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Identification of the Ca^{2+} entry pathway involved in deoxygenation-induced phosphatidylserine exposure in red blood cells from patients with sickle cell disease

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Abstract Phosphatidylserine (PS) exposure in red blood cells (RBCs) from sickle cell disease (SCD) patients is increased compared to levels in normal individuals and may participate in the anaemic and ischaemic complications of SCD. Exposure is increased by deoxygenation and occurs with elevation of intracellular Ca^{2+} to low micromolar levels. The Ca^{2+} entry step has not been defined but a role for the deoxygenation-induced pathway, P_{sickle}, is postulated. Partial P_{sickle} inhibitors 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS), 4,4′-dithiocyano-2,2′-stilbene-disulphonic acid (DIDS) and dipyridamole inhibited deoxygenation-induced PS exposure (DIDS IC_{50}, 118 nM). Inhibitors and activators of other pathways (including these stimulated by depolarisation, benzodiazepines, glutamate and stretch) were without effect. Zn^{2+} and Gd^{3+} stimulated PS exposure to high levels. In the case of Zn^{2+}, this effect was independent of oxygen (and hence HbS polymerisation and RBC sickling) but required extracellular Ca^{2+}. The effect was completely abolished when Zn^{2+} (100 μM) was added to RBCs suspended in autologous plasma, implying a requirement of high levels of free Zn^{2+}.

Keywords Sickle cell disease · Red blood cell · Phosphatidylserine · Deoxygenation · Calcium · Cation channel

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Introduction

Phosphatidylserine (PS) is an aminophospholipid which is normally confined to the inner leaflet of lipid bilayers [40]. In red blood cells (RBCs), this is particularly important because its exposure is prothrombotic, encourages phagocytosis and increases RBC adherence [7, 29, 59, 63, 64]. In healthy individuals, usually <1 % RBCs expose PS [40]. Programmed externalisation is thought to occur in damaged or senescent RBCs to aid their removal from the circulation. This process has been termed eryptosis [42, 45] and is analogous to the apoptosis of nucleated cells. Elevated PS exposure is also seen in a number of disease states, including sickle cell disease (SCD) [17, 66].

In SCD, patients show various complications which fall into two main groups: a chronic anaemia and acute vaso-occlusive disorders (such as pain, stroke, acute chest syndrome and osteonecrosis). PS exposure is observed in a high, but variable, percentage of RBCs (2–10 % is often quoted [15, 17, 40, 41, 66, 67]). PS may therefore contribute to both the anaemic and ischaemic complications of SCD [29]. The mechanism by which it becomes externalised has therefore received considerable attention [1, 17, 40].

Asymmetrical distribution of PS is usually maintained by high activity of an ATP-dependent aminophospholipid translocase (or flippase) in conjunction with relatively low activity of a Ca^{2+}-activated scrambling process (or scramblase activity) [4, 25]. The presence of the abnormal haemoglobin, HbS, in patients' RBCs somehow perturbs this equilibrium to favour PS exposure. HbS molecules are able to aggregate on deoxygenation forming rigid polymers which distort RBC shape, alter rheology and stimulate a number of other deleterious sequelae [29]. Deoxygenation also stimulates PS exposure [6, 49, 66]. Damage to the cytoskeleton may free PS from anchor sites resulting in its higher mobility [22,

26, 52]. In addition, however, deoxygenation-induced PS exposure is Ca^{2+} dependent requiring elevation of the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) to low micromolar levels [66]. The high cation permeability of HbS-containing RBCs may be involved in this process [24, 31, 46]. In particular, a role has been postulated for the deoxygenation-induced cation conductance (sometimes termed P_{sickle}) which is activated upon deoxygenation, HbS polymerisation and RBC shape change [32, 46, 50]. This pathway is permeable to divalent cations including Ca^{2+} and can therefore mediate Ca^{2+} entry. Elevated $[Ca^{2+}]_i$ both inhibits the flippase and also activates the scramblase [25]. A number of other RBC cationic permeabilities have also been described, however, including conductances activated by depolarisation, glutamate, benzodiazepines and stretch, as well as following infection with intraerythrocytic parasites like malaria [3, 35] (although the latter is more widely considered to increase activity of anion-selective pathways [39]). One or more of these, instead of, or as well as P_{sickle}, may contribute to the Ca^{2+} entry involved in deoxygenation-induced PS exposure. Clear identification of the pathway(s) involved would be beneficial as it would direct the search for potential inhibitors amenable to clinical use for amelioration of the complications of SCD.

In this study, we use various inhibitors and agonists of RBC pathways in an attempt to clarify the Ca^{2+} entry step which results in deoxygenation-induced PS exposure in RBCs from SCD patients. Results indicate that partial inhibitors of P_{sickle} reduced deoxygenation-induced PS exposure, consistent with mediation of Ca^{2+} entry via this pathway, but those inhibitors and activators of several other potential entry pathways were without effect.

