

Inhibitors of second messenger pathways and Ca^{2+} -induced exposure of phosphatidylserine in red blood cells of patients with sickle cell disease

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Abstract The present work investigates the contribution of various second messenger systems to Ca^{2+} -induced phosphatidylserine (PS) exposure in red blood cells (RBCs) from sickle cell disease (SCD) patients. The Ca^{2+} dependence of PS exposure was confirmed using the Ca^{2+} ionophore bromo-A23187 to clamp intracellular Ca^{2+} over 4 orders of magnitude in high or low potassium-containing (HK or LK) saline. The percentage of RBCs showing PS exposure was significantly increased in LK over HK saline. This effect was reduced by the Gardos channel inhibitors, clotrimazole and charybdotoxin. Nevertheless, although Ca^{2+} loading in the presence of an outwardly directed electrochemical gradient for K^+ stimulated PS exposure, substantial exposure still occurred in HK saline. Under the conditions used inhibitors of other second messenger systems (ABT491, quinacrine, acetylsalicylic acid, 3,4-dichloroisocoumarin, GW4869 and zVAD-fmk) did not inhibit the relationship between $[\text{Ca}^{2+}]$ and PS exposure. Inhibitors of phospholipase A2, cyclooxygenase, platelet-activating factor, sphingomyelinase and caspases, therefore, were without effect on Ca^{2+} -induced PS exposure in RBCs, incubated in either HK or LK saline.

Keywords Sickle cell disease · Red blood cell · Phosphatidylserine · Calcium · Second messengers

Introduction

Aminophospholipids including phosphatidylserine (PS) are normally confined to the inner leaflet of the plasma membrane [18]. Exposure is a pathophysiological process associated with apoptosis as PS is recognised by macrophages and other targets. A similar occurrence happens in red blood cells (RBCs) although in this case the term eryptosis is sometimes used, with that of apoptosis more appropriate for nucleated cells [18, 21]. Thus, in healthy individuals, only a small percentage of circulating RBCs show externalisation of PS. This is important because PS is prothrombotic and exposure causes RBC removal and thrombus formation [2, 11]. Increased PS exposure does occur in various disease states including sickle cell disease (SCD), in which an increased, but variable, percentage of RBCs show externalised PS, associated with both the anaemic and ischaemic complications of the condition [5, 18, 19, 26]. As such, the mechanism of exposure has received considerable attention.

In normal RBCs, asymmetrical distribution of PS is maintained by an active aminophospholipid translocase (or flippase) which transports aminophospholipids including PS from outer to inner leaflet using energy from ATP hydrolysis [10]. In addition, there is low activity of a Ca^{2+} -dependent scrambling process (or scramblase), of which the transporter responsible remains unclear. In RBCs from SCD patients, PS exposure is associated by a rise in the activity ratio of scramblase to flippase [1] This can be caused by a number of factors but elevation of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) has been proposed as a significant precipitant. High $[\text{Ca}^{2+}]_i$ will both inhibit the flippase and activate the scramblase. Recently members of the TMEM16 protein family have been identified. They may function as Ca^{2+} -dependent phospholipid scramblases as well as Ca^{2+} -dependent Cl^- channels [35, 36], and as such may be important in PS exposure.

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RBCs from SCD patients contain the abnormal haemoglobin HbS, in which the normal amino acid at the sixth position of the β chain is replaced with valine [3]. This substitution allows neighbouring HbS molecules to aggregate on deoxygenation, forming long polymers which distort RBC shape (forming the eponymous sickles and other bizarre morphologies) and have other deleterious sequelae. A further important difference with normal RBCs is a high membrane permeability to cations [14, 27, 37]. One pathway that is activated on HbS polymerisation and sickling, and which is not seen in normal RBCs, is a deoxygenation-induced cation conductance sometimes termed P_{sickle} [13, 27, 28]. P_{sickle} activity has several consequences, one of which is entry of Ca^{2+} . P_{sickle} is therefore associated with deoxygenation-induced elevation of $[\text{Ca}^{2+}]$ [32]. It may therefore encourage PS exposure. Indeed, we have shown previously that deoxygenation-induced PS exposure requires extracellular Ca^{2+} at physiological concentrations, that Ca^{2+} entry and elevation of $[\text{Ca}^{2+}]_i$ is required, and that inhibitors of the P_{sickle} pathway also reduce deoxygenation-induced PS exposure [4, 39]. PS exposure in response to elevation of Ca^{2+} shows a biphasic response with high and low affinity components, requiring $[\text{Ca}^{2+}]_i$ of about 1 and 100 μM , respectively [39].

