Deoxygenation-Induced Non-Electrolyte Pathway in Red Cells from Sickle Cell Patients

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Key Words

Sickle cell disease • Erythrocytes • Non electrolytes • Permeability

Abstract

Red cells from patients with sickle cell disease contain HbS rather than the normal HbA (here termed HbS cells). On deoxygenation, HbS cells exhibit a distinctive solute permeability pathway, P_{sickle} , activated stochastically, and partially inhibited by DIDS and dipyrindamole. It is often referred to as a cation channel although its permeability characteristics remain vague and its molecular identity is unknown. We show that, in contrast to normal red cells, a proportion of HbS cells underwent haemolysis when deoxygenated in isosmotic non-electrolyte solutions. Haemolysis was stochastic: cells unlysed after an initial deoxygenation pulse showed lysis when harvested, reoxygenated and subsequently exposed to a second period of deoxygenation. O_2 dependence of haemolysis was similar to that of P_{sickle} activation. Haemolysis was accom-

panied by high rates of sucrose influx, and both haemolysis and sucrose influx were inhibited by DIDS and dipyrindamole. Sucrose influx was only detected as ionic strength was reduced below 80 mM. These findings are consistent with the postulate that deoxygenation of HbS cells, under certain conditions, activates a novel non-electrolyte pathway. Their significance lies in understanding the nature of the deoxygenation-induced permeability in HbS cells, together with its relationship with novel pathways induced by a variety of manipulations in normal red cells.

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Introduction

Red cells from homozygous HbSS patients contain an abnormal haemoglobin (Hb), HbS, rather than the normal HbA [1]. (HbS-containing red cells are here termed HbS cells, and those with HbA, HbA cells.) The molecular difference between HbA and HbS is simple, namely a

substitution of glutamic acid by valine at position 6 on the beta chain of Hb [2, 3]. The resulting vascular pathology is remarkably extensive, however, producing the clinical condition known as sickle cell disease (SCD) [4]. When deoxygenated, HbS can form rigid polymers, which distort the red cell into a number of peculiar forms, including the sickle shape from which the disease takes its name. HbS cells have a much reduced lifespan, contributing to the anaemia characteristic of SCD. In addition, vascular occlusion is promoted, especially during sickle cell crises, with sequelae including ischaemia, pain, organ dysfunction, and ultimately death [1, 5, 6]. To date, therapy remains supportive and is largely aimed at addressing the anaemia and pain resulting from the condition.

The detailed pathophysiology of SCD remains obscure but dehydration of HbS cells is central [7-10]. Unlike HbA cells, HbS cells are liable to rapid and irreversible shrinkage with osmotically obliged water following solute loss. Because the lag time to HbS polymerisation upon deoxygenation is inversely proportional to a very high power of [HbS] [11], shrunken cells are much more likely to assume bizarre shapes and lodge in the circulation. Considerable effort has been expended on understanding the mechanism of solute loss, with the possibility that specific inhibitors might provide potential chemotherapeutic avenues for treating SCD [2, 9, 10].

Three pathways are involved in solute loss from HbS cells [9, 10, 13]: the KCl cotransporter (KCC), the Gardos channel and P_{sickle} . KCC mediates coupled K^+ and Cl^- loss, with higher activities and unusual regulation observed in HbS cells compared to HbA cells [7, 14-16]. The Gardos channel is a Ca^{2+} -activated conductive K^+ pathway [17]. P_{sickle} is a deoxygenation-induced pathway, whose main importance is that it mediates Ca^{2+} entry and subsequent Gardos channel activation [9, 18-20]. Its molecular nature remains enigmatic, although activation is associated with HbS polymerisation and cell shape change [21-23]. Whilst it has been described as a conductive cation pathway [18], its properties remain poorly defined and its molecular identity is unknown.

As part of our study of the permeability characteristics and activation properties of P_{sickle} , we combined deoxygenation of HbS cells with incubation in isosmotic non-electrolyte solutions. Results from both haemolysis assays and sucrose influx suggest that, as well as forming a cation pathway, deoxygenation of HbS cells also induces non-electrolyte permeability. Like P_{sickle} , this permeability was also inhibited by DIDS and dipyrindamole. The significance of these findings is discussed.

