Original Paper

Protein Kinase Ca and P-Type Ca^{2+} Channel $\text{Ca}_v2.1$ in Red Blood Cell Calcium Signalling

Lisa Wagner-Britz^{a,c} Jue Wang^{b,c} Lars Kaestner^b Ingolf Bernhardt^a

^aLaboratory of Biophysics, Faculty of Natural and Technical Sciences III, Saarland University, Campus, 66123 Saarbrücken, Germany; ^bInstitute for Molecular Cell Biology and Research Centre for Molecular Imaging and Screening, School of Medicine, Saarland University, Building 61, 66421 Homburg, Germany; ^cequal contribution

Key Words

Red blood cells • Ca^{2+} content • Phosphatidylserine • Protein kinase C • P-type Ca^{2+} channel • Flow cytometry • Fluorescence imaging

Abstract

Background/Aims: Protein kinase Ca ($\text{PKC}\alpha$) is activated by an increase in cytosolic Ca^{2+} in red blood cells (RBCs). Previous work has suggested that $\text{PKC}\alpha$ directly stimulates the $\text{Ca}_v2.1$ channel, whereas other studies revealed that $\text{Ca}_v2.1$ is insensitive to activation by PKC. The aim of this study was to resolve this discrepancy. **Methods:** We performed experiments based on a single cell read-out of the intracellular Ca^{2+} concentration in terms of Fluo-4 fluorescence intensity and phosphatidylserine exposure to the external membrane leaflet. Measurement modalities included flow cytometry and live cell imaging. **Results:** Treatment of RBCs with phorbol 12-myristate 13-acetate (PMA) led to two distinct populations of cells with an increase in intracellular Ca^{2+} : a weak-responding and a strong-responding population. The EC_{50} of PMA for the number of cells with Ca^{2+} elevation was $2.7 \pm 1.2 \mu\text{M}$; for phosphatidylserine exposure to the external membrane surface, it was $2.8 \pm 0.5 \mu\text{M}$; and for RBC haemolysis, it was $2.9 \pm 0.5 \mu\text{M}$. Using pharmacological manipulation with the $\text{Ca}_v2.1$ inhibitor ω -agatoxin TK and the broad protein kinase C inhibitor Gö6983, we are able to show that there are two independent PMA-activated Ca^{2+} entry processes: the first is independent of $\text{Ca}_v2.1$ and directly $\text{PKC}\alpha$ -activated, while the second is associated with a likely indirect activation of $\text{Ca}_v2.1$. Further studies using lysophosphatidic acid (LPA) as a stimulation agent have provided additional evidence that $\text{PKC}\alpha$ and $\text{Ca}_v2.1$ are not directly interconnected in a signalling chain. **Conclusion:** Although we provide evidence for a lack of interaction between $\text{PKC}\alpha$ and $\text{Ca}_v2.1$ in RBCs, further studies are required to decipher the signalling relationship between LPA, $\text{PKC}\alpha$ and $\text{Ca}_v2.1$.

Copyright © 2013 S. Karger AG, Basel

Introduction

An increase in the normally low intracellular Ca^{2+} content of human red blood cells (RBCs) [1] activates a number of subsequent cellular mechanisms, leading to changes in

Ingolf Bernhardt
and Lars Kaestner

Lab of Biophysics, Faculty of Natural and Technical Sciences III, Saarland University, Campus, 66123 Saarbrücken (Germany) E-Mail i.bernhardt@mx.uni-saarland.de and Institute for Molecular Cell Biology and Research Centre for Molecular Imaging and Screening, School of Medicine, Saarland University, Building 61, 66421 Homburg (Germany), E-Mail lars_kaestner@me.com

cellular properties [2]. This finding is in line with numerous signalling components and cascades identified recently that have provided insight into the cellular responses of human RBCs towards extracellular stimuli [3-7].

The Ca $^{2+}$ -induced changes include the activation of the phospholipid scramblase [8] and the inhibition of the aminophospholipid translocase [9, 10], resulting in the exposure of phosphatidylserine (PS) to the external membrane leaflet [9-13] and the formation of microvesicles [11, 14]. In addition, increased intracellular Ca $^{2+}$ content activates the Ca $^{2+}$ -activated K $^{+}$ channel (Gardos channel) [15, 16], resulting in an efflux of KCl and osmotically obliged water, which is accompanied by RBC shrinkage [17, 18].

