Original Paper

Measurements of Intracellular Ca^{2+} Content and Phosphatidylserine Exposure in Human Red Blood Cells: Methodological Issues

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Key Words

Red blood cells • Ca^{2+} content • Phosphatidylserine exposure • Lysophosphatidic acid • Flow cytometry • Fluorescence imaging

Abstract

Background/Aims: The increase of the intracellular Ca^{2+} content as well as the exposure of phosphatidylserine (PS) on the outer cell membrane surface after activation of red blood cells (RBCs) by lysophosphatidic acid (LPA) has been investigated by a variety of research groups. Carrying out experiments, which we described in several previous publications, we observed some discrepancies when comparing data obtained by different investigators within our research group and also between batches of LPA. In addition, we found differences comparing the results of double and single labelling experiments (for Ca^{2+} and PS). Furthermore, the results of PS exposure depended on the fluorescent dye used (annexin V-FITC *versus* annexin V alexa fluor® 647). Therefore, it seems necessary to investigate these methodological approaches in more detail to be able to quantify results and to compare data obtained by different research groups. **Methods:** The intracellular Ca^{2+} content and the PS exposure of RBCs separated from whole blood have been investigated after treatment with LPA (2.5 μM) obtained from three different companies (Sigma-Aldrich, Cayman Chemical Company, and Santa Cruz Biotechnology Inc.). Fluo-4 and x-rhod-1 have been used to detect intracellular Ca^{2+} content, annexin V alexa fluor® 647 and annexin V-FITC have been used for PS exposure measurements. Both parameters (Ca^{2+} content, PS exposure) were studied using flow cytometry and fluorescence microscopy. **Results:** The percentage of RBCs showing increased intracellular Ca^{2+} content as well as PS exposure changes significantly between different LPA manufacturers as well as on the condition of mixing of LPA with the RBC suspension. Furthermore, the percentage of RBCs showing PS exposure is reduced in double labelling compared to single labelling experiments and depends also on the fluorescent dye used. Finally, data on Ca^{2+} content are slightly affected whereas PS exposure data are not affected significantly by the measuring

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method (flow cytometry, fluorescence microscopy). **Conclusion:** The LPA batch used and the mixing procedure of LPA and the RBC suspension has to be taken into consideration when comparing results of intracellular Ca^{2+} content and PS exposure of RBCs after LPA activation. In addition, one should consider that the results of single and double labelling experiments might be different depending on the fluorescent dyes used.

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Introduction

In previous papers we have demonstrated that lysophosphatidic acid (LPA) or prostaglandin E_2 (PGE_2) open the non-specific, voltage-dependent cation (NSVDC) channel, which leads to an enhanced intracellular Ca^{2+} content of red blood cells (RBCs) [1, 2], although the molecular regulation mechanism of the Ca^{2+} entry still remains elusive. Both substances are local mediators released from platelets after their activation within the coagulation cascade. An increase of the intracellular Ca^{2+} content of RBCs can be also observed after activation of protein kinase α ($\text{PKC}\alpha$), e.g. by phorbol-12 myristate-13 acetate (PMA). Two independent Ca^{2+} entry processes were reported, the first is P-Type $\text{Ca}_v2.1$ channel independent and the second is associated with a likely indirect activation of $\text{Ca}_v2.1$ [3]. A higher intracellular Ca^{2+} content leads to an activation of the scramblase, which in turn results in a significant exposure of phosphatidylserine (PS) in the outer membrane leaflet [4-6]. We also demonstrated that intracellular increase in Ca^{2+} induced by LPA leads to cell-cell adhesion of human RBCs [7, 8]. It has been reported that PS exposure in the outer membrane leaflet of the RBC membrane is of importance for the adhesion of RBCs to the endothelium in certain diseases (e.g. [9-12]). An active role of RBCs in thrombus formation has been proposed by Andrews and Low [13] whilst a correlation between decreased haematocrit and longer bleeding times has also been reported [14]. A signalling cascade was proposed by Kaestner et al. [2].

