Protein Kinase Cα and P-Type Ca\textsuperscript{2+} Channel Ca\textsubscript{\text{v}}2.1 in Red Blood Cell Calcium Signalling

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Abstract

Background/Aims: Protein kinase Cα (PKCα) is activated by an increase in cytosolic Ca\textsuperscript{2+} in red blood cells (RBCs). Previous work has suggested that PKCα directly stimulates the Ca\textsubscript{\text{v}}2.1 channel, whereas other studies revealed that Ca\textsubscript{\text{v}}2.1 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\textsuperscript{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\textsuperscript{2+}: a weak-responding and a strong-responding population. The EC\textsubscript{50} of PMA for the number of cells with Ca\textsuperscript{2+} elevation was 2.7±1.2 \textmu M; for phosphatidylserine exposure to the external membrane surface, it was 2.8±0.5 \textmu M; and for RBC haemolysis, it was 2.9±0.5 \textmu M. Using pharmacological manipulation with the Ca\textsubscript{\text{v}}2.1 inhibitor \textomega-agatoxin TK and the broad protein kinase C inhibitor Gö6983, we are able to show that there are two independent PMA-activated Ca\textsuperscript{2+} entry processes: the first is independent of Ca\textsubscript{\text{v}}2.1 and directly PKCα-activated, while the second is associated with a likely indirect activation of Ca\textsubscript{\text{v}}2.1. Further studies using lysophosphatidic acid (LPA) as a stimulation agent have provided additional evidence that PKCα and Ca\textsubscript{\text{v}}2.1 are not directly interconnected in a signalling chain. Conclusion: Although we provide evidence for a lack of interaction between PKCα and Ca\textsubscript{\text{v}}2.1 in RBCs, further studies are required to decipher the signalling relationship between LPA, PKCα and Ca\textsubscript{\text{v}}2.1.

Introduction

An increase in the normally low intracellular Ca\textsuperscript{2+} content of human red blood cells (RBCs) [1] activates a number of subsequent cellular mechanisms, leading to changes in...
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\textsuperscript{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\textsuperscript{2+} content activates the Ca\textsuperscript{2+}-activated K\textsuperscript{+} 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-\alpha-lysophosphatidic acid (LPA) is an example of a physiological stimulation leading to an increase in intracellular Ca\textsuperscript{2+} [19, 20]. The characterisation of the Ca\textsuperscript{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\textsuperscript{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\textsubscript{V}2.1 channel [26, 27]. This knowledge is based on pharmacological flow cytometric measurements and on Western blots of Ca\textsubscript{V}2.1.

All PKCs are powerful kinases, and several isoforms have been shown to be present in RBCs: PKC\textalpha, a member of the conventional PKC subgroup (cPKC); PKC\zet, which belongs to the atypical PKC subgroup (aPKC); and PKC\iota and PKC\mu, which are both members of the 4th 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\textalpha in RBCs.

As mentioned above, Ca\textsubscript{V}2.1 was shown to be present in RBCs by Western blotting [27], and functional studies with the specific inhibitor \omega-agatoxin TK support the molecular data [26]. However, investigations of determinants of PKC (two subgroups)-dependent modulation of a family of neuronal Ca\textsuperscript{2+} channels revealed that Ca\textsubscript{V}2.1 activity is insensitive to activation by these PKC subgroups (including PKC\textalpha) [31]. Therefore, we wondered how closely PKC\textalpha and Ca\textsubscript{V}2.1 signalling are interconnected in RBCs. Of the PKC isoforms mentioned above, only PKC\textalpha can be activated by phorbol 12-myristate 13-acetate (PMA) [32]. PMA is a powerful pharmacological tool that activates PKC\textalpha in terms of translocation from the cytosol to the plasma membrane even in the absence of Ca\textsuperscript{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 2x10\textsuperscript{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\textsuperscript{4} g for 10 s.

For experiments, RBCs were diluted to a haematocrit of 0.1% in HPS containing an additional 2 mM CaCl\textsubscript{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\textsubscript{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\textsubscript{2}. 


Flow cytometry analysis. RBCs diluted in HPS to a haematocrit of 1% were stained with 10 µM Fluo-4 AM (45 min, 37°C) to monitor intracellular Ca$^{2+}$ content. To visualise PS exposure, approximately 10$^6$ RBCs were incubated with 4.5 µl of annexin V-FITC (20 min, room temperature) in 500 µl HPS containing an additional 2 mM CaCl$_2$.

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, 3x10$^4$ events were collected.

Live cell imaging. Fluo-4 AM was used as the indicator to measure intracellular Ca$^{2+}$. Live cell imaging was performed to monitor intracellular Ca$^{2+}$ kinetics in individual cells when exposed to PMA or LPA. After being withdrawn and treated as described above, 2x10$^7$ RBCs in 1 ml slightly modified HPS (containing in mM: 135 NaCl, 5.4 KCl, 10 glucose, 10 HEPES, 1.8 CaCl$_2$ and 1 MgCl$_2$, with pH adjusted to 7.35 with NaOH) were loaded with Fluo-4 AM at a concentration of 5 µM for 1 h at 37°C. Cells were then washed 3 times in HPS as just described. Each 10$^6$ 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 535-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 µM for both substances. At 1 µM, the Ca$^{2+}$ response was at a plateau, i.e., a further increase in the concentration did not lead to a further reduction of the Ca$^{2+}$ 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 Ca$^{2+}$). Cells were pre-treated with Fluo-4 AM for 1 h at 37°C. Cells were then washed 3 times in HPS, loaded with Fluo-4 AM, and treated for 15 min before PMA or LPA stimulation. The video-imaging set-up has been previously described [34]. Images were collected every 5 s for a total period of 15 min. A 535-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 µM for both substances. At 1 µM, the Ca$^{2+}$ response was at a plateau, i.e., a further increase in the concentration did not lead to a further reduction of the Ca$^{2+}$ 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 Ca$^{2+}$).
processes in PMA-induced Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 µM. Figure 2A depicts the dose-response relationship of the SRR after 30 min PMA treatment (EC\(_{50}\) = 2.7±1.2 µM). The percentage of cells displaying elevated Fluo-4 intensity increased with PMA dose up to 10 µM PMA, reaching a value of approximately 45% (the increase between 6 and 10 µM 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 Ca\(^{2+}\) content and the level of this cellular Ca\(^{2+}\) content slightly increased.