PS exposure in normal RBCs has also been extensively investigated in the context of eryptosis [21]. Several other second messenger pathways besides high $[\text{Ca}^{2+}]_i$ have been implicated including cyclooxygenase activity and prostaglandin E_2 (PGE_2) [23], phospholipase A2 (PLA2), platelet-activating factor (PAF) [23], sphingomyelinase activation and ceramide production [24, 25], caspases [29], and shrinkage [20, 22, 25, 31, 33]. These other pathways may interact with entry of Ca^{2+} via P_{sickle} . For example, it has been proposed in normal RBCs that PGE_2 may open non-specific cation channels to mediate Ca^{2+} entry [15–17]. In addition, there is evidence that Ca^{2+} -induced PS exposure also involves PKC, likely $\text{PKC}\alpha$ [38]. It is therefore important to establish the extent to which Ca^{2+} -induced PS scrambling is an indirect phenomenon involving any of these other pathways. In this study, we investigated this possibility using inhibitors of other second messenger pathways. Results suggest that neither cyclooxygenase, PLA2, PAF, sphingomyelinase, nor caspases are involved. Activation of the Ca^{2+} -activated K^+ channel (or Gardos channel [9]), with subsequent hyperpolarization or RBC shrinkage, however, do increase PS exposure.

Materials and methods

Solutions and chemicals

The main experimental saline comprised (in mM): NaCl 140, KCl 4, MgCl_2 0.15, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10 and inosine 10 with a pH

of 7.4 at 37 °C (low potassium-containing saline, LK saline). For washing RBCs, a high potassium-containing saline (HK saline) was usually used, comprising (in mM) NaCl 54, KCl 90, MgCl_2 0.15, HEPES 10 and inosine 10 with a pH of 7.4 at room temperature. For Ca^{2+} titration curves, EGTA (2 mM) and appropriate concentrations of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) was used to clamp free $[\text{Ca}^{2+}]_o$ at 0, 1, 10, 100 and 1,000 μM , with total $[\text{Ca}^{2+}]_o$ of 0, 1.91, 2.00, 2.10 and 3.00 mM, respectively. RBCs were permeabilised to Ca^{2+} with the ionophore bromo-A23187 (2.5–6 μM) — control experiments were carried out to determine $[\text{A23187}]$ for maximal effects for alteration of $[\text{Ca}^{2+}]_i$, PS exposure and also K^+ flux. In control experiments, in the absence of bromo-A23187, PS exposure over 60 min incubation changed by <2 % ($n=3$) with no systematic correlation between the extent of PS exposure and the extracellular calcium concentration. The ice-cold wash solution for K^+ flux measurements comprised (in mM): MgCl_2 107, 3-(*N*-morpholino)-propanesulphonic acid (MOPS) 10, pH 7.4 at 0 °C. Lactadherin (LA)-binding buffer consisted of LK or HK HBS with the appropriate $[\text{Ca}^{2+}]_o$, 1 mM vanadate and 16 nM LA-FITC. Osmolality of all solutions was 290 ± 5 mOsm kg^{-1} . Lactadherin-FITC was obtained from Haematologic Technologies Inc. (Vermont, USA) supplied via Cambridge Bioscience (Cambridge, UK), anti-glycophorin A-PE from Becton Dickinson Biosciences (California, USA), HEPES and bromo-A23187 from Calbiochem (Merck, Darmstadt, Germany), the pan caspase inhibitor zVAD-fmk from Enzo Life Sciences Ltd (Essex, UK) and $^{86}\text{Rb}^+$ from PerkinElmer (MA, USA). All other chemicals came from Sigma-Aldrich (Poole, Dorset, UK). Stock solutions of ABT491 and quinacrine were made in water; those of clotrimazole, charybdotoxin, diclofenac, acetylsalicylic acid, 3,4-dichloroisocoumarin, GW4869 and zVAD-fmk in DMSO (final concentration, 0.5 %). Where it was used as a solvent, control RBCs were treated with the same [DMSO]. Control experiments were carried out to investigate the effect of inhibitors on intracellular Ca^{2+} . For these, RBCs were loaded with 5 μM fluo-4-AM for 45 min at 37 °C in the dark (1.5 % Hct) in HK saline, washed once into HK saline containing 1 μM Ca^{2+} , pretreated with second messenger inhibitors prior to addition of ionophore. Mean fluorescence was then measured by FACS (see below) and did not vary by >6 % compared to controls in the absence of second messenger inhibitors, indicative of the lack of effect of inhibitors on $[\text{Ca}^{2+}]_i$, with the exception of RBCs incubated with GW4869 in which mean fluorescence increased by 37 %; however, in this case RBCs showed some evidence of clumping.

RBCs preparation

Blood samples were collected from HbSS SCD patients, with consent and ethical permission (11/LO/0065), in the

anticoagulant EDTA. RBCs were washed three times into HK HBS for 3 min at $600\times g$ and subsequently twice into LK or HK HBS with 2 mM EGTA to remove contaminant Ca^{2+} . RBC suspensions, final haematocrit (Hct) of 0.5 % (except in Fig. 7, where Hct was initially 5 %), were incubated at 37 °C for 30 or 60 min in the absence or presence of various second messenger inhibitors, followed by treatment with bromo-A23187 (nominally 2.5–6 μM with separate batches titrated to establish the optimal concentration, final [DMSO] <0.5 %) at the indicated free $[\text{Ca}^{2+}]_o$ for 30 min at 37 °C. Vanadate (1 mM) was present in the last step to inhibit both the flippase and the plasma membrane calcium pump (PMCA).