Materials and methods

Solutions and chemicals

The main experimental saline comprised (in millimolar): NaCl 140, KCl 4, $CaCl_2$ 1.1, $MgCl_2$ 0.15, *N*-2-hydroxyethylpiperzine-*N*′-2-ethanesulphonic acid (HEPES) 10 and inosine 10 with a pH of 7.4 at 37 °C (low potassium-containing saline, LK HBS). For washing red blood cells (RBCs), a high potassium-containing saline (HK HBS) was usually used, comprising (in millimolar) NaCl 54, KCl 90, $MgCl_2$ 0.15, HEPES 10 and inosine 10 with a pH of 7.4 at room temperature. For experiments carried out in plasma, the RBC wash solution comprised $NaHCO_3$ 24 mM and NaCl 137 mM equilibrated with air and 5 % CO_2, pH 7.4. The ice-cold wash solution for K^+ flux measurements comprised (in millimolar) $MgCl_2$ 107, 3-(*N*-morpholino)-propanesulphonic acid (MOPS) 10, pH 7.4 at 0 °C. Fluorescein isothiocyanate-conjugated lactadherin (LA-FITC)-binding buffer consisted of LK HBS with 1 mM vanadate and 16 nM LA-FITC. Osmolality of all solutions was 290±5 mOsm kg^{-1}. LA-FITC was obtained from Haematologic Technologies Inc. (VT, USA) supplied via Cambridge Bioscience (Cambridge, UK), phycoerythrin (PE)-conjugated anti-glycophorin A from Becton Dickinson Biosciences (CA, USA), HEPES and 4,4′-dithiocyano-2,2′-stilbene-disulphonic acid (DIDS) from Calbiochem (Merck, Darmstadt, Germany) and $^{86}Rb^+$ from PerkinElmer (MA, USA). Diazepam and PK11195 were gifts from Dr. Guillaume Bouyer (CNRS, Roscoff, France). All other chemicals came from Sigma-Aldrich (Poole, Dorset, UK).

RBC preparation

Blood samples were collected from HbSS SCD patients, with consent and ethical permission (11/LO/0065), in the anticoagulant EDTA, except for the experiments in which RBCs were incubated in autologous plasma. In this case, heparin was used as an anticoagulant to prevent chelation of Ca^{2+} and/or Zn^{2+} (Fig. 6). In Fig. 6, only RBCs from normal (HbAA) individuals were used. RBCs were washed three times into HK HBS for 3 min at 600 g and then subsequently twice into LK HBS, unless otherwise indicated, to give a final haematocrit (Hct) of 5 %. RBC suspensions were then deoxygenated for 20 min in Eschweiler tonometers flushed with warmed humidified N_2, by which time PS exposure was unchanged relative to that measured in untreated RBCs (as shown previously [66]). They were then diluted into test tubes also pre-equilibrated with N_2 (final Hct 0.5 %) in the absence or presence of potential inhibitors/activators of PS exposure. Incubation was carried out at 37 °C for up to 60 min in the presence of 1 mM vanadate to inhibit both the flippase and the plasma membrane calcium pump. Solutions of DIDS (stock 5–50 mM in DMSO) were made up fresh every other day as stability decreased after 3 days resulting in strong stimulation of PS exposure, probably through the action of a breakdown product(s). Controls were exposed to the same final concentration of solvent (DMSO or H_2O, 0.2 %) but otherwise treated in the same way. For experiments with glutamate receptor agonists, *N*-methyl-D-aspartate (NMDA) and homocysteine, RBCs were kept oxygenated and incubated in Eppendorf tubes (up to 60 min, 37 °C). For experiments to test the effect of plasma on the action of Zn^{2+}, RBCs were re-suspended in autologous plasma after washing, equilibrated with air and 5 % CO_2 delivered using a Wösthoff gas mixer (Bochum, Germany) at 37 °C to give a final pH 7.4. Normal total plasma Zn^{2+} levels are about 10–15 μM [16, 28, 48]. This was not measured, rather additional Zn^{2+} at the concentration indicated was added directly to plasma and thus represented the minimum total concentration present.

Labelling of PS exposure

Samples of 5 μl aliquots (10^5 RBCs) were placed in 250 μl of LA-FITC binding buffer. PS labelling was carried out in

the dark, because of the high sensitivity of FITC to light, at room temperature for 10 min. RBCs were then pelleted by centrifugation for 10 s at 16,100×*g* and washed once into LK HBS to remove unbound LA-FITC. Unlike annexin-V, LA-FITC binds to PS in a Ca^{2+}-independent manner [14, 61]. Control experiments showed that binding was irreversible. Samples were then kept on ice until analysed by flow cytometry (FACS). Inhibitors/activators were tested (at 100 μM) for self-fluorescence using unlabelled RBCs. The percentage of RBCs exposing PS is usually normalised to values measured in control RBCs prior to addition of inhibitors, as the absolute magnitude of exposure varied between samples. These control values are given in the figure legends.

FACS acquisition and analysis

Externalised PS was measured in the FL-1 channel, with an emission wavelength for FITC of 519 nm, of a fluorescence-activated flow cytometer (FACSCalibur, Becton Dickinson, BD) and analysed with BD CellQuest Pro software using the protocol as previously published [66]. In control experiments, forward scatter (FSC, size) and side scatter (SSC, granularity) gates for RBCs were identified using a PE-labelled anti-glycophorin A assay. FSC was set with threshold at 512. Measurements were taken under logarithmic gain with voltages set at FSC, E00; SSC, 235; FL-1, 688; FL-2, 630; and FL-3, 590. Compensation was set as FL-1, 2.0 % of FL-2; FL-2, 19.6 % FL-1; FL-2, 0.0 % FL-3; and FL-3, 8.5 % FL-2 to minimise the effect of over spill of fluorescence to adjacent channels. For each measurement 10,000 events were gated. All gated LA-FITC-labelled cells were additionally cross checked against overlap into FL-2 and FL-3 fluorescent channels and for spill out of the size gate and if these occurred events were excluded from analysis. The percentage of such resolved events was noted and for most experiments was ≤1 %. On FL-1/FL-2 dot plot graph, the cut-off quadrants, for negative fluorescent gate, were set using unlabelled cells as $x=20$ and $y=20$. The PS-positive cells were represented as the percentage of all gated RBCs with sufficient externalised PS to appear positive fluorescently in the FL-1 channel [14] and thus fallen into the quadrant ($x>20$ and $y<20$).