Materials and Methods

Solutions and Chemicals

All chemicals used were obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated. 3-[N-morpholino] propane sulfonic acid (MOPS)-buffered saline (MBS), used to wash all blood samples, comprised (in mM): NaCl (145), MOPS (10), glucose (5), pH 7.4, at 37°C. MOPS-buffered nitrate saline (MBNS), used to deplete red cells of intracellular chloride, comprised $NaNO_3$ (145), MOPS (10), glucose (5), pH 7.4, at 37°C. For determination of haemolysis, a solution of MOPS (10) and glucose (5) was made isosmotic with the non-electrolyte (sucrose or taurine, 300 mM) under investigation, and pH adjusted to 7.4 at 37°C. All haemolysis and radioisotope influx solutions were further supplemented with ouabain (0.1 mM), bumetanide (0.01 mM) and clotrimazole (0.01 mM) from concentrated stock solutions in dimethyl sulphoxide (DMSO). Ice-cold stop solution, used to terminate red cell haemolysis, comprised MOPS-buffered sucrose (400 mM). Ice-cold $MgCl_2$ solution, used to wash red cells following radioisotopic sucrose influx comprised (in mM) $MgCl_2$ (107), MOPS (10), pH 7.4.

Blood

Samples of normal (HbAA) and sickle (HbSS) red cells were collected with ethical consent by venepuncture of consenting volunteers. Blood was collected in heparin-containing syringes and stored at 4°C until required (normally within 72 h). Prior to use, blood samples were washed 3 times by centrifugation ($850 \times g$, 5 min, 4°C) in MBS to remove platelets and plasma. Where required, red cells were washed an additional 3 times in MBNS to achieve Cl^- substitution. After the final wash red cell samples were left as a packed preparation (approximately 90% haematocrit).

Measurements of red cell haemolysis

2.5-5 ml of isosmotic haemolysis solution (containing sucrose or taurine) was transferred to an Eschweiler tonometer (Eschweiler GmbH & Co., Kiel, Germany) suspended in a thermally regulated water bath and supplemented with ouabain, bumetanide and clotrimazole as described above. The tonometer was connected to a humidified gas supply (N_2 , air, or an intermediate O_2 tension made using a Wösthoff gas mixing pump) and allowed to equilibrate at the relevant O_2 tension for at least 10 minutes at 37°C. To start the haemolysis assay 0.1-0.2 ml of washed packed cells in MBNS were transferred to the tonometer at time 0. The contents of the tonometer were continuously gently mixed and flushed with the appropriate gas or gas mixture. After pre-determined intervals, 0.1-0.2 ml of the cell suspension was removed and added to 0.8 ml of ice-cold stop solution in an Eppendorf tube and immediately centrifuged ($10,000 g$, 20s). The supernatant was transferred to a cuvette or 96-well plate and the extent of haemolysis was determined by measuring optical density at 540 nm. Maximal (100%) lysis was measured by transferring 0.1-0.2 ml of cell suspension to 0.8 ml Triton X-100, centrifugation and determination of the optical density (540 nm) of the supernatant.

Fig. 1. Haemolysis of human red cells incubated in isosmotic sucrose solutions. Red cells were incubated in tonometers under air (oxy) or N_2 (deoxy) for 90 min at approximately 4% haematocrit. At the time intervals indicated, samples were removed and percentage lysis determined spectrophotometrically. (a) Normal red cells (HbA cells); (b) red cells from sickle cell patients (HbS cells). Ouabain (100 μ M), bumetanide (10 μ M) and clotrimazole (10 μ M) were present throughout. Data indicate mean $\pm$ SEM, $n = 5$.

Fig. 2. Haemolysis of human red cells incubated in isosmotic taurine solutions. Red cells were incubated in tonometers under air (oxy) or N_2 (deoxy) for 90 min at approximately 4% haematocrit. At the time intervals indicated, samples were removed and percentage lysis determined spectrophotometrically. (a) Normal red cells (HbA cells); (b) red cells from sickle cell patients (HbS cells). Ouabain (100 μ M), bumetanide (10 μ M) and clotrimazole (10 μ M) were present throughout. Data indicate mean $\pm$ SEM, $n = 3$.