Labelling of PS exposure

For PS labelling, 5- μl aliquots (10^5 RBCs) of each sample were placed in 250 μl of LA-FITC binding buffer and incubated in the dark at room temperature for 10 min. RBCs were then pelleted by centrifugation for 10 s at $16,100\times g$, washed once in LK or HK HBS to remove unbound LA-FITC and kept on ice until flow cytometry analysis. Unlike annexin-V, LA-FITC binds to PS in a Ca^{2+} -independent manner and control experiments showed that binding was irreversible. Inhibitors were tested for self-fluorescence at their highest concentration with unlabelled RBCs.

FACS acquisition and analysis

Externalised PS was measured with LA-FITC using an excitation wavelength of 488 nm in the FL1 channel with an emission wavelength of 519 nm on a fluorescence-activated flow cytometer (FACSCalibur, Becton Dickinson, BD) and analysed with the BD CellQuest Pro software using a protocol published previously [4]. Measurements were taken using a logarithmic gain. Forward scatter (FSC, size) and side scatter (SSC, granularity) gates for RBCs were identified in control experiments using anti-glycophorin A-PE labelled RBCs. The positive fluorescent gate was set using RBCs unlabelled with LA-FITC. For each measurement 10,000 events were gated. PS positive cells were defined as all events falling within the preset FSC, SSC and positive fluorescent gates.

K^+ flux measurements

Potassium fluxes were measured using $^{86}\text{Rb}^+$ as a tracer for K^+ [6, 34]. RBCs (Hct about 20 %) were first equilibrated in Eschweiler tonometers (Kiel, Germany) in air at 37 °C, pH 7.4, in HK saline with added CaCl_2 (10 μM) in which glucose (5 mM) was substituted for inosine and MOPS for HEPES. Aliquots were then diluted 10-fold into test tubes containing the same saline with added A23187 (nominally 4 μM). Bumetanide (10 μM) and ouabain (100 μM) were present to inhibit K^+ transport through the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$

cotransport and Na^+/K^+ pump. Influx was started by adding $^{86}\text{Rb}^+$ (final activity about 0.05 MBq ml^{-1}) and was performed in the absence and presence of clotrimazole (5 μM) over 10 min, to give the activity of the Ca^{2+} -activated K^+ (or Gardos) channel after which RBCs were washed four times in ice-cold isotonic MgCl_2 wash solution, the final cell pellet lysed with Triton X-100 (0.1 %), protein precipitated with 5 % trichloroacetic acid and activity was measured as Čerenkov radiation by liquid scintillation (Packard Tri-carb 2100TR). Microhaematocrit determination was used to measure Hct.

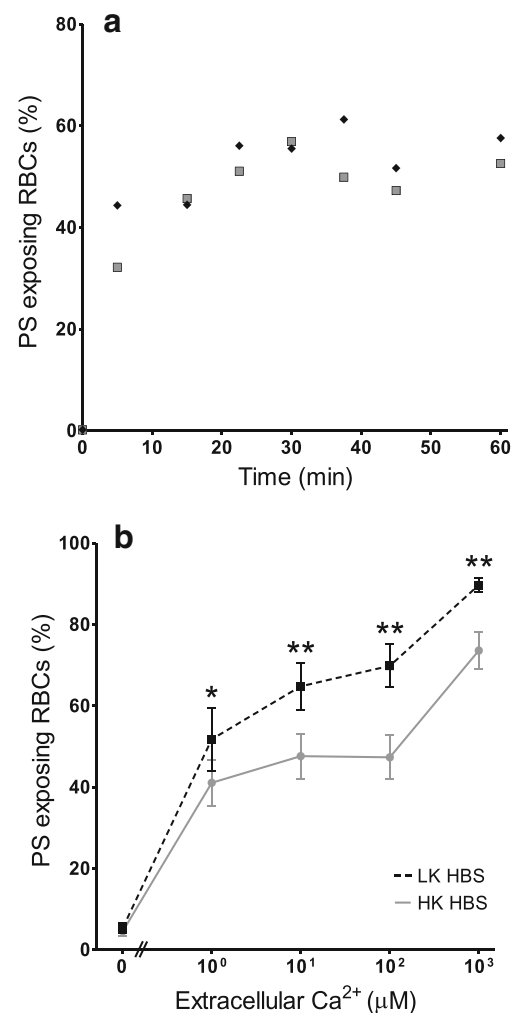


Fig. 1 Ca^{2+} -induced exposure of phosphatidylserine (PS) in red blood cells (RBCs) from patients with sickle cell disease (SCD). **a** Time course: RBCs were treated with the ionophore bromo-A23187 (2.5–6 μM) for up to 60 min in high potassium-containing (HK) saline with 100 μM extracellular $[\text{Ca}^{2+}]_o$ ($[\text{Ca}^{2+}]_o$). Symbols represent duplicate measurements from a single sample. **b** Ca^{2+} dependence: RBCs (0.5 % haematocrit, Hct; final [DMSO] <1 %) were treated with ionophore (2.5–6 μM) for 30 min, in HK or low potassium-containing (LK) saline, with $[\text{Ca}^{2+}]_o$ varied between 0 μM and 1 mM. Symbols represent means \pm SEM, $n=12$. * $p < 0.05$ and ** $p < 0.002$ between PS exposure in RBCs incubated in HK or LK saline

Statistics

With the exception of those shown in Figs. 1a and 7, all data are presented as means±SEM for blood samples of *n* different SCD patients. Statistical significance was tested with Student's *t*-test, and a *p* value <0.05 was considered significant.