K^+ flux measurements

Potassium fluxes were measured using $^{86}Rb^+$ as a tracer for K^+ [18, 62]. In these experiments, RBCs (Hct about 20 %) were first equilibrated in Eschweiler tonometers (Kiel, Germany) in air or N_2 at 37 °C, pH 7.4, in LK saline in which Cl^- was substituted with NO_3^- and MOPS for HEPES. Cl^- substitution prevented K^+ transport through the Na^+-K^+-$2Cl^-$ cotransporter and K^+-Cl^- cotransporter. Aliquots were then diluted tenfold into test tubes containing the same saline and also pre-equilibrated with air or N_2 for flux measurements. Bumetanide (10 μM), ouabain (100 μM) and clotrimazole (5 μM) were all also present to inhibit K^+ transport through the Na^+-K^+-$2Cl^-$ cotransport, Na^+/K^+ pump and Ca^{2+}-activated K^+ channel. $^{86}Rb^+$ was then added into a KCl solution to give a final $[K^+]$ of 7.5 mM. Influx was performed over 10 min, after which RBCs were washed four times in ice-cold isotonic $MgCl_2$ wash solution, lysed using Triton X-100 (0.1 %) and protein was precipitated with trichloroacetic acid (5 %) and 5 min centrifugation at 13,000×*g*. $^{86}Rb^+$ (K^+) in the supernatant was then counted by liquid scintillation as Čerenkov radiation (Tri-Carb 2100TR, PerkinElmer, MA, USA). The difference in K^+ influx at the two gas tensions provides a measure of the deoxygenation-induced cation conductance, P_{sickle}.

RBC morphology

Suspensions of RBCs (0.2 % Hct) were fixed in saline in the presence of glutaraldehyde (0.3 %) and viewed under a phase field mode on Leica DM6000B Microscope (Leica Microsystems Ltd, UK). From each sample, at least eight different frames (×20) were saved using a digital camera (AF600DFC) and Leica LAS AF Lite software to determine the percentage of sickled cells. At least 500 cells were counted for each sample.

Statistics

Unless otherwise indicated, data are presented as means ± S.E.M. for *n* individuals. Statistical significance was tested with paired Student's *t* test; $p<0.05$ was accepted as significant. All graphs were made using GraphPad Prism 5 (San Diego, CA). Samples from at least five individuals were collected for each of experiments. Calculations were made either using Microsoft Office Professional 2010 Excel software or GraphPad Prism 5.

Results

Effect of oxygen tension on PS exposure in RBCs from SCD patients

In control experiments, PS exposure in RBCs from SCD patients was 3.3±0.2 % at time 0, increasing after deoxygenation for 30 and 60 min to 7.5±0.4 and 10.5±0.6 % (means ± S.E.M., $n=124$), respectively. The lowest PS exposure values were 0.5, 1.7 and 1.9 % at time 0 and after 30 and 60 min deoxygenation, respectively, while the highest values were 14.3, 27.5 and 26.2 %. In separate experiments, PS exposure in RBCs kept oxygenated did not change

