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Activation of protein kinase C by phorbol ester increases red blood cell scramblase activity and external phosphatidylserine

Latorya A. Barber¹, Mary B. Palascak¹, Xiaoyang Qi¹, Clinton H. Joiner², and Robert S. Franco¹

¹Division of Internal Medicine-Hematology/Oncology, University of Cincinnati College of Medicine, Cincinnati, OH

²Department of Pediatrics, Division of Hematology Oncology, Children's Healthcare of Atlanta, Aflac Cancer and Blood Disorders Center, Emory University, Atlanta, GA, USA

Abstract

Externalization of phosphatidylserine (PS) is thought to contribute to sickle cell disease (SCD) pathophysiology. The red blood cell (RBC) aminophospholipid translocase (APLT) mediates the transport of PS from the outer to the inner RBC membrane leaflet to maintain an asymmetric distribution of PL, while phospholipid scramblase (PLSCR) equilibrates PL across the RBC membrane, promoting PS externalization. We previously identified an association between PS externalization level and PLSCR activity in sickle RBC under basal conditions. Other studies showed that activation of protein kinase C (PKC) by PMA (phorbol-12-myristate-13-acetate) causes increased external PS on RBC. Therefore, we hypothesized that PMA-activated PKC stimulates PLSCR activity in RBC and thereby contributes to increased PS externalization. In the current studies, we show that PMA treatment causes immediate and variable PLSCR activation and subsequent PS externalization in control and sickle RBC. While TfR+ sickle reticulocytes display some endogenous PLSCR activity, we observed a robust activation of PLSCR in sickle reticulocytes treated with PMA. The PKC inhibitor, chelerythrine (Chel), significantly inhibited PMA-dependent PLSCR activation and PS externalization. Chel also inhibited endogenous PLSCR activity in sickle reticulocytes. These data provide evidence that PKC mediates PS externalization in RBC through activation of PLSCR.

Keywords

red blood cell; protein kinase C; scramblase; phorbol ester; phosphatidylserine; sickle cell disease

Abnormal activation of phospholipid scramblase (PLSCR) (1) allows equilibration of phosphatidylserine (PS) and other phospholipids (PL) between the inner and outer red blood cell (RBC) membrane leaflets. Conversely, the normally active aminophospholipid

Correspondence: Robert S. Franco, PhD, Division of Hematology-Oncology, University of Cincinnati College of Medicine, 3125 Eden, Street ML 0508, Cincinnati, Ohio 45267-0508, USA. Tel: +513 558 3241; Fax: +513 558 6703; robert.franco@uc.edu.

Author contributions

LAB collected laboratory data and contributed to writing the manuscript; MBA collected laboratory data; XQ made a critical review of the manuscript; CHJ helped design the research and made a critical review of the manuscript; and RSF designed the laboratory studies and contributed to writing the manuscript.

translocase (APLT) maintains an asymmetric distribution of PS in the RBC membrane (2–4). When APLT is impaired, there is a correlation between PLSCR activity and PS externalization in sickle RBC (5). PLSCR is inactive in PS negative mature sickle RBC and increasingly active as PS externalization levels increase. However, the mechanism of PLSCR activation in sickle RBC is poorly understood. Intracellular Ca^{2+} inhibits APLT at ~90 nM (6) and activates PLSCR at a much higher concentration (~50 μM) (7). With this high Ca^{2+} requirement, it is unlikely that transient, sickling-mediated Ca^{2+} elevation alone activates PLSCR *in vivo* (8), suggesting that additional mechanisms contribute to activation of PLSCR in PS-exposing RBC.

There is considerable evidence that protein kinase C (PKC) is involved in PS externalization. Addition of phorbol ester to the leukemic cell lines NB4 and HL60 activates PKC- δ and leads to increased PLSCR activity (9). However, there is no detectable PKC- δ in RBC, which contain the α , ζ , ι , and μ isoforms (10, 11). Of these, PKC- α is the only isoform that translocates to the membrane and becomes activated upon addition of phorbol ester (11). *In vivo* activation of PKC- α requires diacylglycerol (DAG) and Ca^{2+} binding in the presence of PS. The phorbol ester, phorbol-12-myristate-13-acetate (PMA), is a potent PKC activator that mimics DAG binding but does not require Ca^{2+} . Upon PMA treatment, PKC- α translocates to the RBC membrane, becomes activated, and phosphorylates a variety of RBC membrane proteins (12–17). Treatment of RBC with PMA led to PS externalization (18) that was prevented by chelerythrine (Chel), an inhibitor specific to conventional PKC isoforms including PKC- α . Chel inhibits PKC activity by binding on the catalytic domain at the site of substrate phosphorylation. As there is a potential PKC phosphorylation site on PLSCR (19), these data raised the possibility that PKC participates in PS externalization through direct PLSCR activation (20, 21). In the studies described here, we evaluated PLSCR activity and PS externalization in normal and sickle RBC and sickle reticulocytes treated with PMA in the presence or absence of Chel. Sickie reticulocytes were included in these studies as they are the starting point for mature RBC in the circulation, and abnormalities in this immature phase may carry over into the mature cells.