The increase of the intracellular Ca^{2+} content as well as the external presentation of PS after stimulation of RBCs by LPA or PMA has been investigated by many research groups [3, 7, 8, 15-23]. These investigations were mainly carried out to investigate physiological parameters affecting the PS exposure. Translocation of PS from the inner to the outer membrane leaflet of RBCs is a typical sign of eryptosis (a term introduced by Lang [24]), defining the suicidal death of RBCs. A large variety of physiological parameters as well as substances have been described to induce eryptosis (e.g., [25-31]).

The experiments on PS exposure performed in our research group have been carried out by several investigators. During these studies we observed quantitative discrepancies with respect to different investigators but also with respect to the LPA batches used. In addition, we realized differences comparing the results of double and single labelling experiments (for Ca^{2+} and PS). Furthermore, the results of PS exposure depended on the fluorescent dye used (annexin V-FITC *versus* annexin V alexa fluor® 647). We report here about the methodological approaches of the measurements of intracellular Ca^{2+} content as well as PS exposure to understand the challenge and to be able to compare results obtained by different research groups.

Material and Methods

Blood and solution

Human venous blood from healthy donors was obtained from the Institute of Sports and Preventive Medicine of Saarland University and the Institute of Clinical Haematology and Transfusion Medicine of Saarland University Hospital. EDTA or heparin was 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 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, the RBCs were re-suspended in HPS and stored at 4°C until the beginning of the experiment. The experiment was started immediately after resuspension of the RBCs.

Single labelling experiments

Measurement of intracellular Ca^{2+} content: RBCs were loaded with 1 μM fluo-4 AM or 1 μM x-rhod-1 AM from a 1 mM stock solution in dimethyl sulfoxide (DMSO) in 2 ml HPS. The extracellular Ca^{2+} concentration was 2 mM, i.e. CaCl_2 was added to the HPS. Cells were incubated at a haematocrit of 0.1 % in the dark for 30 min at 37°C with continuous shaking. Then the cells were washed again (16,000 g for 10 s), with an ice-cold HPS, re-suspended and measured as a control (at room temperature), or incubated with LPA (2.5 μM) at 37°C to activate Ca^{2+} uptake. Stock solutions for LPA from different companies (for comparison, 1 mM) were prepared in phosphate buffered saline. The incubation times with LPA were 1, 15 and 30 min. After incubation the cells were washed again (16,000 g for 10 s) with an ice-cold HPS, re-suspended and measured at room temperature.

Measurement of PS exposure: The cells were prepared as for measurement of the Ca^{2+} content. To detect the PS exposure, either annexin V-FITC or annexin V alexa fluor® 647, at a concentration of 4.5 μM was used. Annexin V binds to PS in the outer layer of the membrane and is coupled with a fluorescent dye (FITC or alexa fluor® 647), which can be measured by flow cytometry and fluorescence microscopy [32]. First a control measurement without LPA was performed. After that, LPA (2.5 μM) was added to activate the Ca^{2+} uptake. The cells were then incubated at 37°C for 1, 15 and 30 min. After incubation the cells were washed again (16,000 g for 10 s) with an ice-cold HPS and re-suspended. Finally, annexin V-FITC or annexin V alexa fluor® 647 was added to the cells. The staining was performed at a haematocrit of 0.1% in HPS solution with the addition of 2 mM Ca^{2+} at room temperature for 10 min. The measurements were also performed at room temperature.

The procedure to prepare the RBCs for measurement of intracellular Ca^{2+} content as well as PS exposure is based on the protocols of Nguyen et al. [15] and Wesseling et al. [16]. The time intervals between various incubation steps were kept as short as possible.

Double labelling experiments

For double labelling experiments we followed the protocols developed by Nguyen et al. [15] and Wesseling et al. [16]. The combinations of the fluorescent dyes applied were: (i) fluo-4 and annexin V alexa fluor® 647 or (ii) x-rhod-1 and annexin V-FITC to detect Ca^{2+} and PS, respectively.

For double labelling experiments the same procedure was used as for single labelling experiments to measure the Ca^{2+} content, i.e. for Ca^{2+} loading the extracellular Ca^{2+} concentration was 2 mM. The cells were stimulated with LPA and incubated at 37°C for 1, 15 and 30 min. After the last re-suspension of the RBCs for Ca^{2+} measurements (see above) annexin V-FITC or annexin V alexa fluor® 647 (4.5 μM) was added to detect PS and the cells incubated at room temperature for 10 min. Each sample has been measured first using flow cytometry and subsequently using fluorescence microscopy.