RBC stimulation with oleoyl-L- α -lysophosphatidic acid (LPA) is an example of a physiological stimulation leading to an increase in intracellular Ca $^{2+}$ [19, 20]. The characterisation of the Ca $^{2+}$ entry and the consequent cell behaviour have been described in numerous reports [21-25]. The question of how exposure to LPA leads to Ca $^{2+}$ entry remains far from clear. Current knowledge assumes a signalling cascade in which an unspecified isoform of protein kinase C (PKC) activates a P-type Ca v 2.1 channel [26, 27]. This knowledge is based on pharmacological flow cytometric measurements and on Western blots of Ca v 2.1.

All PKCs are powerful kinases, and several isoforms have been shown to be present in RBCs: PKC α , a member of the conventional PKC subgroup (cPKC); PKC ζ , which belongs to the atypical PKC subgroup (aPKC); and PKC ι and PKC μ , which are both members of the 4 th subgroup [28]. Members of the novel PKC (nPKC) subgroup could not be found in RBCs [28]. However, evidence was recently provided that such biochemical investigations may be contaminated by polymorphonuclear neutrophil granulocytes [29, 30] and thus overestimate the number of PKC isoforms present in RBCs. However, based on pharmacological experiments that use phorbol esters [22, 27], there is no doubt about the abundance of PKC α in RBCs.

As mentioned above, Ca v 2.1 was shown to be present in RBCs by Western blotting [27], and functional studies with the specific inhibitor ω -agatoxin TK support the molecular data [26]. However, investigations of determinants of PKC (two subgroups)-dependent modulation of a family of neuronal Ca $^{2+}$ channels revealed that Ca v 2.1 activity is insensitive to activation by these PKC subgroups (including PKC α) [31]. Therefore, we wondered how closely PKC α and Ca v 2.1 signalling are interconnected in RBCs. Of the PKC isoforms mentioned above, only PKC α can be activated by phorbol 12-myristate 13-acetate (PMA) [32]. PMA is a powerful pharmacological tool that activates PKC α in terms of translocation from the cytosol to the plasma membrane even in the absence of Ca $^{2+}$, which is required for binding to the C2 domain of the cPKCs under physiological conditions [33].

Materials and Methods

Preparation and treatment of RBCs

RBCs from healthy adult donors were used. Blood samples were obtained from the Institute of Sports and Preventive Medicine (Saarland University, Saarbrücken) and from the Institute of Clinical Haematology and Transfusion Medicine (Saarland University Hospital, Homburg). Blood was withdrawn by venipuncture into citrate-coated tubes or with heparin as anticoagulant and used within one day. The blood samples were stored at 4°C until use. RBCs were isolated by centrifugation at 2×10^3 g for 3.5 min, followed by the aspiration of buffy coat and plasma. The remaining RBCs were washed three times in a HEPES-buffered physiological solution (HPS) containing (in mM): 145 NaCl, 7.5 KCl, 10 glucose and 10 HEPES, at pH 7.4. The final centrifugation was performed at 10^4 g for 10 s.

For experiments, RBCs were diluted to a haematocrit of 0.1% in HPS containing an additional 2 mM CaCl $_2$ and incubated with A23187 or PMA. For the LPA experiments, RBCs were diluted in HPS at the same haematocrit; LPA was added first, and within a few seconds, 2 mM CaCl $_2$ was added. A23187, LPA, and PMA were dissolved in DMSO at 1 mM and stored at -20°C. Incubation was carried out at 37°C, and after their respective incubation times, cells were washed in HPS containing an additional 2 mM CaCl $_2$.

Flow cytometry analysis

For flow cytometry analysis, RBCs diluted in HPS to a haematocrit of 1% were stained with 10 μ M Fluo-4 AM (45 min, 37°C) to monitor intracellular Ca²⁺ content. To visualise PS exposure, approximately 10⁶ RBCs were incubated with 4.5 μ l of annexin V-FITC (20 min, room temperature) in 500 μ l HPS containing an additional 2 mM CaCl₂.