Materials and methods

Materials

NBD-PC (1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids, Inc. Anti-TfR (transferrin receptor)-phycoerythrin/Cy5 was from BD Pharmingen. Annexin V-FITC or annexin-phycoerythrin (PE) was from Invitrogen. Phorbol-12-myristate-13-acetate (PMA), DMSO, and chelerythrine chloride were purchased from EMD Biosciences. All other materials were from Sigma-Aldrich.

Blood samples

Blood from normal volunteers or patients with SCD (homozygous HbS) was drawn into EDTA (ethylenediaminetetraacetic acid) after obtaining informed consent. Patients with SCD who had received a blood transfusion within the past 3 months or were taking hydroxyurea or had a painful episode within the previous 30 d were excluded from study.

RBC were washed three times in phosphate-buffered saline (PBS; 2.67 mM KCl, 1.47 mM KH_2PO_4 , 137.93 mM NaCl, 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; pH 7.2) and resuspended in PBS at a hematocrit of 30%.

RBC PLSCR activity

Phospholipid scramblase activity was determined by flow cytometric measurement of the internalization of a fluorescent analogue of phosphatidylcholine (NBD-PC), as previously described (5, 8, 22–24). Internal NBD-PC was determined after residual probe in the outer leaflet was removed with BSA extraction. RBC with active PLSCR have more NBD-PC fluorescence after extraction, whereas RBC in which PLSCR is inactive have little or no NBD-PC fluorescence. Using a gate that included all RBC, we obtained a mean fluorescence (MF) value for pre- and postextraction samples and expressed PLSCR activity as NBD-PC internalization fraction (IF) calculated for each sample by dividing postextraction MF by pre-extraction MF.

PMA treatment

Phorbol-12-myristate-13-acetate treatment of RBC was performed as previously described (18). Normal or sickle RBC were suspended at 0.1% hematocrit in 1 mL EGTA buffer (100 mM KCl, 45 mM NaCl, 20 mM HEPES, 10 mM glucose, 0.2 mM MgCl_2 , and 1 mM EGTA) containing either 10 μM Chel or an equivalent volume (2 μL) of H_2O at room temperature. The suspensions were transferred to a 37°C water bath, and 1 μM final concentration NBD-PC was added to each. NBD-PC was allowed to incorporate into the RBC membranes at 37°C in the presence or absence of Chel for 30 min. During this NBD-PC incorporation incubation, samples of each suspension were taken every 6 min, immediately placed on ice, and separated into two equal aliquots. One aliquot was subjected to BSA extraction. After 30 min of NBD-PC incorporation, each cell suspension was divided into two equal parts for further incubation at 37°C with and without PMA. PMA was reconstituted with DMSO just prior to use and added to one part at a final concentration of 6 μM . An equal volume of DMSO was added to the second part. Both suspensions, with and without PMA, were incubated at 37°C, and samples of each were taken at 0, 3, 6, 9, and 12 min for normal RBC and 0, 3, 6, 15, and 30 min for sickle RBC. Samples from each time point were immediately placed on ice and separated into two equal aliquots. One aliquot was subjected to BSA extraction.

For experiments in which PLSCR activity was determined for reticulocytes (cells that were positive for transferrin receptor, TfR), sickle RBC were labeled with anti-TfR- PE/Cy5 (5) prior to the NBD-PC internalization assay and inhibitor pretreatment. For experiments in which both PLSCR activity and external PS were determined, sickle RBC were incubated with annexin V-PE (5) after completion of the NBD-PC internalization assay. Three color flow cytometric analysis was performed on sickle RBC samples labeled with anti-TfR, NBD-PC, and annexin V to measure the NBD-PC internalization fraction for each cell subpopulation of interest.