Flow cytometry

Intracellular free Ca^{2+} content as well as PS exposure was measured by flow cytometry (FACS Calibur and Cell Quest Pro software, Becton Dickinson Biosciences, Franklin Lakes, USA) as described before [15, 16, 33]. Ca^{2+} was measured in the FL-1 channel (excitation at 488 nm, emission at 520/15 nm) for fluo-4 and in the FL-2 channel (excitation at 550 nm, emission at 585/42 nm) for x-rhod-1. PS exposure in case of annexin V-FITC was detected also in the FL-1 channel. In case of annexin V alexa fluor® 647 it was detected in the FL-4 channel (excitation at 633 nm, emission at 661/16 nm) with a xenon diode. In all cases the negative and positive gates were identified based on control experiments (without stimulating substances) and A23187 (2 μM), respectively. Compensation was not necessary since there was no overlapping. The relative fluorescence intensity was analysed using the mean value of 30,000 cells from each blood sample. For each condition at least three different blood samples were used.

Fluorescence microscopy

The RBCs were monitored using an inverted fluorescence microscope (Eclipse TE2000-E, Nikon, Tokyo, Japan) as described before [15, 16]. The diluted RBC samples (approximately 0.025% haematocrit)

were placed on a cover slip in a dark room at room temperature. Images were taken with an electron multiplication CCD camera (CCD97, Photometrics, Tucson, USA) using a 100x1.4 (NA) oil immersion lens with infinity corrected optics. From each RBC sample, 5 images from different positions of the cover slip randomly chosen were taken using the imaging software VisiView (Visitron Systems, Puchheim, Germany). Each image consists of one transmitted light (exposure time 200 ms) and one (for single labelling) or two (for double labelling) fluorescence shot (exposure time 4 s). Fluo-4 and annexin V-FITC were 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. X-rhod-1 and annexin V alexa fluor® 647 were excited at a centre wavelength of 543 nm and 661 nm, respectively. Emission was recorded at 610/20 nm for x-rhod-1 and 660/20 nm for annexin V alexa fluor® 647.

Reagents

If not mentioned otherwise, all chemicals used were purchased from Sigma-Aldrich (Munich, Germany). LPA was obtained from Sigma-Aldrich, Cayman Chemical Company (Ann Arbor, USA), and Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Fluo-4 AM and annexin V-FITC were obtained from Molecular Probes (Eugene, USA) and annexin V alexa fluor® 647 from Roche Diagnostics GmbH (Mannheim, Germany).

Statistical significance

Data are presented as mean values $\pm$ SD of at least 3 independent experiments. The significance of differences was tested by ANOVA. Statistical significance of the data was defined as follows: $p > 0.05$ (n.s.); $0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)

Results and Discussion

Comparison of RBC activation using LPA from different companies

In our experiments described in previous papers [3, 15, 16, 21] we realised that RBC stimulation with LPA from different batches obtained from Sigma-Aldrich led to significant different results of the intracellular Ca^{2+} content as well as PS exposure. To investigate this phenomenon in more detail we performed experiments with LPA from 4 different batches, 2 from Sigma-Aldrich, one from Cayman Chemical Company, and one from Santa Cruz Biotechnology Inc. All 4 LPA probes were freshly ordered, re-frozen only once and were applied to the same blood on the same day and using the same HPS. In addition, all experiments were carried out by the same investigator using identical experimental conditions (see below). The results for double labelling measurement of intracellular Ca^{2+} content (with x-rhod-1) and PS exposure (with annexin V-FITC) in RBCs at LPA incubation times of 1 min, 15 min and 30 min using flow cytometry are shown in Fig. 1. The control data, i.e. Ca^{2+} content and PS exposure of RBCs without LPA activation, are shown at time zero. In all cases of LPA activation there is a significant increase in the percentage of RBCs showing increased intracellular Ca^{2+} content (Fig. 1A) and PS exposure (Fig. 1B) compared to control. Interestingly, there are significant differences in the percentages of RBCs responding with increased intracellular Ca^{2+} content as well as PS exposure depending on the LPA batch used. The highest LPA activation was obtained using one batch from Sigma-Aldrich (batch 1) and from Cayman Chemical Company (not significantly different) followed by a batch from Santa Cruz Biotechnology Inc. (significantly different from the two batches mentioned before). LPA from the second batch from Sigma-Aldrich (batch 2) resulted in much less stimulation (significantly different from all others) at any time point measured. In addition, we found that the LPA activation efficiency within a single batch decreased with the number of times the LPA stock solution was frozen and thawed. After 3 times thawing, the activation efficiency for Ca^{2+} measurements decreased by about 37% and 26% for fluo-4 and x-rhod-1, respectively. At the same time the activation efficiency for PS exposure decreased by 55%, both for annexin V-FITC and annexin V alexa fluor® 647. The findings are of importance comparing results of RBC activation with LPA from different batches of Sigma-Aldrich as