Results

Ca²⁺ dependence of PS exposure of RBCs from patients with SCD

The Ca²⁺ dependence of PS scrambling was confirmed using the ionophore bromo-A23187 to clamp intracellular [Ca²⁺]_i. Due to the Donnan ratio, this method clamps [Ca²⁺]_i at about double the value of [Ca²⁺]_o [7, 30]. PS scrambling occurred within minutes with a plateau achieved by 20 min (Fig. 1a). In all subsequent experiments, therefore, RBCs were treated with ionophore for 30 min before labelling for PS. Elevation of [Ca²⁺]_i, as well as causing PS scrambling, will also lead to activation of the Ca²⁺-activated K⁺ channel (or Gardos channel), and possibly a Ca²⁺-dependent Cl⁻ channel [35]. In low potassium-containing (LK) salines, Gardos channel activation

will result in loss of K⁺ and shrinkage and hyperpolarisation of the RBC membrane [12]. High potassium-containing salines (HK saline) were used to prevent these secondary effects. Typically, a biphasic response was apparent in both HK and LK salines with the major component of PS scrambling (accounting for about two-thirds of the total) occurring with high Ca²⁺ affinity (about 1 μM) and a minor component with low Ca²⁺ affinity (about 100 μM, Fig. 1b), as previously reported [39]. PS exposure was significantly increased in LK HBS. The Ca²⁺ affinities, however, appeared unaltered (Fig. 1b). These Ca²⁺ titration curves of PS exposure were subsequently used to investigate the effect of inhibitors of other second messengers, with RBCs left as untreated controls or treated with second messenger inhibitors for 30 min prior to and also during alteration of [Ca²⁺]_i with ionophore.

The effect of inhibitors of second messengers in HK saline

The effect of diclofenac (10–500 μM) and acetylsalicylic acid (50–200 μM) as inhibitors of cyclooxygenase, ABT491 (1–50 μM) as an antagonist of PAF receptors, quinacrine (25 and 100 μM) as a non-specific PLA2 inhibitor, and two sphingomyelinase inhibitors, 3,4-dichloroisocoumarin (20 and 200 μM) — a serine proteinase inhibitor — and GW4869 (100

Table 1 Effect of second messenger inhibitors on phosphatidylserine (PS) exposure in red blood cells (RBCs) from sickle cell disease (SCD) patients

Second messenger System	Inhibitor	[Ca ²⁺] _i (μM) in HK saline				
		0 % PS Exposure	1	10	100	1000
Cyclooxygenase	Acetylsalicylic acid					
	Control	4±0	53±11	52±7	61±6	80±6
	200 μM (<i>n</i> =3)	4±1	55±6	58±5	58±5	82±7
	Diclofenac					
	Control	4±0	31±9	34±5	45±7	68±4
	500 μM (<i>n</i> =4)	4±1	44±7*	47±7*	52±3	87±2*
Platelet activating factor	ABT491					
	Control	5±2	28±2	33±6	36±2	77±2
	50 μM (<i>n</i> =3)	4±1	29±4	42±3	44±5	72±12
Phospholipase A ₂	Quinacrine					
	Control	2±0	25±5	29±9	36±3	67±9
	100 μM (<i>n</i> =3)	6±1	33±5	38±5	40±2	73±4
Sphingomyelinase	3,4-Dichloro-isocoumarin					
	Control	4±0	53±7	51±5	61±4	79±6
	200 μM (<i>n</i> =4)	5±0	49±3	56±3	52±5	81±4
	GW4869					
	Control	3±0	21±6	30±2	33±4	68±8
	10 μM (<i>n</i> =5)	17±5*	50±7*	47±10	57±5*	77±4

RBCs were treated with the ionophore bromo-A23187 (2.5–6 μM) for 30 min in HK saline in the absence of [Ca²⁺]_o and with [Ca²⁺]_o varied between 1 μM and 1 mM. Inhibitors were absent (controls) or added at the concentrations indicated for 30 min prior to, and during, ionophore treatment (0.5 % Hct, final [DMSO] <1 %). Values represent means±SEM of percentage of RBCs with exposed PS for *n* individuals. * *p*<0.05 compared to controls