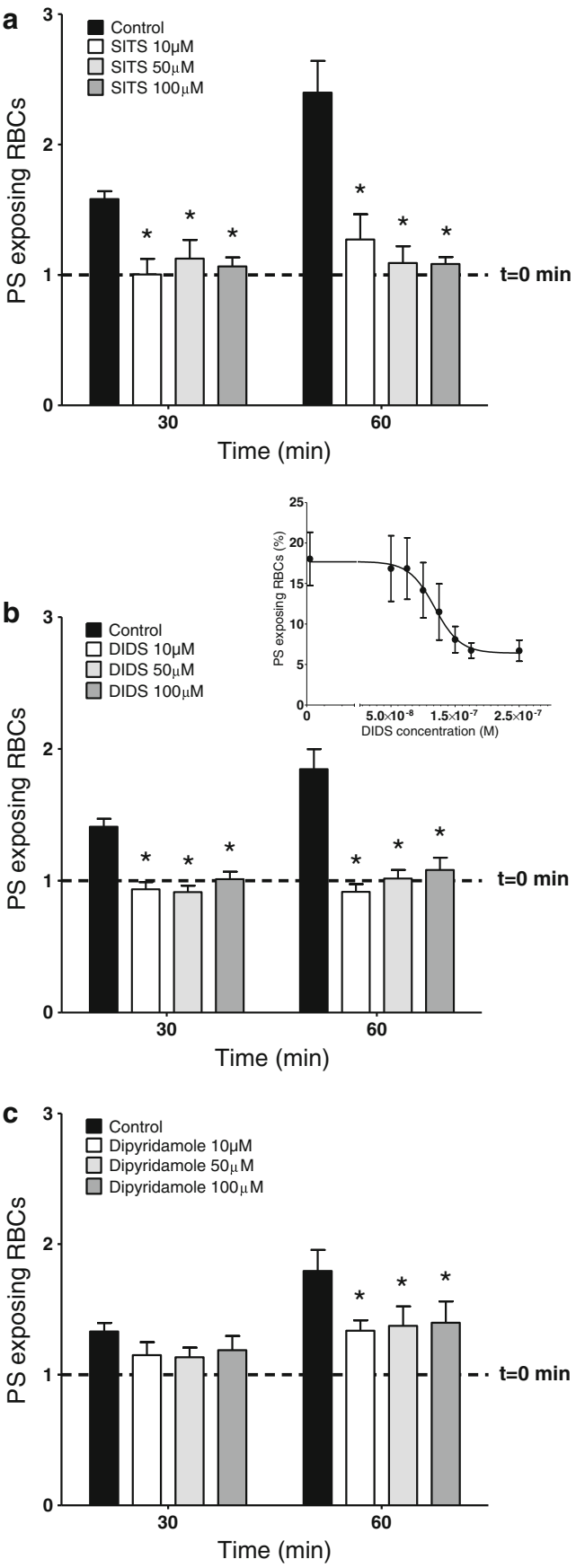

Fig. 1 Effect of P_{sickle} inhibitors on deoxygenation-induced phosphatidylserine (*PS*) exposure in red blood cells (*RBCs*) from SCD patients. **a** PS exposure is normalised to exposure in RBCs at time 0, just before addition of SITS, which was 2.8±0.8 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of SITS at 10, 50 and 100 μM. Data represent means ± S.E.M., $n=5$. *$p<0.05$ compared to deoxygenated RBCs in the absence of SITS. **b** PS exposure is normalised to exposure in RBCs at time 0, just before addition of DIDS, which was 4.3±0.5 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of DIDS at 10, 50 and 100 μM. Data represent means ± S.E.M., $n=8$. *$p<0.002$ compared to deoxygenated RBCs in the absence of DIDS. *Inset* represents the concentration dependence of DIDS inhibition of deoxygenation-induced PS exposure in RBCs from SCD patients. Percentage PS exposure is shown after 60 min deoxygenation in RBCs treated with DIDS at the concentrations indicated. PS exposure at time 0, just before addition of DIDS, was 4.8±0.7 %. IC_{50} for DIDS was 118±10 nM. Data represent means ± S.E.M., $n=9$. **c** PS exposure is normalised to exposure in RBCs at time 0, just before addition of dipyridamole, which was 3.8±0.4 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (lighter histograms) of dipyridamole at 10, 50 and 100 μM. Data represent means ± S.E.M., $n=7$. *$p<0.02$ compared to deoxygenated RBCs in the absence of dipyridamole

(3.1±0.5 % at time 0 and 3.8±0.7 and 4.1±0.6 % after 30 and 60 min of incubation, respectively, $n=13$). We have previously reported similar findings [66].

Effects of inhibitors of P_{sickle}

Although there are no specific inhibitors of P_{sickle}, stilbenes [30] and the pyrimidine derivative dipyridamole [33] have been shown to inhibit the deoxygenation-induced cation permeability of RBCs from SCD patients. In the first series of experiments, therefore, the effects of 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS), 4,4′-dithiocyano-2,2′-stilbene-disulphonic acid (DIDS) and dipyridamole were tested on deoxygenation-induced PS exposure (Fig. 1). Each of the three reagents significantly inhibited the deoxygenation-induced PS exposure at both 30 and 60 min, at all concentrations used (10, 50 and 100 μM). The effect of DIDS was explored in more detail. Significant inhibition was observed by 100 nM ($p<0.005$), with an IC_{50} of 118±10 nM ($n=9$), and was largely complete by 250 nM.

It was possible that these reagents acted indirectly through inhibition of HbS polymerisation and sickling. This was tested using morphological studies to assess the percentage of RBCs which underwent sickling following deoxygenation. After 60 min deoxygenation, in the absence and presence of DIDS (100 μM), sickling was 61±8 and 62±5 % (means ± S.E.M., $n=4$, not significant (N.S.)), respectively. A possible explanation for the effect of DIDS on PS exposure via inhibition of sickling was therefore discounted.

Effect of inhibitors of the non-specific cation channels of RBCs

A non-selective cation conductance has been described in RBCs from normal individuals, activated by a number of manoeuvres including osmotic or oxidative shock, or Cl^- substitution with gluconate (which will also cause RBC shrinkage) [19, 43]. Amiloride derivatives represent effective inhibitors of this pathway [44]. The effect of ethylisopropylamiloride (EIPA) was therefore tested on the deoxygenation-induced PS exposure of RBCs from patients with SCD but no significant effect was observed (Fig. 2).

Effects of inhibitors of unusual RBC cation pathways

Recently, it has been proposed that RBCs express a conductance mediated via the peripheral benzodiazepine receptor (PBR) [9]. This pathway has multiple conductance states and is permeable to a number of ions including anions. It has also been postulated that RBCs respond to glutamate receptor agonists, such as NMDA and homocysteine, with the possibility that these ligand-gated channels may also be present [51]. Various agonists and antagonists of these pathways were investigated (Fig. 3). In experiments on deoxygenation-induced PS exposure, however, neither the PBR agonist diazepam (10–100 μM; Fig. 3a) nor its antagonist PK11195 (10–100 μM; Fig. 3b) had any significant effects. When tested on oxygenated RBCs, the glutamate receptor agonists NMDA and homocysteine (both 100 μM; Fig. 3c) did not affect PS exposure. Similarly, the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801, 50 μM) had no effect on the deoxygenation-induced PS exposure (data not shown).