Fig. 1. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content (measured using x-rhod-1) and (B) responding with increased PS exposure (measured using annexin V-FITC) after activation with LPA ($2.5 \mu\text{M}$) obtained from different companies depending on time using flow cytometry. White square: LPA from Sigma-Aldrich (batch 1); dark square: LPA from Sigma-Aldrich (batch 2); dark circle: LPA from Cayman Chemical Company; dark triangle: LPA from Santa Cruz Biotechnology Inc. $N = 3$ (90,000 cells), error bars = S.D. (only half error bar is shown for convenience). Significant differences for LPA batches at a certain time, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)); for Ca^{2+} content, time point 1 min: * Cayman and Sigma 2 vs. Santa Cruz, *** Cayman and Sigma 1 vs. Sigma 2; time point 15 min: ** Santa Cruz vs. Sigma 2, *** Cayman vs. Sigma 2, Sigma 1 vs. Sigma 2; time point 30 min: ** Santa Cruz vs. Sigma 2, Sigma 1 vs. Sigma 2, *** Cayman vs. Sigma 2; for PS exposure, time point 1 min: * Cayman vs. Sigma 2; time point 15 min: ** Santa Cruz vs. Sigma 2, *** Cayman vs. Sigma 2, Sigma 1 vs. Sigma 2; time point 30 min: * Cayman vs. Santa Cruz, ** Sigma 1 vs. Sigma 2, *** Cayman vs. Sigma 2. Significant differences for LPA batches for PS exposure depending on time: LPA from Sigma 1: * 1 min vs. 30 min, ** 1 min vs. 15 min; LPA from Cayman: *** 1 min vs. 15 min and 30 min.

well as from different companies. For our investigations reported in the present paper we divided the obtained LPA into aliquots to avoid repeated thawing.

Comparison of RBC activation using LPA with different mixing conditions

To study a possible effect of the experimenter on the results obtained, we performed experiments with two different ways of mixing the RBC suspensions after LPA activation. Double labelling experiments for Ca^{2+} content (using x-rhod-1) and PS exposure (using annexin V-FITC) after activation of RBCs with LPA obtained from Cayman Chemical Company were compared following simple shaking by hand and with vortexing the RBC suspensions (both in Eppendorf tubes, 5 s each). The results are presented in Fig. 2. One can observe a significant higher percentage of RBCs responding with increased intracellular Ca^{2+} content as well as PS exposure when the suspension is vortexed after addition of LPA compared with simple shaking by hand. Similar relationships were obtained when the fluorescent dye combination fluo-4 (to detect Ca^{2+}) and annexin V alexa fluor® 647 (to detect PS) has been used (data not shown). These results suggest that a mechanosensitive channel, like the recently reported Piezo1, may contribute to the LPA-induced Ca^{2+} entry ([34] and references therein).