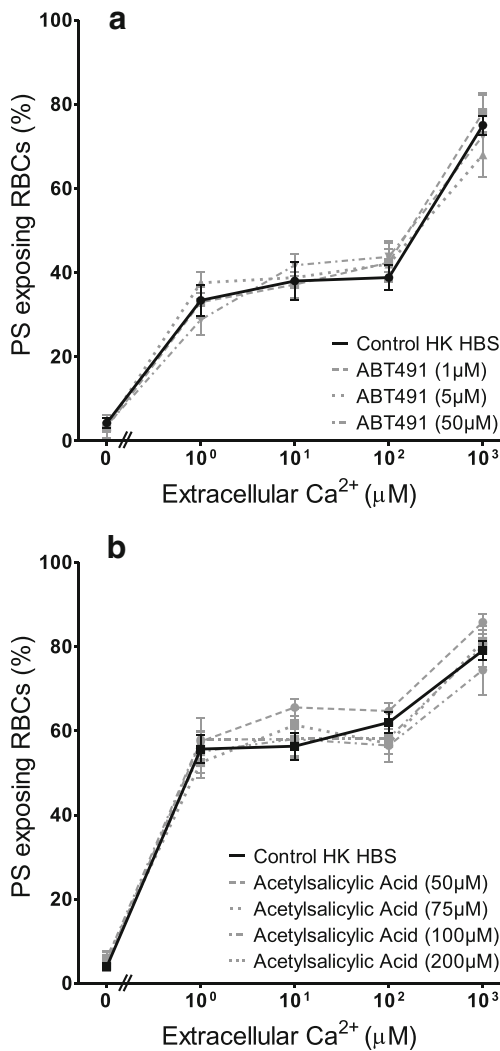


Fig. 2 Effect of platelet-activating factor (*PAF*) and cyclooxygenase inhibitors on Ca^{2+} -induced PS exposure in RBCs from SCD patients. RBCs were incubated in HK saline in the presence or absence of inhibitor for 30 min prior to treatment with ionophore for 30 min (as in Fig. 1b). **a** Effect of treatment with the *PAF* inhibitor ABT491 (1–50 μM). **b** Effect of treatment with the cyclooxygenase inhibitor acetylsalicylic acid (50–200 μM). Symbols represent means \pm SEM, $n=3$, except for 5 μM ABT491, where $n=2$. There was no significant difference between values in the presence or absence (controls) of the inhibitors

nM–10 μM), both active against neutral sphingomyelinase, were tested on PS scrambling in HK saline. Results are summarised in Table 1 for the highest inhibitor concentration used while Fig. 2 shows the effect of ABT491 and of acetylsalicylic acid over the full range of concentrations used. None of these reagents decreased the extent of PS exposure in the Ca^{2+} titration curves. Diclofenac and GW4896, however, showed a significant increase in PS exposure at some, but not all, concentrations (Table 1). For GW4896, this did not require the presence of Ca^{2+} . In the presence of GW4896 at 10 μM there was some indication of RBC clumping which may have artefactually increased PS exposure. Such increases were not

present for the alternative cyclooxygenase and sphingomyelinase inhibitors, acetylsalicylic acid and 3,4-dichloroisocoumarin.

The effect of Gardos channel activity on PS exposure of RBCs from SCD patients

In the next series of experiments, the effect of Ca^{2+} -induced K^+ efflux via the Gardos channel was investigated. When RBCs were incubated with the Gardos channel inhibitor clotrimazole (5–20 μM , results shown for 20 μM only), PS

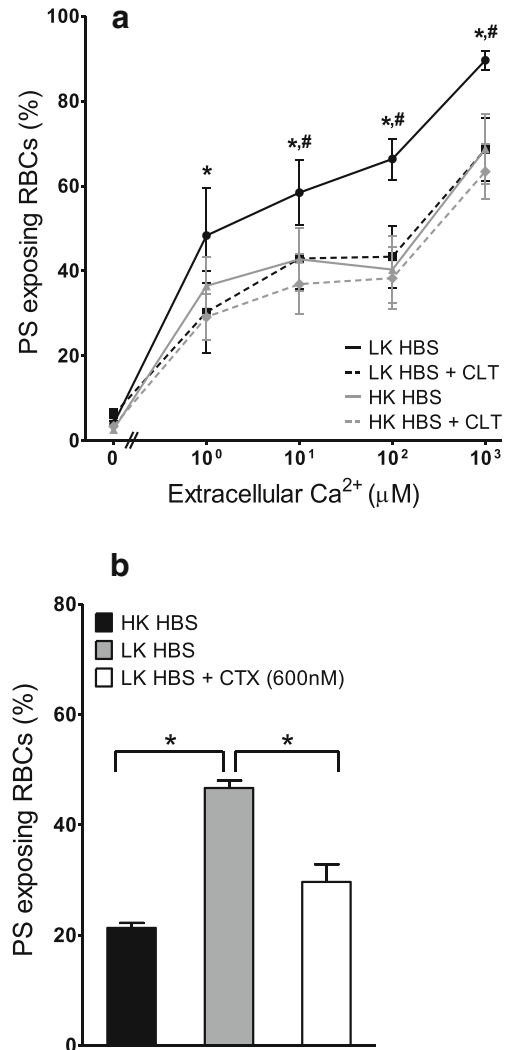


Fig. 3 Involvement of the Ca^{2+} -activated K^+ channel (Gardos channel) in Ca^{2+} -induced PS exposure in RBCs from SCD patients. RBCs were treated with ionophore for 30 min (as in Fig. 1b). **a** RBCs were incubated in either HK or LK HEPES-buffered saline (*HBS*) in the absence and presence of 20 μM clotrimazole (*CLT*). Symbols represent means \pm SEM, $n=5$; black circles LK *HBS*, black squares LK *HBS* with *CLT* (20 μM), grey triangles HK *HBS* and grey diamonds HK *HBS* with *CLT* (20 μM). $*p<0.03$ between PS exposure in RBC incubated in LK saline in the presence and absence of *CLT*, $\#p<0.05$ between LK and HK saline. **b** RBCs were incubated in HK or LK saline, in the absence and presence of charybdotoxin (600 nM). Histograms represent means \pm SEM, $n=3$. $*p<0.05$