Results

NBD-PC internalization into normal and sickle RBC after PMA treatment

Figure 1 shows examples of flow cytometric histograms for pre- and postextraction samples of normal and sickle RBC after treatment with PMA (or DMSO control) in the presence or absence of Chel. The black peaks in the top two rows, representing postextraction NBD-PC fluorescence for control samples treated with DMSO or DMSO + Chel, represent background fluorescence. In these samples, the NBD-PC was extracted from the outer RBC leaflets indicating that no NBD-PC was internalized in the presence or absence of Chel pretreatment. In response to PMA, there was an immediate and progressive increase in postextraction NBD-PC fluorescence (black peaks) indicating rapid internalization of NBD-PC into the inner RBC membrane. We observed two postextraction NBD-PC internalization peaks at 3 min in PMA-treated normal and sickle RBC. This suggests that cells within the same sample can undergo different degrees of PLSCR activation. When RBC were treated with PMA in the presence of Chel, the PMA-induced widening of the postextraction fluorescence peak was inhibited and a longer time was required for increased postextraction fluorescence. Taken together, these data are consistent with PLSCR activation by PKC after stimulation by PMA. While this activation occurred in both normal and sickle RBC, it was more pronounced in sickle cells.

Internalization fractions were calculated from the pre- and postextraction histograms in Fig. 1 as described in Methods; a stable ratio indicates an inactive PLSCR and an increasing ratio reflects an activated PLSCR. Figure 2A shows internalization fractions for a representative sample of normal RBC after addition of PMA. The NBD-PC internalization fraction increased linearly during the first 6 min after PMA addition, implying rapid activation of PLSCR. This activation was partially prevented by Chel. Figure 2B and C shows representative examples of NBD-PC internalization in sickle RBC before and after addition of PMA. Time points prior to PMA addition were examined as, in contrast to normal RBC, preliminary experiments demonstrated endogenous PLSCR activity in some sickle RBC subpopulations. In mature (TfR⁻) sickle RBC (Fig. 2B), there was little or no endogenous PLSCR activity and no effect of Chel prior to PMA addition (-24-0 min). After PMA addition, there was rapid PLSCR activation that was partially prevented by Chel. In TfR⁺ sickle reticulocytes (Fig. 2C), in the absence of PMA, there was endogenous PLSCR activity which increase progressively throughout the incubation; Chel inhibited this endogenous activity. Addition of PMA to TfR⁺ sickle reticulocytes caused a marked increase in PLSCR activity that was resistant to inhibition by Chel at the concentration used.

Figure 3A summarizes PLSCR activity *before* PMA treatment in mature (TfR⁻) sickle RBC and TfR⁺ sickle reticulocytes. PLSCR activity is expressed as the change (slope) in the internalization fraction over time. During this pretreatment period, the presence of Chel (black) had no effect on PLSCR activity in mature (TfR⁻) sickle RBC. PLSCR activity was higher in TfR⁺ sickle reticulocytes than in TfR⁻ sickle RBC and was inhibited by Chel, suggesting its dependence on PKC even in the absence of PMA. Fig. 3B shows PLSCR activation in normal and sickle RBC and TfR⁺ sickle reticulocytes *after* PMA treatment. Chel significantly inhibited activation of PLSCR after PMA addition to normal and mature

(TfR-) sickle RBC. Sickle RBC had significantly higher PLSCR activity after PMA treatment than normal RBC, and there was greater variability in the degree of PLSCR activation in sickle RBC. PMA treatment of TfR+ sickle reticulocytes significantly increased PLSCR activity compared to endogenous levels, as it did in both normal and mature SS RBC. However, Chel did not inhibit this PMA-stimulated activity in reticulocytes as it did in mature RBC, suggesting that pathways other than Chel-sensitive PKC were involved in this effect of PMA.

PS externalization in normal and sickle RBC treated with PMA

As PS externalization can occur as a result of PLSCR activation, we next examined the effect of PMA-induced PLSCR activity on PS externalization.

Incubation of normal or sickle RBC at 37°C for 30 min in the absence of PMA gave no change in external PS (with or without Chel (Fig. 4A). After PMA-dependent activation of PLSCR, external PS increased significantly in both normal and sickle RBC (Fig. 4A and B). Unexpectedly, a greater effect was observed in normal cells than sickle cells, despite lower PLSCR activation in normal cells (Figs 2 and 3). PS externalization was partially prevented by pretreatment of normal and sickle RBC with Chel.

Discussion

The collapse of normal phospholipid asymmetry in pathologic cells is facilitated by PLSCR (25) and may or may not be accompanied by APLT inhibition. We recently examined the balance of APLT and PLSCR activities in sickle RBC subpopulations (in the absence of PMA) and found that mature (i.e., non-reticulocyte) sickle RBC with high external PS had increased PLSCR and decreased APLT activity (5). These data were consistent with the concept that PS externalization in RBC depends on the relative activities of PLSCR and APLT. We also found active APLT and active PLSCR in sickle reticulocytes with and without external PS (5).