For flow cytometry measurements, the stained RBCs were analysed using the FL-1 channel (excitation wavelength 488 nm, emission wavelength 530 nm) on a 'FACSCalibur' with 'CellQuest Pro' software (Becton Dickinson Biosciences, San Jose, CA, USA). For each experiment, 3 $\times$ 10⁴ events were collected.

Live cell imaging

Fluo-4 AM was used as the indicator to measure intracellular Ca²⁺. Live cell imaging was performed to monitor intracellular Ca²⁺ kinetics in individual cells when exposed to PMA or LPA. After being withdrawn and treated as described above, 2 $\times$ 10⁷ RBCs in 1 ml slightly modified HPS (containing in mM: 135 NaCl, 5.4 KCl, 10 glucose, 10 HEPES, 1.8 CaCl₂ and 1 MgCl₂, with pH adjusted to 7.35 with NaOH) were loaded with Fluo-4 AM at a concentration of 5 μ M for 1 h at 37°C. Cells were then washed 3 times in HPS as just described. Each 10⁶ cells were plated on a coverslip in slightly modified HPS, waiting 15 min for cell sedimentation and dye de-esterification. The video-imaging set-up has been previously described [34]. Images were collected every 5 s for a total period of 15 min. A 505-nm long-pass dichroic mirror separated the emission light from the excitation light (central wavelength 480 nm) and a 535/40-band-pass filter was used as an emission filter. A local perfusion system was utilised to quickly exchange solutions in the field of view and to apply the desired concentrations of substances. For experiments including Gö6983 and/or ω -agatoxin TK, the applied concentration of the antagonists was determined in preliminary experiments to be 1 μ M for both substances. At 1 μ M, the Ca²⁺ response was at a plateau, i.e., a further increase in the concentration did not lead to a further reduction of the Ca²⁺ response. Cells were pre-treated with these antagonists for 15-20 min before PMA or LPA stimulation. For the control, the fluorescence intensity of Fluo-4 was measured in slightly modified HPS as defined above (in the presence of 1.8 mM Ca²⁺). Every experiment was performed with at least three different blood samples and repeated at least three times.

Images were processed in ImageJ (Wayne Rasband, National Institute of Health), and traces were performed using Igor Pro software (WaveMetrics Inc., Lake Oswego, OR, USA).

Sources of reagents

A23187, LPA, and PMA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-4, AM and annexin V-FITC were obtained from Invitrogen (Carlsbad, CA, USA). Gö6983 and ω -agatoxin TK were purchased from Tocris (Bristol, UK) and Pepta Nova (Sandhausen, Germany), respectively.

Statistics

Data are presented as the mean values $\pm$ S.D. of at least 3 different blood samples. Statistical analysis was performed using Student's t-test when Gaussian distributed. Otherwise, a Mann-Whitney test was performed. The statistical significance of the data is indicated as (***): $p \leq 0.001$, (**): $p \leq 0.01$, (*): $p \leq 0.05$, not significant: $p > 0.05$.

Results

Recent investigations have explicitly worked out heterogeneously reacting populations of RBCs upon pharmacological stimulation [35]. When stimulating PKC α with PMA, a Ca²⁺ entry is known to happen [22, 27]. Therefore, as a first approach, we wanted to probe the distribution of subpopulations of RBC after their stimulation with PMA. Figure 1 shows histograms of RBCs that were stimulated with PMA (Fig. 1A) in comparison to a straight Ca²⁺ entry that was mediated by the Ca²⁺ ionophore A23187 [36] (Fig. 1B) and stimulation with the physiologically occurring blood plasma component LPA (Fig. 1C). PMA stimulation leads to two distinct RBC populations of reacting cells that we term "weak responding RBCs" (WRR) and "strong responding RBCs" (SRR). These two populations comprise all RBCs (there were no non-responding cells) and suggest the involvement of two distinct

