidylserine exposure on red blood cells. *Biochemistry* 2002;41:12562-12567.
- 23 Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M, Eisele K, Podolski M, Huber SM, Wieder T, Lang F: Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. *Am J Physiol* 2006;290:C244-C253.
- 24 Govekar RB, Zingde SM: Protein kinase C isoforms in human erythrocytes. *Ann Hematol* 2001;80:531-534.
- 25 Van Dijck PW, De Kruijff B, Van Deenen LL, De Gier J, Demel RA: The preference of cholesterol for phosphatidylcholine in mixed phosphatidylcholine-phosphatidylethanolamine bilayers. *Biochim Biophys Acta* 1976;455:576-587.
- 26 Saito M, Tanaka Y, Ando S: Thin-layer chromatography-densitometry of minor acidic phospholipids: application to lipids from erythrocytes, liver, and kidney. *Anal Biochem* 1983;132:376-383.
- 27 Kaestner L, Tabellion W, Weiss E, Bernhardt I, Lipp P: Calcium imaging of individual erythrocytes: problems and approaches. *Cell Calcium* 2006;39:13-19.
- 28 Gardos G: The permeability of human erythrocytes to potassium. *Acta Physiol Hung* 1956;10:185-189.
- 29 Hoffman JF, Joiner W, Nehrke K, Potapova O, Foye K, Wickrema A: The hSK4 (KCNN4) isoform is the Ca^{2+} -activated K^+ channel (Gardos channel) in human red blood cells. *Proc Natl Acad Sci USA* 2003;100:7366-7371.
- 30 Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca^{2+} -activated K^+ channels in human erythrocyte apoptosis. *Am J Physiol* 2003;285:C1553-C1560.
- 31 Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM, Lang F: Mechanisms of suicidal erythrocyte death. *Cell Physiol Biochem* 2005;15:195-202.
- 32 Foeller M, Huber SM, Lang F: Erythrocyte programmed cell death. *IUBMB Life* 2008;60:661-668.
- 33 Lord JM, Pongracz J: Protein kinase C: a family of isoenzymes with distinct roles in pathogenesis. *Clin Mol Pathol* 1995;48:M57-M64.
- 34 Fathallah H, Sauvage M, Romero JR, Canessa M, Giraud F: Effects of PKC α activation on Ca^{2+} pump and $\text{K}(\text{Ca})$ channel in deoxygenated sickle cells. *Am J Physiol* 1997;273:C1206-C1214.
- 35 Tiffert T, Bookchin RM, Lew VL: Calcium homeostasis in normal and abnormal human red cells; in Bernhardt I, Elorry JC (eds): *Red cell membrane transport in health and disease*. Berlin, Springer, 2003, pp 373-405.
- 36 Gilboa H, Chapman BE, Kuchel PW: ^{19}F NMR magnetization transfer between 5-FBAPTA and its complexes. An alternative means for measuring free Ca^{2+} concentration, and detection of complexes with protein in erythrocytes. *NMR Biomed* 1994;7:330-338.
- 37 Duranton C, Huber S, Tanneur V, Lang K, Brand V, Sandu C, Lang F: Electrophysiological properties of the plasmodium falciparum-induced cation conductance of human erythrocytes. *Cell Physiol Biochem* 2003;13:189-198.
- 38 Andrews DA, Yang L, Low PS: Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. *Blood* 2002;100:3392-3399.
- 39 Del Carlo B, Pellegrini BM, Pellegrino M: Modulation of Ca^{2+} -activated K^+ channels of human erythrocytes by endogenous protein kinase C. *Biochim Biophys Acta* 2003;1612:107-116.
- 40 Anderson DR, Davis JL, Carraway KL: Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease. *J Biol Chem* 1977;252:6617-6623.
- 41 Hatanaka M, Yoshimura N, Murakami T, Kannagi R, Murachi T: Evidence for membrane-associated calpain I in human erythrocytes. Detection by an immunoelectrophoretic blotting method using monospecific antibody. *Biochemistry* 1984;23:3272-3276.
- 42 Lofvenberg L, Backman L: Calpain-induced proteolysis of beta-spectrins. *FEBS Lett* 1999;443:89-92.
- 43 Lang F, Lang KS, Wieder T, Myssina S, Birka C, Lang PA, Kaiser S, Kempe D, Duranton C, Huber SM: Cation channels, cell volume and the death of an erythrocyte. *Pflügers Arch* 2003;447:121-125.
- 44 Lang F, Gulbins E, Szabo I, Lepple-Wienhues A, Huber SM, Duranton C, Lang KS, Lang PA, Wieder T: Cell volume and the regulation of apoptotic cell death. *J Mol Recognit* 2004;17:473-480.
- 45 Wolfs JLN, Comfurius P, Bekers O, Zwaal RFA, Balasubramanian K, Schroit AJ, Lindhout T, Bevers EM: Direct inhibition of phospholipid scrambling activity in erythrocytes by potassium ions. *Cell Mol Life Sci* 2009;66:314-323.
- 46 Van Meer G, Holthuis JC: Sphingolipid transport in eukaryotic cells. *Biochim Biophys Acta* 2000;1486:145-170.
- 47 Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF: Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. *Biochemistry* 1992;31:6355-6360.
- 48 Daleke DL: Regulation of phospholipid asymmetry in the erythrocyte membrane. *Curr Opin Hematol* 2008;15:191-195.
- 49 Makhro A, Wang J, Vogel J, Boldyrev AA, Gassmann M, Kaestner L, Bogdanova A: Functional NMDA receptors in rat erythrocytes. *Am J Physiol Cell Physiol* 2010;298:C1315-C1325.
- 50 Choi JW, Herr DR, Noguchi K, Yung YC, Lee C, Mutoh T, Lin M, Teo ST, Park KE, Mosley AN, Chun J: LPA receptors: subtypes and biological actions. *Annu Rev Pharmacol Toxicol* 2010;50:157-186.
- 51 Eichholtz T, Jalink K, Fahrenfort I: The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* 1993;291:677-680.
- 52 Mandal D, Moitra PK, Saha S, Basu J: Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett* 2002;513:184-188.
- 53 Wolfs JLN, Comfurius P, Bevers EM, Zwaal RFA: Influence of erythrocyte shape on the rate of Ca^{2+} -induced scrambling of phosphatidylserine. *Mol Membr Biol* 2003;20:83-91.
- 54 Bosman GJCGM, Cluitmans JCA, Groenen YAM, Were JM, Willekens FLA, Novotny VMJ: Susceptibility to hyperosmotic stress-induced phosphatidylserine exposure increases during red blood cell storage. *Transfusion* 2011;51:1072-1087.
- 55 Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S, Jendrosseck V, Ishii S, Shimizu T, Waidmann M, Hessler G, Huber SM, Lang F, Wieder T: Stimulation of erythrocyte ceramide formation by platelet-activating factor. *J Cell Sci* 2005;118:1233-1243.
- 56 Kiedaisch V, Akel A, Niemoeller OM, Wieder T, Lang F: Zinc-induced suicidal erythrocyte death. *Am J Clin Nutr* 2008;87:1530-1534.
- 57 Telen MJ: Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. *Semin Hematol* 2000;37:130-142.
- 58 Setty BN, Kulkarni S, Stuart MJ: Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. *Blood* 2002;99:1564-1571.