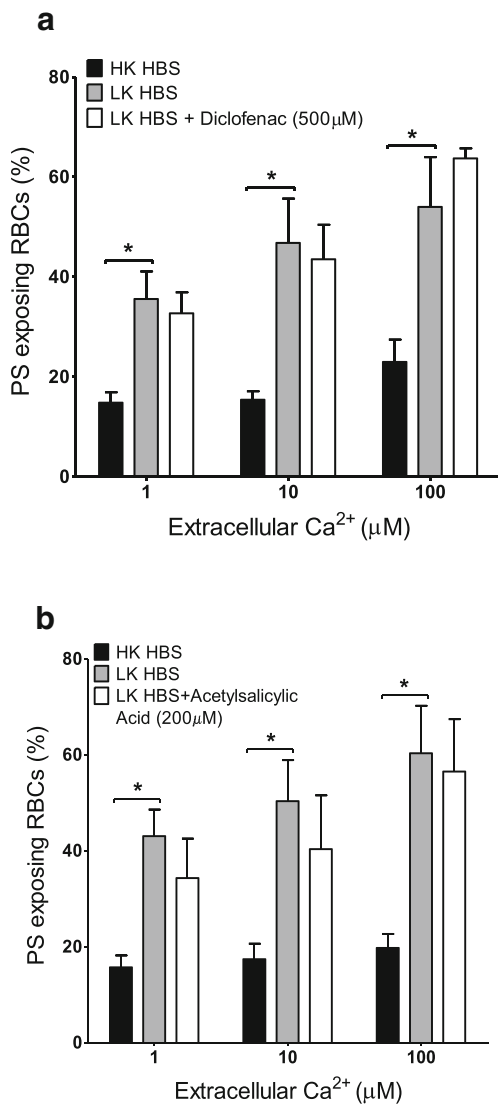


Fig. 4 Effect of cyclooxygenase inhibitors on Ca²⁺-induced PS exposure in RBCs from SCD patients. RBCs were incubated in HK saline, LK saline or LK saline plus inhibitor for 30 min before treatment with ionophore for 30 min (as in Fig. 1b). **a** Effect of diclofenac (500 μM). **b** Effect of acetylsalicylic acid (200 μM). Histograms represent means±SEM, n=3. *p<0.05

exposure in LK saline was reduced (Fig. 3a). Clotrimazole, however, had no effect on PS exposure in RBCs incubated in HK saline (Fig. 3a). Charybdotoxin (600 nM), a spider venom which inhibits the Gardos channel with higher affinity than clotrimazole, was also tested. Similar to clotrimazole, PS exposure in LK saline was reduced to levels observed in HK saline (Fig. 3b). The inhibitory effect of these drugs on Ca²⁺-activated K⁺ fluxes across the RBC membrane was confirmed. With A23187-treated RBCs and [Ca²⁺]_o of 10 μM Ca²⁺, K⁺ influx increased to 386±79 mmol (l cells h)⁻¹ being reduced to 18±2 (means±SEM, n=9) in the presence of 5 μM clotrimazole, an inhibition of 95 %. These findings are consistent with activation of the Gardos channel with an outwardly

directed K⁺ gradient responsible for elevation of PS exposure in LK saline in RBCs permeabilised to Ca²⁺, as has been observed in RBCs from normal individuals [22, 31].

The effect of inhibitors of second messengers in LK saline

Although Gardos channel activity likely accounts for the higher PS exposure in LK saline (compared to HK saline), other second messenger pathways may also be involved. The various inhibitors were therefore tested on the augmented PS exposure observed in LK saline. PS exposure was measured in LK saline in ionophore-treated RBCs at 1, 10 and 100 μM