Fig. 1. Histograms of flow cytometry analysis of intracellular Ca²⁺ content in RBCs. (A) Representative histogram showing the classification of RBCs into two populations after treatment with 6 μ M PMA, revealing diverse intracellular Ca²⁺ content (Fluo-4 intensity). Left curve (black): untreated RBCs; right curve (grey): RBCs after 30 min of PMA treatment. A large population of weak responding RBCs (WRR) and a small population of strong responding RBCs (SRR) can be observed. (B) Representative histogram of the Fluo-4 intensity after 2 μ M A23187 stimulation for 30 min. Left curve (black): untreated RBCs; right curve (grey): RBCs after treatment, representing a single population with elevated intracellular Ca²⁺ content. (C) Representative histogram showing the classification of RBCs into two populations after treatment with 2.5 μ M LPA for 30 min, revealing a diverse intracellular Ca²⁺ content. Left curve (black): untreated RBCs; right curve (grey): RBCs after LPA stimulation. A small population of WRR and a large population of SRR can be observed.

processes in PMA-induced Ca²⁺ entry. The SRR Fluo-4 intensity is close to the population of A23187-stimulated cells, which can be considered a positive control close to the level of Fluo-4-saturated Ca²⁺ entry (Fig. 1B). LPA stimulation does not resemble PMA stimulation in respect to the distribution of WRR and SRR, but the distribution range in terms of Fluo-4 intensities is very similar when comparing LPA and PMA treatment (Fig. 1C).

While there are broad characterisations of A23187- [22, 23, 25, 37] and LPA- [11, 20] induced Ca²⁺ influx into RBCs available in the literature, reports on the PMA stimulation of RBCs are rather sparse [22, 27, 38]. Therefore, a set of experiments was performed to investigate the effect of various doses of PMA on Ca²⁺ content, PS exposure and RBC haemolysis, as shown in Fig. 2. Figure 2 illustrates the dose response relationship of the parameters mentioned above for PMA concentrations up to 10 μ M. Figure 2A depicts the dose-response relationship of the SRR after 30 min PMA treatment ($EC_{50} = 2.7 \pm 1.2$ μ M). The percentage of cells displaying elevated Fluo-4 intensity increased with PMA dose up to 10 μ M PMA, reaching a value of approximately 45% (the increase between 6 and 10 μ M was not significant). The dependence of the fluorescence intensities on PMA concentration after 30 min of incubation for the WRR and SRR are shown in Fig. 2B as red triangles and blue circles, respectively. In both cell populations, a slight increase in Fluo-4 intensity with PMA concentration can be observed. With higher PMA concentrations, both the number of cells with elevated Ca²⁺ content and the level of this cellular Ca²⁺ content slightly increased. To show one of the physiological effector mechanisms of the Ca²⁺ increase, Fig. 2C illustrates that increasing PMA concentrations led to significantly larger populations of RBCs with exposed PS. However, with respect to a putative signalling pathway, how the different populations emerge remains an interesting question. The EC_{50} of PMA is 2.8 ± 0.5 μ M and 2.9 ± 0.8 μ M for the effector parameter considered in Fig. 2C (PS exposure) and for haemolysis (Fig. 2D), respectively. To maintain the balance between induced-Ca²⁺ entry (EC_{50} of 2.7 ± 1.2 μ M) and limited haemolysis, a concentration of 3 μ M PMA was selected for further kinetic experiments.

Fig. 2. Flow cytometry characterisation of intracellular Ca²⁺ content and PS exposure in RBCs treated with PMA. (A) Number of RBCs with elevated Fluo-4 intensity (strong-responding RBCs - SRR) after stimulation with different PMA concentrations for 30 min. (B) Fluo-4 intensity of weak-responding RBCs (WRR) (red triangles) and of SSR (blue circles) after stimulation with different PMA concentrations for 30 min. (C) Annexin V-FITC-positive RBCs after treatment with different PMA concentrations for 30 min. (D) Number of haemolysed RBC after treatment with different PMA concentrations for 30 min. RBC number was determined by cell counts per time unit compared to the same sample before the addition of PMA. All measurements are the mean values ($\pm$ S.D.) of 5 different blood samples.