To show one of the physiological effector mechanisms of the Ca\(^{2+}\) 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 µM and 2.9±0.8 µM for the effector parameter considered in Fig. 2C (PS exposure) and for haemolysis (Fig. 2D), respectively. To maintain the balance between induced-Ca\(^{2+}\) entry (EC\(_{50}\) of 2.7±1.2 µM) and limited haemolysis, a concentration of 3 µM PMA was selected for further kinetic experiments.
Because ω-agatoxin TK was reported to inhibit PMA-induced Ca$^{2+}$ 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 µM PMA with and without pre-incubation with 1 µM ω-agatoxin TK. Examining the cell populations, PMA provokes an increase in intracellular Ca$^{2+}$. For the first 3 min, however, there is no change in the intracellular Ca$^{2+}$ whether the cells were pre-incubated with ω-agatoxin TK or not, which suggests an initial process that is independent of the CaV2.1 channel. After a PMA incubation time of 3 min (4 min of total experiment time), the net Ca$^{2+}$ entry of the two groups diverges: the Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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
subsequent different compensation for the Ca\textsuperscript{2+} entry by the Ca\textsuperscript{2+}-ATPase [39, 40]. Later, the Ca\textsuperscript{2+} traces diverge, revealing a significant increase in the population without Gö6983 (Fig. 4A). This result shows that under LPA stimulation, PKC\(\alpha\)-mediated Ca\textsuperscript{2+} influx does occur, but there are PKC\(\alpha\)-independent contributions to the Ca\textsuperscript{2+} influx.

Because (i) ω-agatoxin TK-blocked Ca\textsuperscript{2+} entry is a secondary effect of PKC\(\alpha\) activation and (ii) PKC\(\alpha\)-mediated Ca\textsuperscript{2+} influx is only one contribution to LPA-induced Ca\textsuperscript{2+} entry, we wondered what would happen if we blocked both the PKC\(\alpha\) activity and the Ca\textsubscript{2.1} 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 Ca\textsuperscript{2+} entry completely (Fig. 4B).

**Fig. 4.** Kinetics of LPA-induced Ca\textsuperscript{2+} 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 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/F\textsubscript{o} 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.
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 PKCα stimulation resemble processes that occur under LPA stimulation. This assumption holds especially true when considering that the relatively high haemolysis rate after 6 µM PMA treatment may specifically reduce the SRR population. PMA stimulates cPKCs and nPKCs and not the 4th subfamily [42]. Therefore, the pharmacological manipulation of PKC within this paper can be considered an interaction with PKCα.

Although PMA imitates the naturally occurring diacylglycerol (DAG), it is worth mentioning that PMA activates PKCα directly, while the presence/binding of Ca²⁺ is compulsory for the physiological activation of PKCα (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 PKCα because the initial Ca²⁺ entry required for PKCα 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 µM 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⁺ entry with a second phase of Ca²⁺ increases during the first 3 min of stimulation (Fig. 3A). This saturation is close to 40 µM 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 µM. Interestingly, the PS exposition Ca²⁺ effector reaches values close to full saturation at 10 µM PMA stimulation for 30 min (Fig. 2C) with an EC₅₀ of 2.8±0.5 µM. 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 µM 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 µM 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 µM for the Ca²⁺ increase, 2.8 µM for the PS exposure and 2.9 µM 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 Caᵥ2.1 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 PKCα-mediated Ca²⁺ influx, which is not through Caᵥ2.1 channels, and the activation of Caᵥ2.1 can be assumed to be a secondary effect of PKCα activation. In the context of a previous study, this finding is in line with a lack of interaction between PKCα and Caᵥ2.1 [31], thus indicating an indirect effect of PKCα on Caᵥ2.1 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 PKCα-independent Ca²⁺ entry with a second phase of PKCα-mediated Ca²⁺ influx. Conceptually, this is reasonable because PKCα requires Ca²⁺ to be activated (see above).

When RBCs are pre-incubated with Gö6983 and ω-agatoxin TK, stimulation with 5 µM LPA does not provoke any increase in intracellular Ca²⁺ (green trace in Fig. 4A). From these
experimental data, we deduce that PKCα-mediated Ca\textsuperscript{2+} influx and Ca\textsubscript{v}2.1 channel activity are two different routes of Ca\textsuperscript{2+} entry.

In summary, we were able to show that PKCα activation in RBCs leads to two independent Ca\textsuperscript{2+} entry processes. The first is Ca\textsubscript{v}2.1 independent, and the second is associated with a likely indirect activation of Ca\textsubscript{v}2.1 (Fig. 3). Further studies using LPA as a stimulation agent provided further evidence that PKCα and Ca\textsubscript{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\textsuperscript{2+} influx in RBCs.

References