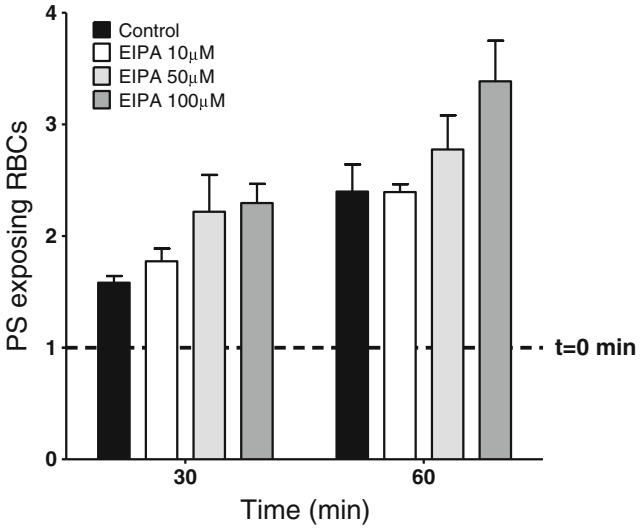

Fig. 2 Effect of EIPA on deoxygenation-induced PS exposure in RBCs from SCD patients. PS exposure is normalised to exposure in RBCs at time 0, just before addition of EIPA, which was 2.8±0.8 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of EIPA at 10, 50 and 100 μM. Data represent means ± S.E.M., n=5. All N.S. compared to deoxygenated RBCs in the absence of EIPA

Effects of inhibitors of stretch-activated channels

Under certain conditions, the aminoglycoside antibiotics, streptomycin and gentamicin [13], and the heavy metals Zn^{2+} and Gd^{3+} [10, 13, 50] reduce the permeability of RBCs from SCD patients, possibly acting as stretch-activated channel blockers [60]. Long-term treatment with Zn^{2+} has also been observed to induce PS exposure in normal human RBCs [38]. Their effects on deoxygenation-induced PS exposure were investigated (Figs. 4 and 5). In the presence of streptomycin, PS exposure increased, but not significantly (Fig. 4).

Both Zn^{2+} (up to 100 μM) and Gd^{3+} (up to 50 μM) markedly increased PS exposure (Fig. 5a, b). Higher concentrations of Gd^{3+} (100 μM) caused RBC aggregation and its effects could not be investigated using FACS. The action of Zn^{2+} (100 μM) was studied in more detail (Table 1). Zn^{2+} significantly increased PS exposure in both oxygenated and deoxygenated RBCs. Its effect was therefore different to the deoxygenation-induced PS exposure, requiring HbS polymerisation and sickling. The effect, however, was also dependent on the presence of extracellular Ca^{2+} (1.1 mM) and largely abolished when it was omitted (Table 1). Additionally, similar effects were observed for both the RBCs from SCD patients and from healthy individuals (cf Figs. 5a (HbSS)) and Fig. 6 (HbAA). In corresponding K^+ flux measurements, Zn^{2+} increased the deoxygenation-induced K^+ influx from 0.59±0.14 to 1.02±0.13 mmol(l cells h)$^{-1}$ (means ± S.E.M., n=3; p<0.002) thus almost doubling the activity of the P_{sickle}-like activity.

As negatively charged plasma proteins are likely to chelate Zn^{2+}, in the final series of experiments, the effect of Zn^{2+} was determined in RBCs (in this case from normal HbAA individuals suspended in autologous plasma, Fig. 6). In these experiments, PS exposure was measured in the absence and presence of added Zn^{2+} (100 μM). In saline, as in Fig. 5a, Zn^{2+} resulted in considerable PS exposure. In plasma, however, an increase in the percentage of RBCs showing PS exposure remained modest even after 60 min of incubation. Similar findings were observed with RBCs from SCD patients (data not shown).

Discussion

The present findings further define the Ca^{2+} entry step responsible for deoxygenation-induced PS exposure in RBCs from SCD patients. In particular, partial P_{sickle} inhibitors (the