Because ω -agatoxin TK was reported to inhibit PMA-induced Ca²⁺ influx in RBCs, [27] we aimed to study this process using live cell imaging. Figure 3A depicts these experiments, wherein cells were imaged under control conditions (slightly modified HPS) and under stimulation with 3 μ M PMA with and without pre-incubation with 1 μ M ω -agatoxin TK. Examining the cell populations, PMA provokes an increase in intracellular Ca²⁺. For the first 3 min, however, there is no change in the intracellular Ca²⁺ whether the cells were pre-incubated with ω -agatoxin TK or not, which suggests an initial process that is independent of the Ca_v2.1 channel. After a PMA incubation time of 3 min (4 min of total experiment time), the net Ca²⁺ entry of the two groups diverges: the Ca²⁺ level remains roughly constant for ω -agatoxin TK pre-incubated cells, while the signal from pure PMA stimulation increases further. The significant differences between the Ca²⁺ signal immediately after 2 min of stimulation (3 min of total experiment time) and after 10 min of stimulation (11 min of total experiment time) are depicted in Fig. 3B, strengthening the impression given from the traces observed in Fig. 3A. The average Ca²⁺ signals in the presence and absence of ω -agatoxin TK appear very similar in RBCs whether they were stimulated with PMA or LPA (data not shown).

To investigate whether a signalling difference remains between the PMA and LPA stimulations, we performed measurements in which RBCs were probed with LPA and pre-incubated with or without the broad PKC (cPKC, nPKC, aPKC) inhibitor Gö6983 (grey trace in Fig. 4A). After initial fluctuations with very little to no difference in the first 4 min after the beginning of the stimulation, there was no significant difference in LPA-induced Ca²⁺ influx 5 min after stimulation (6 min of total experiment time) in the presence or absence of Gö6983 (Fig. 4B). The initial differences may be due to the differing stimulation strength and

Fig. 3. Kinetics of PMA induced Ca²⁺ entry in the presence and absence of ω -agatoxin TK. (A) Average traces of single RBCs (all cells, including WRR and SRR) derived from live cell imaging experiments as self-ratio values. Labelled lines above the traces indicate the stimulation regime. The traces are the mean values of 3 independent experiments, and the numbers in brackets at the end of the colour legend refer to the number of cells measured. (B) Statistical analysis of F/F₀ fluorescence intensity values at different time points of the traces depicted in (A). The numbers below the boxes refer to the number of cells tested.

Fig. 4. Kinetics of LPA-induced Ca²⁺ entry in the presence and absence of PKC inhibitor Gö6983 and ω -agatoxin TK. (A) Average traces of single cells derived from live cell imaging experiments are presented as self-ratio values. Labelled lines above the traces indicate the stimulation regime. The traces are the mean values of 3 independent experiments, and the numbers in brackets at the end of the colour legend refer to the number of cells measured. (B) Statistical analysis of F/F₀ fluorescence intensity values at different time points of the traces depicted in (A). The numbers below the boxes refer to the number of cells tested.

subsequent different compensation for the Ca²⁺ entry by the Ca²⁺-ATPase [39, 40]. Later, the Ca²⁺ traces diverge, revealing a significant increase in the population without Gö6983 (Fig. 4A). This result shows that under LPA stimulation, PKC α -mediated Ca²⁺ influx does occur, but there are PKC α -independent contributions to the Ca²⁺ influx.

Because (i) ω -agatoxin TK-blocked Ca²⁺ entry is a secondary effect of PKC α activation and (ii) PKC α -mediated Ca²⁺ influx is only one contribution to LPA-induced Ca²⁺ entry, we wondered what would happen if we blocked both the PKC α activity and the Ca_v2.1 channel. Therefore, we stimulated RBCs with LPA and pre-incubated with Gö6983 and ω -agatoxin TK (green trace in Fig. 4A). This combined inhibition abolished Ca²⁺ entry completely (Fig. 4B).