We now show that treatment with PMA, a known activator of PKC, causes a rapid increase in PLSCR activity, estimated by the rate of NBD-PC internalization, in normal and sickle RBC. The activity of PLSCR after PMA was higher and more variable in sickle RBC than in normal RBC and may be related to variability in PS externalization in sickle RBC subpopulations. The PKC inhibitor Chel caused a significant but not complete inhibition of PMA-mediated PLSCR activation in both normal RBC and mature sickle RBC, raising the possibility of both PKC-dependent and PKC-independent activation of PLSCR. Among the potential explanations for greater PLSCR activation in sickle RBC is the higher PKC content in sickle RBC membranes compared to normal RBC (10, 26).

These studies confirm that without PMA, sickle reticulocytes have significantly increased PLSCR activity compared to mature sickle RBC, and this activity is inhibited by Chel. After PMA stimulation, there is further activation of PLSCR in sickle reticulocytes. However, Chel did not inhibit the PMA-mediated activation in these cells. This observation suggests that either PMA treatment causes an overwhelming PKC activation in sickle reticulocytes so that even after partial inhibition by Chel, there is still activation of PLSCR, or a different

mechanism is in place in reticulocytes. Normal reticulocytes were characterized with respect to both APLT and PLSCR activities in a previous publication from our group (5) and found to be similar to normal mature RBC.

Despite higher PLSCR activation in sickle RBC, there was a greater increase in PMA-induced PS externalization in normal RBC. As sickle RBC contain a much higher proportion of young cells than normal RBC, the presence of highly active APLT in younger sickle RBC may have suppressed PS externalization even though PLSCR was activated. Additionally, increased PS externalization after PMA could be influenced by other factors in abnormal sickle subpopulations. For example, there is evidence that cytosolic PKC content and activity is decreased in dense sickle RBC (26), and this would offset the known decreased level of APLT in these cells to determine external PS after addition of PMA.

We confirm that treatment of RBC with PMA results in an increased level of external PS (18). PS externalization followed, and was most likely caused by, rapid activation of PLSCR by PKC-mediated phosphorylation. This mechanism was supported by the partial prevention of PLSCR activation and PS externalization of the PKC inhibitor Chel. These studies suggest that inhibition of RBC PKC may be a useful therapeutic strategy in SCD and that identification of the *in vivo* pathway(s) of PKC-mediated PLSCR activation is needed.

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Figure 1.

Example of flow cytometric histograms measuring NBD-PC fluorescence of pre- and postextraction normal (A) and sickle (B) RBC. Gray peaks represent NBD-PC fluorescence of pre-extraction samples, while black peaks represent postextraction samples. In this example, cells were treated with PMA or DMSO control for 12 or 15 min in the presence or absence of chelerythrine chloride (Chel). PLSCR activity is estimated by the proportion of internalized (postextraction) NBD-PC fluorescence divided by pre-extraction NBD-PC fluorescence present on the cells.

Figure 2. Phospholipid scramblase activation in normal and sickle RBC and TfR+ sickle reticulocytes in response to PMA treatment. NBD-PC internalization fractions in response to PMA treatment (at time = 0) in representative samples of (A) normal RBC, (B) mature (TfR-) sickle RBC, and (C) TfR+ sickle reticulocytes.

Figure 3.

Rate of change of NBD-PC internalization fraction. Average slopes of NBD-PC internalization (A) in mature (TfR-) sickle RBC and TfR+ sickle reticulocytes ($n = 4$) prior to addition of PMA between -6 min and 0 min and (B) in normal RBC, TfR- mature sickle RBC and TfR+ sickle reticulocytes ($n = 7$) after addition of PMA between 0 min and 6 min. Error bars represent one standard deviation from mean values. Statistical comparisons between samples $\pm$ Chel unless indicated by horizontal bars. (*, $P < 0.05$; **, $P < 0.01$).

Figure 4.

PS Externalization in normal and sickle RBC in response to PMA treatment. External PS, determined by Annexin V binding, in normal (A) and sickle RBC (B) in response to PMA in the presence and absence of Chelerythrine Chloride. For normal RBC, $n = 6$ or 7 ; sickle RBC, $n = 7$. Error bars represent one standard deviation from mean values. Statistical comparisons were made between PMA and PMA + Chel treatments. (*, $P < 0.05$; **, $P < 0.01$).