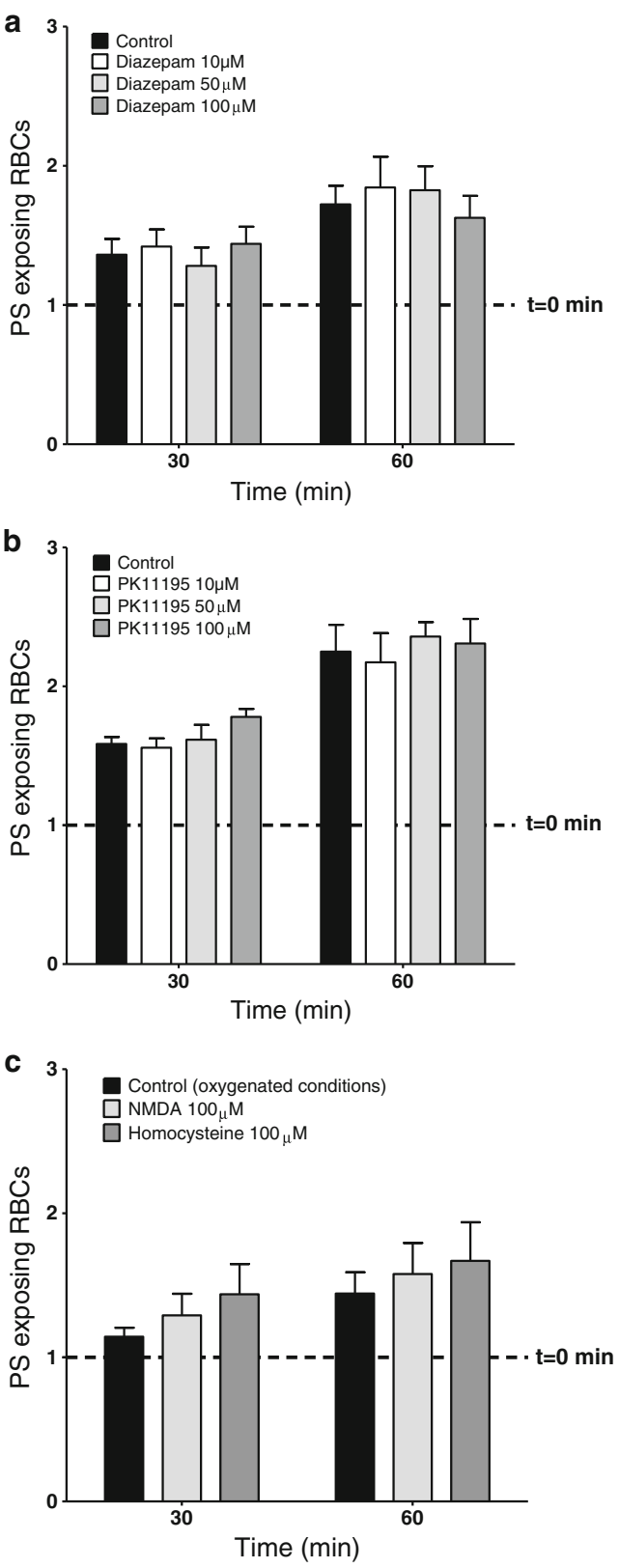

Fig. 3 Effect of modulators of novel RBC cation channels on PS exposure in RBCs from SCD patients. **a** PS exposure is normalised to exposure in RBCs at time 0, just before addition of diazepam, which was 5.3±1.0 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of diazepam at 10, 50 and 100 μM. Data represent means ± S.E.M., n=6. All N.S. compared to deoxygenated RBCs in the absence of diazepam. **b** PS exposure is normalised to exposure in RBCs at time 0, just before addition of PK11195, which was 3.0±0.5 % (indicated by the *dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of PK11195 at 10, 50 and 100 μM. Data represent means ± S.E.M., n=7. All N.S. compared to deoxygenated RBCs in the absence of PK11195. **c** PS exposure is normalised to exposure in oxygenated RBCs at time 0, just before addition of NMDA or homocysteine, which was 4.0±0.9 % (indicated by the *dotted line*), and after 30 and 60 min of incubation in the absence (*black histograms*) or presence (*lighter histograms*) of NMDA or homocysteine (both 100 μM). Data represent means ± S.E.M., n=6. All N.S. compared to oxygenated RBCs in the absence of NMDA or homocysteine

stilbenes, SITS and DIDS and the pyrimidine derivative dipyridamole) were similarly active against PS externalisation. By contrast, modulators of other pathways (including inhibitors of the non-specific cation channel and agonists or blockers of glutamate- and benzodiazepine-gated channels) were without effect. Heavy metals Zn^{2+} and Gd^{3+}, rather than inhibiting PS exposure, caused increased levels of externalisation, an effect which in the case of Zn^{2+} was shown to be Ca^{2+} dependent but independent of oxygen tension, HbS polymerisation and RBC sickling.

RBCs from SCD patients have been known to show increased solute permeability for over 50 years. Seminal experiments by Tosteson and colleagues showed that deoxygenated RBCs from SCD patients lost K^+ at greater rates than they gained Na^+, resulting in net solute loss and shrinkage [65]. This is particularly important as the lag time to HbS polymerisation following deoxygenation is inversely proportional to a very high power of the concentration of HbS [20]. Modest dehydration will markedly increase the likelihood of sickling as RBCs traverse hypoxic areas of the vasculature. Since then, the nature of the altered permeability has been much studied and several pathways have been identified [23, 31, 46]. Amongst these are the KCl cotransporter (likely KCC1 and KCC3 isoforms) and the Ca^{2+}-activated K^+ channel (or Gardos channel). In addition to increased permeability to univalent cations, RBCs from SCD patients also show elevated permeability to both Ca^{2+} and Mg^{2+} [21, 55, 58]. The pathway(s) responsible for passage of these ions remains less certain, but a pre-eminent role for the deoxygenation-induced cation conductance termed P_{sickle} has been proposed [31, 46].

Although the permeability characteristics of P_{sickle} have been extensively investigated, its molecular identity remains enigmatic [31, 32]. As a flux pathway, its activation is associated with deoxygenation, HbS polymerisation and RBC shape change [31, 53, 58]. It is permeable to both univalent and divalent cations including Ca^{2+}, shows a distinct pH dependence for both activation and permeation [34]

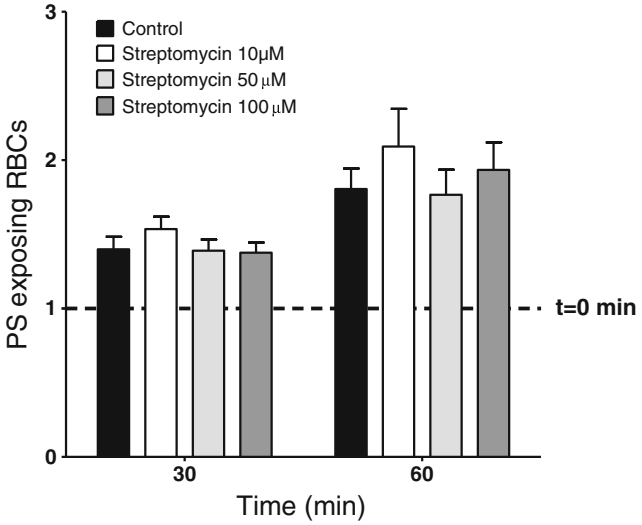

Fig. 4 Effect of streptomycin on deoxygenation-induced PS exposure in RBCs from SCD patients. PS exposure is normalised to exposure in RBCs at time 0, just before addition of streptomycin, which was 5.7±0.8 % (*indicated by the dotted line*), and after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of streptomycin at 10, 50 and 100 μM. Data represent means ± S.E.M., n=9. All N.S. compared to deoxygenated RBCs in the absence of streptomycin