Discussion

The similarity in intensity distribution (WRR and SRR) between PMA stimulation (Fig. 1A) and LPA stimulation (Fig. 1C) leads to the assumption that the two processes induced by the artificial PKC α stimulation resemble processes that occur under LPA stimulation. This assumption holds especially true when considering that the relatively high haemolysis rate after 6 μ M PMA treatment may specifically reduce the SRR population. PMA stimulates cPKCs and nPKCs [41]. It has been shown that PKC α is the only isoform among the cPKCs and nPKCs that are found in RBCs (cf. Introduction) [28]. Gö6983 is a broad PKC inhibitor that affects cPKCs, nPKCs and aPKCs but not the 4th subfamily [42]. Therefore, the pharmacological manipulation of PKC within this paper can be considered an interaction with PKC α .

Although PMA imitates the naturally occurring diacylglycerol (DAG), it is worth mentioning that PMA activates PKC α directly, while the presence/binding of Ca²⁺ is compulsory for the physiological activation of PKC α (e.g., by LPA) [33, 43]. In this way, the PMA activation of RBCs, as characterised in Fig. 2, is more direct than any physiological stimulation of PKC α because the initial Ca²⁺ entry required for PKC α activation is circumvented. However, PMA stimulation leads to an increase in the intracellular Ca²⁺ content of RBCs, and not all cells respond equally. This finding is in agreement with a recent investigation of RBC heterogeneity upon hormonal stimulation [35]. Up to 10 μ M PMA, the Ca²⁺ increase (as indicated by both the number of SRRs and the Ca²⁺ content-related fluorescence) in the RBCs did not saturate, except in the intensity of the SRRs, which is most likely related to the saturation of the Ca²⁺ dye Fluo-4. This saturation is close to 40 μ M Ca²⁺ *in vitro* [44], but the Ca²⁺ sensitivity of Fluo-4 decreases by a factor of approximately 3 *in vitro* [45], leading to Ca²⁺ saturation at approximately 120 μ M. Interestingly, the PS exposition Ca²⁺ effector reaches values close to full saturation at 10 μ M PMA stimulation for 30 min (Fig. 2C) with an EC₅₀ of 2.8 $\pm$ 0.5 μ M. This result leads to the conclusion that the Ca²⁺ increase in the WRR is sufficient for PS exposition.

However, the rate of haemolysis cannot be neglected: at saturation values (> 10 μ M PMA), it is approximately 30%. This rate is too high to be acceptable in routine measurements and requires lowering the PMA concentrations towards the EC₅₀ value; consequently, it was set to 3 μ M for the experiments presented in Fig. 3.

The dose-response curve of haemolysis (Fig. 2D) was very similar to the PS exposure curve, suggesting a relationship of haemolysis to lipid remodelling in the RBC plasma membrane. Although the EC₅₀ values of 2.7 μ M for the Ca²⁺ increase, 2.8 μ M for the PS exposure and 2.9 μ M for haemolysis fit together, the cell numbers (at EC₅₀) in these processes (30%, 50% and 18%, respectively) point to a more complex interdependence.

Considering the kinetic traces of the Ca²⁺ increase in PMA-stimulated RBCs (Fig. 3A), one can identify two phases. The initial Ca²⁺ increase during the first 3 min of stimulation (4 min of total experiment time) is independent of ω -agatoxin TK, i.e., it is not mediated by the Ca_v2.1 channel (cf. Introduction). The following phase shows a continuing increase in the absence of ω -agatoxin TK (almost saturating after 10 min) and no further increase in the presence of ω -agatoxin TK, which suggests an initial PKC α -mediated Ca²⁺ influx, which is not through Ca_v2.1 channels, and the activation of Ca_v2.1 can be assumed to be a secondary effect of PKC α activation. In the context of a previous study, this finding is in line with a lack of interaction between PKC α and Ca_v2.1 [31], thus indicating an indirect effect of PKC α on Ca_v2.1 channel activity.

To probe the similarity between PMA and LPA stimulation of RBCs, we performed LPA stimulation in the presence and absence of Gö6983 (Fig. 4A). Instead of a total inhibition of the intracellular Ca²⁺ increase, we found that there was no difference in intracellular Ca²⁺ between the two conditions after 5 min of stimulation (6 min of total experiment time) (Fig. 4B). This result points to an initial PKC α -independent Ca²⁺ entry with a second phase of PKC α -mediated Ca²⁺ influx. Conceptually, this is reasonable because PKC α requires Ca²⁺ to be activated (see above).