Comparison of RBC activation using LPA between single and double labelling. Effect of different fluorescent dyes

The intracellular Ca^{2+} content and the PS exposure of RBCs can be measured on the basis of single as well as double labelling experiments. Furthermore, different fluorescent dyes are available for the detection of either parameter. It was shown that fura-2 cannot be used for Ca^{2+} measurement in RBCs because of the haemoglobin absorption [35]. Commonly used for the Ca^{2+} and PS measurements are fluo-4 (or fluo-3) and annexin V-FITC, respectively [36-39]. However, for double labelling experiment the combination of these

Fig. 2. Percentage of RBCs responding with increased intracellular Ca^{2+} content (detected with x-rhod-1) as well as PS exposure (detected with annexin V-FITC) after activation with LPA obtained from Cayman Chemical Company ($2.5 \mu\text{M}$) for 1 min with and without shaking by hand or vortexing the RBC suspensions using flow cytometry. Left 3 columns: RBCs with increased intracellular Ca^{2+} content. Right 3 columns: RBCs with increased PS exposure: White bars: control measurements (without LPA activation), grey bars: shaking the RBC suspensions by hand for 5 s, black bars: vortexing the RBC suspensions for 5 s. $N = 3$ (90,000 cells), error bars = S.D. (only half error bar is shown for convenience). Significant differences, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***) for Ca^{2+} content: ** control vs. LPA with shaking by hand, LPA with shaking by hand vs. LPA with vortexing, *** control vs. LPA with vortexing. For PS exposure: * control vs. LPA with shaking by hand, ** LPA with shaking by hand vs. LPA with vortexing, *** control vs. LPA with vortexing.

Fig. 3. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content and (B) with increased PS exposure after 1 min activation with LPA ($2.5 \mu\text{M}$) obtained from Cayman Chemical Company using flow cytometry. Comparison between single labelling (SL) and double labelling (DL) experiments using x-rhod-1 and fluo-4 for Ca^{2+} detection and annexin V-FITC and annexin V alexa fluor[®] 647 for PS detection. All experiments were done with vortexing the RBC suspensions. (A): Black column: SL, fluo-4, dark grey column: DL, fluo-4 and annexin V alexa fluor[®] 647 (AV647), light grey column: SL, x-rhod-1, white column: DL, x-rhod-1 and annexin V-FITC (AV-FITC). (B): Black column: SL, annexin V alexa fluor[®] 647 (AV647), dark grey column: DL, annexin V alexa fluor[®] 647 (AV647) and fluo-4, light grey column: SL, annexin V-FITC (AV-FITC), white column: DL, annexin V-FITC (AV-FITC) and x-rhod-1. $N = 3$ (90,000 cells), error bars = S.D. (only half error bar is shown for convenience). Significant differences, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)) are shown in the figure.

dyes is not suitable since they are both derivatives of fluorescein and thus their absorption and emission spectra are similar. Therefore, we compared results obtained on the basis of single labelling (fluo-4 and x-rhod-1 for Ca^{2+} , annexin V alexa fluor[®] 647 and annexin V-FITC for PS) and double labelling experiments using a combination of fluo-4 (for Ca^{2+}) / annexin V alexa fluor[®] 647 (for PS) and x-rhod-1 (for Ca^{2+}) / annexin V-FITC (for PS). The data after 1 min LPA activation are shown in Fig. 3. For these experiments LPA from Cayman Chemical Company was used. Fig. 3A indicates that for intracellular Ca^{2+} content there is no significant

Fig. 4. Percentage of RBCs (A) responding with increased intracellular Ca^{2+} content and (B) with increased PS exposure after 30 min activation with LPA (2.5 μM) obtained from Sigma-Aldrich (batch 1). Comparison between flow cytometry (left 4 columns) and fluorescence microscopy (right 4 columns) measurements based on single labelling (SL) and double labelling (DL) experiments using x-rhod-1 and fluo-4 for Ca^{2+} detection and annexin V-FITC and annexin V alexa fluor[®] 647 for PS detection. All experiments were done with vortexing the RBC suspensions. (A): Black column: SL, fluo-4, dark grey column: DL, fluo-4 and annexin V alexa fluor[®] 647 (AV647), light grey column: SL, x-rhod-1, white column: DL, x-rhod-1 and annexin V-FITC (AV-FITC). (B): Black column: SL, annexin V alexa fluor[®] 647 (AV647), dark grey column: DL, annexin V alexa fluor[®] 647 (AV647) and fluo-4, light grey column: SL, annexin V-FITC (AV-FITC), white column: DL, annexin V-FITC (AV-FITC) and x-rhod-1. N = 3 (90,000 cells), error bars = S.D. (only half error bar is shown for convenience). Significant differences, ANOVA ($0.01 < p \leq 0.05$ (*); $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)) are shown in the figure.