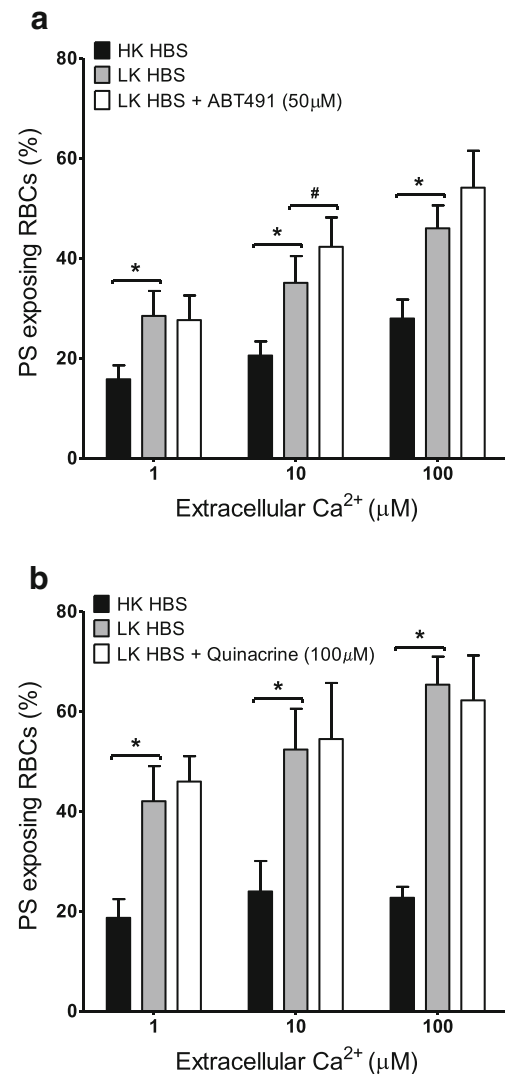


Fig. 5 Effect of platelet-activating factor (PAF) and phospholipase A2 (PLA2) inhibitors on Ca²⁺-induced PS exposure in RBCs from SCD patients. RBCs were incubated in HK saline, LK saline or LK saline plus inhibitor for 30 min before treatment with ionophore for 30 min (as in Fig. 1b). **a** Effect of the PAF inhibitor ABT491 (50 μM). Histograms represent means±SEM, n=7. #p<0.05, *p<0.005. **b** Effect of the PLA2 inhibitor quinacrine (100 μM). Histograms represent means±SEM, n=3. *p<0.03

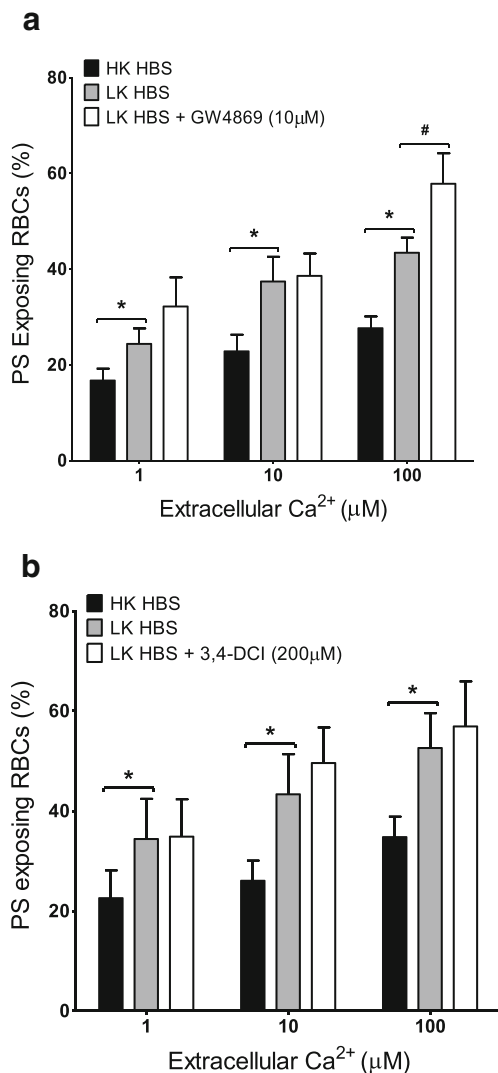


Fig. 6 Effect of sphingomyelinase (*SMase*) inhibitors on Ca²⁺-induced PS exposure in RBCs from SCD patients. RBCs were incubated in HK saline, LK saline or LK saline plus inhibitors for 30 min before treatment with ionophore for 30 min (as in Fig. 1b). **a** Effect of the Mg²⁺-dependent neutral *SMase* inhibitor GW4869 (10 μM). Histograms represent means ± SEM, *n* = 6. **b** Effect of the *SMase* inhibitor 3,4-dicloroisocoumarin (200 μM). Histograms represent means ± SEM, *n* = 6. **p* < 0.03, [#]*p* < 0.05

[Ca²⁺]_o at the highest concentration of inhibitors used in HK saline (Figs. 4, 5 and 6). In all, there was a significant increase in PS exposure when comparing RBCs incubated in LK with those in HK saline. Again, however, there was no significant difference in PS exposure in the absence or presence of diclofenac (500 μM), acetylsalicylic acid (200 μM), quinaquine (100 μM) or 3,4-dichloroisocoumarin (200 μM). While there was an increase in PS exposure (*p* < 0.05) with ABT491 (50 μM) at a free [Ca²⁺]_i of 10 μM and GW4869 (10 μM) at a free [Ca²⁺]_i of 100 μM PS exposure at the other [Ca²⁺]_is was unchanged. However, none of the drugs used caused an inhibition of PS exposure. Finally, the effect of the pan caspase

inhibitor zVAD-fmk (60 μM) was investigated (Fig. 7) in LK saline. Again, PS exposure was unaltered.

Discussion

The present work investigates the contribution of various other second messenger systems to Ca²⁺-induced PS exposure in RBCs from SCD patients. Inhibitors of cyclooxygenase, PAF, PLA2, sphingomyelinase and caspases were without effect. By contrast, the percentage of RBCs showing PS exposure was significantly increased under circumstances which allowed K⁺ exit through the Ca²⁺-activated K⁺ channel (or Gardos channel). This effect was inhibited by the Gardos channel inhibitors, clotrimazole and charybdotoxin.