When RBCs are pre-incubated with Gö6983 and ω -agatoxin TK, stimulation with 5 μ M LPA does not provoke any increase in intracellular Ca²⁺ (green trace in Fig. 4A). From these

experimental data, we deduce that PKC α -mediated Ca $^{2+}$ influx and Ca $_v$ 2.1 channel activity are two different routes of Ca $^{2+}$ entry.

In summary, we were able to show that PKC α activation in RBCs leads to two independent Ca $^{2+}$ entry processes. The first is Ca $_v$ 2.1 independent, and the second is associated with a likely indirect activation of Ca $_v$ 2.1 (Fig. 3). Further studies using LPA as a stimulation agent provided further evidence that PKC α and Ca $_v$ 2.1 are not directly interconnected in a signalling chain (Fig. 4). This finding is in contrast to the common belief suggested in a previous study [27]. Further investigations are required to decipher the complete signalling cascade from LPA stimulation to Ca $^{2+}$ influx in RBCs.

References

- 1 Harrison DG, Long C: The calcium content of human erythrocytes. *J Physiol (Lond)* 1968;199:367–381.
- 2 Bogdanova A, Makhro A, Wang J, Lipp P, Kaestner L: Calcium in red blood cells - a perilous balance. *Int J Mol Sci* 2013;14:9848–9872.
- 3 Andrews DA, Low PS: Role of red blood cells in thrombosis. *Curr Opin Hematol* 1999;6:76–82.
- 4 Kaestner L, Bernhardt I: Ion channels in the human red blood cell membrane: their further investigation and physiological relevance. *Bioelectrochemistry* 2002;55:71–74.
- 5 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 Haemostasis* 2004;92:1269–1272.
- 6 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.
- 7 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.
- 8 Bevers EM, Williamson PL: Phospholipid scramblase: an update. *FEBS letters* 2010;584:2724–2730.
- 9 Wolfs JLN, Comfurius P, Rasmussen JT, Keuren JFW, Lindhout T, Zwaal RFA, Bevers EM: Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction. *Cell Mol Life Sci* 2005;62:1514–1525.
- 10 Kuypers FA, Lewis RA, Hua M, Schott MA, Discher D, Ernst JD, Lubin BH: Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. *Blood* 1996;87:1179–1187.
- 11 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.
- 12 Zwaal RFA, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005;62:971–988.
- 13 Zhou Q, Zhao J, Stout JG, Luhm RA, Wiedmer T, Sims PJ: Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J Biol Chem* 1997;272:18240–18244.
- 14 Gonzalez LJ, Gibbons E, Bailey RW, Fairbo J, Nguyen T, Smith SK, Best KB, Nelson J, Judd AM, Bell JD: The influence of membrane physical properties on microvesicle release in human erythrocytes. *PMC Biophysics* 2009;2:7.
- 15 Gardos G: The permeability of human erythrocytes to potassium. *Acta Physiol Hung* 1956;10:185–189.
- 16 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.
- 17 Maher AD, Kuchel PW: The Gárdos channel: a review of the Ca $^{2+}$ -activated K $^{+}$ channel in human erythrocytes. *Int J Biochem Cell Biol* 2003;35:1182–1197.
- 18 Lang PA, Kaiser SJ, Myssina S, Wieder T, Lang F, Huber SM: Role of Ca $^{2+}$ -activated K $^{+}$ channels in human erythrocyte apoptosis. *Am J Physiol-Cell Physiol* 2003;285:C1553–C1560.
- 19 Yang JJ, Yamato M, Nishida K, Ohki T, Kanzaki M, Sekine H, Shimizu T, Okano T: Cell delivery in regenerative medicine: the cell sheet engineering approach. *J Control Release* 2006;116:193–203.