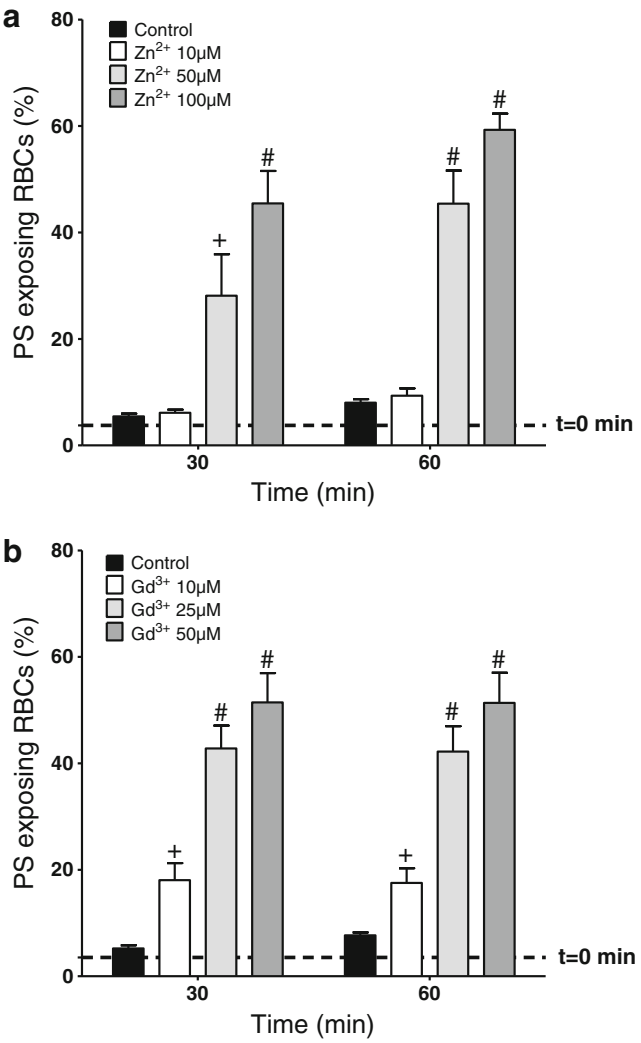

Fig. 5 Effect of heavy metals, Zn^{2+} and Gd^{3+}, on deoxygenation-induced PS exposure in RBCs from SCD patients. **a** PS exposure at time 0, just before addition of Zn^{2+}, was 3.8±0.4 % (indicated by the *dotted line*), and is also shown after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of Zn^{2+} at 10, 50 and 100 μM. Data represent means ± S.E.M., n=7. $^{\#}p<0.001$, $^{+}p<0.03$ compared to deoxygenated RBCs in the absence of Zn^{2+}. **b** PS exposure at time 0, just before addition of Gd^{3+}, was 3.5±0.6 % (indicated by the *dotted line*), and is also shown after 30 and 60 min deoxygenation in the absence (*solid black histograms*) or presence (*lighter histograms*) of Gd^{3+} at 10, 25 and 50 μM. Data represent means ± S.E.M., n=5. $^{\#}p<0.002$, $^{+}p<0.02$ compared to deoxygenated RBCs in the absence of Gd^{3+}

and appears randomly (or "stochastically") activated upon deoxygenation [47]. As for its molecular nature, identification of specific inhibitors has proved elusive. Stilbenes [30] and dipyridamole [33], however, are partial inhibitors, with the latter having been used in clinical trials [12].

Deoxygenation of RBCs from SCD patients is known to result in increased PS exposure [6, 49, 66]. The effect is Ca^{2+} dependent, requiring physiological levels of extracellular Ca^{2+} and elevation of intracellular Ca^{2+} to low micromolar levels [66]. P_{sickle} is therefore an obvious candidate for mediation of Ca^{2+} entry with subsequent PS exposure following from inhibition of the aminophospholipid translocase (or flippase) and stimulation of the RBC scramblase—both now known to occur at low micromolar [Ca^{2+}] [5, 66]. Notwithstanding, various other RBC cation pathways have been described [35] and it is possible that one or more of these are also involved.

The present results support a role for the P_{sickle}-like pathway in deoxygenation-induced PS exposure. Thus, stilbene derivatives and dipyridamole were active against deoxygenation-induced PS exposure, with no effect on morphological sickling (as also observed previously [30]) implying that they do not act via reduction in HbS polymerisation and subsequent RBC shape change. Inhibition by DIDS, in particular, occurred at low concentrations (IC_{50} 118 nM), more similar to those required to inhibit anion flux through the anion exchanger AE1, rather than monovalent cation flux through the P_{sickle} pathway described previously [30]. Altered AE1 behaviour has been postulated to play a role in the conductance of deoxygenated sickle cells [46] and has been shown to mediate a cation permeability [11] or alter the activity of other cation transporters [8], at least when mutated, which may be relevant to the present findings. DIDS may also directly inhibit the scrambling transporter, as well as Ca^{2+} entry, but this occurs at much lower affinity with an IC_{50} of about 10 μM [37]. Here, 250 nM reduced PS exposure during deoxygenation to oxygenated levels.