The assay chosen for the current investigation was a Ca²⁺ titration curve in which RBCs were treated with bromo-A23187 and different levels of extracellular Ca²⁺ to alter the free [Ca²⁺]_i between 0.1 μM and 1 mM. This allows the visualisation of both high and low Ca²⁺ affinities of PS scrambling [4, 39], a process that begins within 5 min and is complete within 20 min. The assay used enables any effects of second messenger inhibitors to be observed as either a change in Ca²⁺ affinity of the scrambling process or as a change of the percentage of RBCs showing PS exposure. Under the conditions chosen, however, Ca²⁺-induced scrambling did not appear to involve cyclooxygenase, PAF, PLA2, sphingomyelinase and caspases in either HK saline or in LK saline. Increases in PS exposure with diclofenac and GW4869 did occur but were variable, inconsistent between HK and LK salines, and not observed with alternative inhibitors of the same

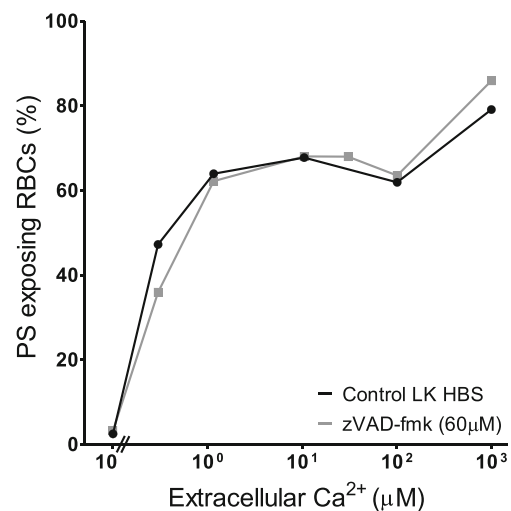


Fig. 7 Effect of the caspase inhibitor zVAD-fmk on Ca²⁺-induced PS exposure in RBCs from SCD patients. RBCs (5 % Hct) were incubated in LK saline for 60 min in the absence or presence of zVAD-fmk (60 μM) prior to treatment with ionophore (30 min, at 0.5 % Hct, as in Fig. 1b). Results are from a single experiment representative of four different SCD patients

pathways (acetylsalicylic acid and 3,4-dichloroisocoumarin), perhaps indicative of a non-specific effect. The time taken for RBCs to traverse the microvasculature in vivo will usually be seconds to minutes, so these findings suggest that relatively brief elevations of intracellular $[Ca^{2+}]$ would probably need to act directly if they are to stimulate the scrambling process.

PS exposure involved in the process of RBC eryptosis has received considerable attention and has emerged as a complex event with many potential stimuli. Cyclooxygenase and PGE₂ [8, 23], PAF and PLA2 [23], sphingomyelinase and ceramide [24, 25] and caspases [29] have all been implicated in PS exposure using inhibitor studies and reagents similar to those described here. These previous reports differ in the use of normal human (HbAA) RBCs, with one exception [26]. They also usually involve relatively prolonged incubation of RBCs with reagents tested on PS exposure over several hours to days (e.g., [25, 26]). The shorter term effects described in the current work may be more relevant to the initial trapping of PS-exposing RBCs via interaction with phagocytes or activated endothelial cells. The Ca^{2+} signal alone would appear to contribute to the initial PS exposure under these conditions, with other important pathways investigated in the present work becoming involved more slowly and playing an additional role in stimulating PS exposure.

Gardos channels have previously been shown to increase PS exposure in response to Ca^{2+} loading in normal (HbAA) human RBCs [22, 31]. In addition, treatment with the K^+ ionophore valinomycin to increase K^+ permeability directly also increases PS exposure [33]. We observed similar effects in RBCs from SCD patients. Thus in contrast to cyclooxygenase, PAF, PLA2, sphingomyelinase and caspase activity, activation of the Gardos channel in the presence of an outwardly directed electrochemical gradient for K^+ was associated with a rise in the percentage of RBCs showing PS exposure. The rise of PS was inhibited by removing the gradient for K^+ loss (HK saline) or by inhibition of the channel directly with either clotrimazole or charybdotoxin, as shown previously in RBCs from normal individuals [22, 31]. When activated, K^+ channels will cause hyperpolarization of the RBC membrane as well as solute loss and shrinkage [12]. It has been suggested that the increased negative potential difference acts to increase Ca^{2+} entry and hence elicit PS exposure [22]. The lack of effect on Ca^{2+} affinity in the titration curves (e.g., Fig. 1b), however, suggests that this is not the case. It is also important to realise, nevertheless, that the considerable PS exposure in HK saline implies that K^+ efflux and its sequelae are not prerequisites of Ca^{2+} -induced PS exposure. Similarly, although high K^+ may directly inhibit scrambling [40], this effect was not sufficient to prevent considerable PS exposure in RBCs from SCD patients.

In conclusion, Ca^{2+} -induced PS exposure in RBCs from SCD patients does not appear to involve a number of second messengers in the time scale of the present experiments. K^+ efflux, probably via shrinkage, however, does increase externalization of PS.

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