difference between single and double labelling experiments. In addition, the results do not depend on the fluorescent dye (or the combination of the fluorescent dyes) used. The values for PS exposure are illustrated in Fig. 3B. It can be seen that the values of double labelling experiments are significantly lower for both fluorescent dyes (annexin V-FITC and annexin V alexa fluor[®] 647 (for PS), in combination with x-rhod-1 and fluo-4 (for Ca^{2+}), respectively) compared to single labelling experiments (annexin V-FITC or annexin V alexa fluor[®] 647 alone (for PS)). In addition, the amount of RBCs showing PS exposure is also significantly different in single labelling experiments when the cells were stained with annexin V-FITC in comparison to annexin V alexa fluor[®] 647 as well as in the corresponding double labelling experiments. Therefore, the combination of x-rhod-1 (to detect Ca^{2+} content) and annexin V-FITC (to detect PS exposure) seems to be more efficient than the combination of fluo-4 and annexin V alexa fluor[®] 647 in double labelling experiments. However, it should be taken into consideration that the PS data obtained in double labeling experiments are only hardly to compare with data obtained from single labeling measurements, because of the Ca^{2+} buffering by the Ca^{2+} fluorophor (see below).

In another set of experiments we investigated the intracellular Ca^{2+} content and PS exposure of RBCs after 30 min LPA activation. For these experiments LPA from one batch obtained from Sigma-Aldrich (batch 1) has been used. Results of single and double labelling experiments using different fluorescent dyes can be seen in Fig. 4. Figure 4A and 4B present data of Ca^{2+} and PS measurements, respectively, in which on the left side flow cytometry data and on the right side data obtained using fluorescence microscopy are shown (for analysing fluorescence microscopy images see below).

Similar to the results obtained after 1 min LPA activation, there are no significant differences in the Ca^{2+} content measured in single labelling experiments (fluo-4 and x-rhod-1

for Ca^{2+}) and the corresponding double labelling experiments using a combination of fluo-4 (for Ca^{2+}) / annexin V alexa fluor® 647 (for PS) and x-rhod-1 (for Ca^{2+}) / annexin V-FITC (for PS). In addition, the results of the experiments using fluo-4 and x-rhod-1 are also not significantly different (Fig. 4A, left). Comparing the data after 1 min and 30 min LPA activation (Figs. 3A and 4A), one can see in all cases a decrease of the Ca^{2+} content after 30 min. Such behaviour has been reported and explained in a recent publication [16]. For PS exposure, nearly the same tendency after 30 min compared to 1 min LPA activation can be seen (Fig. 4B, left). The results of single as well as double labelling experiment using annexin V alexa fluor® 647 for PS detection are again lower compared to the experiments where annexin V-FITC has been used (but only the double labelling data are significantly different). In addition, as for the 1 min LPA activation experiments, the amount of RBCs showing PS exposure is also significantly lower in double labelling compared to single labelling experiments when the cells were stained with annexin V alexa fluor® 647. Comparing the data after 1 min and 30 min LPA activation (Figs. 3B and 4B), one can see in all cases of measurement an increase of the PS exposure after 30 min. Such behaviour has been reported and explained in a recent publication [16].

The reasons for the differences in single labelling *versus* double labelling experiments as presented in Figs. 3 and 4 are multifactorial and can be explained by three major effects: (i) the Ca^{2+} buffering capacities of the Ca^{2+} fluorophores, (ii) the properties of the fluorescent dyes, and (iii) the temporal development of the Ca^{2+} signals.

(i) Fluo-4 and x-rhod-1 have *in vitro* dissociation constants K_d for Ca^{2+} of 345 nM and 700 nM, respectively [40]. In living cells these K_d 's are, dependent on their cellular localisation, and are usually increased [41]. Especially when taking the low resting Ca^{2+} concentration in RBCs of around 60 nM [42] into account, the buffering capacity of the Ca^{2+} fluorophores loaded into the cells is high. The variety of the cellular responses [21] may add to the observed effects. Although the EC_{50} of the scramblase (30–70 μM) compared to the *in vivo* K_d of fluo-4 (1 μM) [43] is several fold higher, there are observations suggesting that scrambling may actually require much less Ca^{2+} [44], and therefore the buffering of the Ca^{2+} is the most likely explanation for a lower PS exposure in the double labelled cells as depicted in Figs. 3B and 4B. The relative decrease in PS exposure of the double labelling compared the single labelling is much higher for fluo-4 compared to x-rhod-1 (Fig. 3B). This observation is also in agreement with the K_d 's of the two fluorophores (see above) and hence their buffering properties.