By contrast, none of the other channel inhibitors/agonists, including those for the more established non-specific cation

Table 1 Effect of Zn^{2+} on phosphatidylserine (PS) exposure in red blood cells (RBCs) from patients with sickle cell disease. All RBCs were incubated in low potassium-containing (LK) saline and treated with Zn^{2+} (100 μM) for the duration indicated. Extracellular Ca^{2+} was also present at 1.1 mM or omitted from saline (with addition of 2 mM EGTA). RBCs were equilibrated with nitrogen (deoxygenated) or air (oxygenated). PS exposures are given as percentage of positive cells. Values represent means ± S.E.M. (n=4). Statistics show p values for RBCs treated with Zn^{2+} compared to paired controls without Zn^{2+} or for Zn^{2+}-treated RBCs in the presence or absence of Ca^{2+}

	1.1 mM $[Ca^{2+}]_o$			0 mM $[Ca^{2+}]_o$		
Time of incubation (min)	Control Mean ± S.E.M.	100 μM Zn^{2+} Mean ± S.E.M.	Student's t test vs control	Control Mean ± S.E.M.	100 μM Zn^{2+} Mean ± S.E.M.	Student's t test vs 1.1 mM $[Ca^{2+}]_o$
Deoxygenated conditions						
0	4.1±1.4	4.5±1.4		4.7±1.4	4.1±1.4	
30	5.2±1.4	45.9±10.9	p<0.02	5.8±1.1	6.6±1.8	p<0.02
60	7.9±1.4	53.0±13.6	p<0.02	7.1±1.0	10.5±4.2	p<0.01
Oxygenated conditions						
0	3.3±0.6	6.2±1.4		4.4±1.0	4.9±1.4	
30	3.8±0.8	52.7±18.2	p<0.05	5.1±1.2	8.1±3.3	p<0.04
60	5.1±1.0	71.7±12.9	p<0.008	5.0±0.8	20.6±11.0	p<0.001

channel [2, 3, 27] or for the more controversial PBR- and glutamate-gated pathways [9, 51], had any effect. Streptomycin, which might act as a stretch-activated channel blocker [13, 60], was also without effect.

Zn^{2+} and Gd^{3+}, which also inhibit the cation conductance of RBCs from SCD patients [10, 50], did not inhibit PS exposure. Instead, they caused greatly increased levels of externalisation—an effect previously described for Zn^{2+} in RBCs from normal individuals following longer term (24 h) exposure [38]. This was not dependent on oxygen tension and appears to be mediated via a different mechanism than the deoxygenation-induced PS exposure under study. Their stimulation also required extracellular Ca^{2+}, as described previously [38]. It would appear that both destabilise the RBC permeability, in a similar way to that described previously for lead and aluminium [45]. A possible action is via titration of the surface negative charges. K^+ influx measurements, however, also showed a rise in the activity of P_{sickle}-like pathway which may also provide increased entry of Ca^{2+} for PS scrambling. Notwithstanding, experiments in plasma suggest that chelation of Zn^{2+} would reduce its free concentration below values required for the rapid PS exposure described here. This effect may be of some significance as some SCD patients are Zn^{2+} deficient and supplementation is sometimes given [56, 57]. Ensuring that free plasma $[Zn^{2+}]$ does not rise excessively is an obvious caveat in these cases.

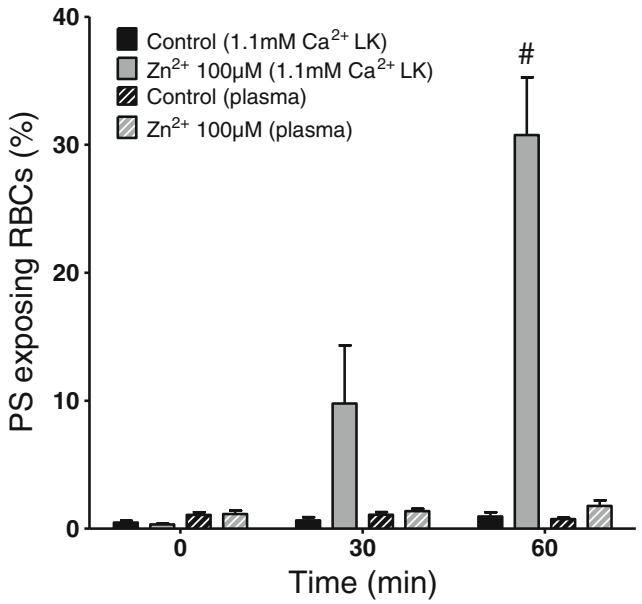

Fig. 6 Effect of Zn^{2+} and autologous plasma on PS exposure in RBCs from normal (HbAA) individuals. RBCs were incubated under oxygenated conditions in LK saline (*solid histograms*) or in autologous plasma (*hatched histograms*), in which case pH was maintained by flushing with warm humidified CO_2 (5 %). Percentage PS exposure is shown at time 0, just before addition of Zn^{2+} (100 μM), and after 30 and 60 min. *Histograms* represent means ± S.E.M., n=4 (paired samples). $^{\#}p$<0.008 compared to values in plasma

Finally, although the present work is concerned with Ca^{2+} entry and deoxygenation-induced PS exposure in RBCs from SCD patients, it is also important to note that in RBCs from normal individuals other pathways may be significant. In this context, it is important to note that the present results concern RBCs from SCD patients whose permeability is altered by the presence of polymerised HbS. Most previous reports on PS exposure have been carried out on RBCs from normal individuals (e.g. [44]). It is likely that Ca^{2+} entry, which triggers subsequent PS exposure, can occur via several different routes. Thus, in addition to the P_{sickle} pathway of sickle cells, normal RBCs show marked Ca^{2+} elevation in response to lysophosphatidic acid [54, 63, 68], possibly via the non-selective cation channel [35, 36, 44]. An alternative

pathway for PS exposure involving protein kinase C has also been proposed [54, 63], as well as the Ca^{2+}-mediated one. The role of these pathways in RBCs from SCD patients remains to be investigated; however, the lack of effect of EIPA on deoxygenation-induced PS exposure argues against the involvement of the non-specific cation channel.

In summary, we show that PS exposure in RBCs from SCD patients is stimulated by deoxygenation, via a pathway likely consistent with mediation via P_{sickle}. Future experiments will be aimed at establishing the molecular identity of this pathway and the establishment of inhibitors with potential for clinical use.

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