- 20 Kaestner L, Tabellion W, Weiss E, Bernhardt I, Lipp P: Calcium imaging of individual erythrocytes: Problems and approaches. *Cell Calcium* 2006;39:13–19.
- 21 Tiffert T, Lew VL: Elevated intracellular Ca²⁺ reveals a functional membrane nucleotide pool in intact human red blood cells. *J Gen Physiol* 2011;138:381–391.
- 22 Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, Bernhardt I: Regulation of phosphatidylserine exposure in red blood cells. *Cell Physiol Biochem* 2011;28:847–856.
- 23 Steffen P, Jung A, Nguyen DB, Müller T, Bernhardt I, Kaestner L, Wagner C: Stimulation of human red blood cells leads to Ca²⁺-mediated intercellular adhesion. *Cell Calcium* 2011;50:54–61.
- 24 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* 2012;87:89–95.
- 25 Muravyov A, Tikhomirova I: Role Ca(2+) in mechanisms of the red blood cells microrheological changes. *Advances in experimental medicine and biology* 2012;740:1017–1038.
- 26 Yang L, Andrews DA, Low PS: Lysophosphatidic acid opens a Ca(++) channel in human erythrocytes. *Blood* 2000;95:2420–2425.
- 27 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.
- 28 Govekar RB, Zingde SM: Protein kinase C isoforms in human erythrocytes. *Ann Hematol* 2001;80:531–534.
- 29 Achilli C, Ciana A, Balduini C, Risso A, Minetti G: Application of gelatin zymography for evaluating low levels of contaminating neutrophils in red blood cell samples. *Anal Biochem* 2011;409:296–297.
- 30 Minetti G, Egée S, Mörsdorf D, Steffen P, Makhro A, Achilli C, Ciana A, Wang J, Bouyer G, Bernhardt I, Wagner C, Thomas S, Bogdanova A, Kaestner L: Red cell investigations: Art and artefacts. *Blood Rev* 2013;27:91–101.
- 31 Stea A, Soong TW, Snutch TP: Determinants of PKC-dependent modulation of a family of neuronal calcium channels. *Neuron* 1995;15:929–940.
- 32 Ono Y, Fujii T, Igarashi K, Kuno T, Tanaka C, Kikkawa U, Nishizuka Y: Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci USA* 1989;86:4868–4871.
- 33 Reither G: PKC : a versatile key for decoding the cellular calcium toolkit. *J Cell Biol* 2006;174:521–533.
- 34 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.
- 35 Wang J, Wagner-Britz L, Bogdanova A, Ruppenthal S, Wiesen K, Kaiser E, Tian Q, Krause E, Bernhardt I, Lipp P, Phillip SE, Kaestner L: Morphologically homogeneous red blood cells present a heterogeneous response to hormonal stimulation. *PLoS ONE* 2013, in press.
- 36 Reed PW, Lardy HA: A23187: a divalent cation ionophore. *J Biol Chem* 1972;247:6970–6977.
- 37 Cuffe A, Seear R, Dyrda A, Bouyer G, Egée S, Esposito A, Skepper J, Tiffert T, Lew VL, Thomas SL: Effects of elevated intracellular calcium on the osmotic fragility of human red blood cells. *Cell Calcium* 2010;47:29–36.
- 38 Wright LC, Chen S, Roufogalis BD: Regulation of the activity and phosphorylation of the plasma membrane Ca(2+)-ATPase by protein kinase C in intact human erythrocytes. *Arch Biochem Biophys* 1993;306:277–284.
- 39 Schatzmann H: The red cell calcium pump. *Ann Rev Physiol* 1983;45:303–312.
- 40 Lew VL, Tsien RY, Miner C, Bookchin RM: Physiological [Ca²⁺]_i level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature* 1982;298:478–481.
- 41 Liu WS, Heckman CA: The sevenfold way of PKC regulation. *Cell Signal* 1998;10:529–542.
- 42 Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ: Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS letters* 1996;392:77–80.
- 43 Lipp P, Hui X, Reither G, Kaestner L: Multi-channel imaging of cellular signaling: Interplay of Ca²⁺ and conventional protein kinase C. *Cold Spring Harb Protoc* 2013; in press.
- 44 Haugland RP: Handbook of fluorescent probes and research products. ed 9 Eugene, Molecular Probes, 2002.
- 45 Thomas D, Tovey SC, Collins TJ, Bootman MD, Berridge MJ, Lipp P: A comparison of fluorescent Ca²⁺ indicator properties and their use in measuring elementary and global Ca²⁺ signals. *Cell Calcium* 2000;28:213–223.