(ii) Other aspects that may contribute to the differences between FITC and alexa fluor® 647 results are the photophysical properties of the dyes. Assuming that the fluorescence quantum yield of a cyanine dye (alexa fluor® 647) may change when the surrounding conditions are changed, e.g. in RBC haemoglobin close to the plasma membrane could be a factor influencing alexa fluor® 647. Isomerisation of double bonds of cyanine dyes is well known [45, 46]. Such changes in combination with wavelength dependencies of the detectors may account, at least partly, for the differences observed for PS detection based on FITC and alexa fluor® 647. Differences in incubation times for single and double labelling experiments as well as the solvent DMSO can be ruled out as sources for different results (we tested this by incubating the RBCs for 30 min in DMSO but without fluo-4, data not shown).

(iii) Apart from the different LPA batches, measurements between Fig. 3 and Fig. 4 differ in their measurement time after LPA stimulation (1 min *versus* 30 min, respectively). After 30 min LPA stimulation, the PS exposure, which is a cumulative process, reaches in the presence of x-rhod-1 the same value as that in the absence of x-rhod-1. As mentioned before, due to its K_d , x-rhod-1 buffers the Ca^{2+} at a higher level compared to fluo-4. This Ca^{2+} level allows a basal scramblase activity, which in combination with the temporal aspects explains the differences observed between Fig. 3B and Fig. 4B.

Furthermore, it is worthwhile to mention that de-esterification of the Ca^{2+} fluorophores may generate formaldehyde and thus affect RBC behaviour, namely by ATP-depletion [47], which can be prevented by the addition of pyruvate [48].

Comparison of RBC activation using LPA between flow cytometry and fluorescence microscopy measurements

In addition to flow cytometry investigations of intracellular Ca^{2+} content and PS exposure of RBCs, the same parameters were estimated from RBCs images of the same samples using fluorescence microscopy. The results obtained are presented for comparison in Fig. 4A and 4B (right). The percentage of RBCs after LPA activation showing an evaluated intracellular Ca^{2+} content is slightly lower when measured using fluorescence microscopy. This is in contrast to previous investigations, where a loss of high Ca^{2+} cells in flow cytometry due to their increased fragility was reported [49]. However, considering the hypothesis mentioned above, that a mechanosensitive channel contributes to the LPA-induced Ca^{2+} entry, the shear stress and pressure in flow cytometers could explain a higher Ca^{2+} content in flow cytometry compared to microscopy [50, 51]. Although there is such a tendency for all conditions measured, only for fluo-4 and single labelling experiments the difference is significant. For PS exposure no differences between flow cytometry and fluorescence microscopy measurements can be seen.

Conclusion

We were able to show that the results of measurements of the Ca^{2+} content as well as PS exposure of RBCs after LPA activation significantly depend on the LPA batches most probably due to the substance stability. We propose to handle that issue by limiting quantitative comparisons of data to those obtained with the same LPA batch. Nevertheless qualitative comparisons cross LPA batches and cross laboratories should still be possible.

We propose that differences in results due to the method of mixing LPA with the RBC suspension are caused by mechanosensitive ion channels. This hypothesis requires further investigations.

In addition, we demonstrated that annexin V-FITC is more sensitive in comparison to annexin V alexa fluor® 647 for the quantification of PS exposure both in single as well as double labelling experiments.

Furthermore, the percentage of RBCs showing PS exposure is reduced in double labelling compared to single labelling experiments, which is most probably caused by the Ca^{2+} buffering capacities of the Ca^{2+} indicators. It is an example how the measurement itself disturbs the process being investigated. It is an almost unavoidable side effect but should be taken into consideration.

Finally, data of Ca^{2+} content are slightly affected by the measuring method (flow cytometry *versus* fluorescence microscopy). Although compared to the issues discussed above, these are minor differences.

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Disclosure Statement

The authors declare no conflict of interest.

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