Determinations of sucrose influx

Radioisotopic ^{14}C -sucrose influx was measured into HbA and HbS cells. Blood samples were washed into MBNS as described above and left as a packed cell preparation. For determinations of sucrose influx, 3.6 ml of buffered isosmotic taurine solution, supplemented with ouabain (0.1 mM), bumetanide (0.01 mM) and clotrimazole (0.01 mM) was added to a tonometer. 0.2 ml of packed cells were added to this solution (to give a final haematocrit of approximately 5%) and the tonometer was flushed with the required gas mixture as appropriate for at least 15 minutes at 37°C. Sucrose influx was started by the addition of 0.2 ml HEPES buffered taurine or mannitol saline supplemented with sucrose and $[^{14}C]$ sucrose such that its final concentration and activity were 5 mM and 0.2 μ Ci ml $^{-1}$. Tonometers were continuously mixed and flushed with the required humidified gas for a further 30 minutes. At the end of this period 3 $\times$ 1 ml samples were removed, washed 3 times in

ice-cold isosmotic $MgCl_2$ stop-solution and processed for scintillation counting. To determine the effect of ionic strength on ^{14}C -sucrose influx into deoxygenated HbS cells, this procedure was repeated for cells suspended in solutions of MBS mixed with isosmotic mannitol. In this way, influx was determined as a function of $[NaCl]$ under isosmotic conditions. Although all influxes shown were carried out over a 30 min period, during which time significant haemolysis would have occurred, preliminary findings showed that shorter influx periods (10 min) gave qualitatively similar results.

Statistics

Unless otherwise stated data are presented as mean $\pm$ SEM from n separate experiments. For certain experiments measurements were made in triplicate and the mean value determined. Significant differences were determined using Student's unpaired t test.

Fig. 3. Stochastic haemolysis of deoxygenated HbS cells in isosmotic non-electrolyte solutions. HbS cell samples were incubated in deoxygenated isosmotic taurine or sucrose solutions for 40 min, after which haemolysis was determined spectrophotometrically (light grey bars). Unlysed red cells were subsequently harvested, reoxygenated and then deoxygenated for a further period of 40 min. Haemolysis following this second deoxygenation pulse was then determined as before (dark grey bars). Ouabain (100 μ M), bumetanide (10 μ M) and clotrimazole (10 μ M) were present throughout. Histograms represent means $\pm$ S.E.M. ($n = 3$).

Results

In the first series of experiments, red cell samples were incubated in MOPS-buffered isosmotic sucrose, whilst being equilibrated with air or nitrogen.

At various time points, percentage lysis was assessed by measuring [Hb] in the cell-free supernatant using a spectrophotometer. Results are shown in Figure 1. Normal HbA red cells showed minimal lysis, which was unaffected by O_2 tension (Figure 1a). Oxygenated HbS cells behaved similarly, although in some samples, lysis was apparent after 40 minutes (Figure 1b). By contrast, when deoxygenated, HbS cells in isosmotic sucrose showed progressive haemolysis over about 40 min, by which time the percentage lysis had reached $48 \pm 12\%$ (mean $\pm$ SEM, $n = 5$). The extent of haemolysis subsequently remained constant at this magnitude, at least for the ensuing 50 min. This pattern of haemolysis was also obtained for deoxygenated HbS cells incubated in isosmotic taurine solution (Figure 2a and b), again with progressive haemo-

Fig. 4. (a) O_2 dependence of haemolysis of HbS cells in isosmotic sucrose solution. HbS cell samples were incubated in tonometers and flushed with humidified gas from a Wösthoff gas mixing pump at the O_2 tension indicated for 40 min. Aliquots were then removed and percentage haemolysis determined spectrophotometrically. (b) For comparison Cl^- independent K^+ influx is also presented for each O_2 tension (see [54]). For measurements, ouabain (100 μ M), bumetanide (10 μ M) and clotrimazole (10 μ M) were present throughout.

lysis of a subpopulation of cells, over about 40 min, to a maximum level, in this case, of $22 \pm 8\%$ ($n = 3$). Again, lysis was negligible in oxygenated HbS cells, or in HbA cells regardless of the O_2 tension (Figure 2).

In these initial experiments, it was evident that a population of deoxygenated HbS cells were protected from lysis. In some experiments, these unlysed red cells were harvested, reoxygenated, and then subsequently exposed to a second period of deoxygenation. A substantial fraction of red cells, which had been insensitive

Fig. 5. Sucrose influx into oxygenated and deoxygenated red cells. Red cells were suspended in saline and gassed for 15 minutes at the O_2 tension indicated. ^{14}C -sucrose (in 5 mM sucrose) was added, and influx then measured over a 30 min period at the same O_2 tension. (a) HbS cells incubated in MOPS-buffered saline at the O_2 tensions indicated. (b) Oxygenated (O) and deoxygenated (D) red cells in isosmotic taurine solution (plus 5 mM sucrose), HbA cells on left, HbS red cells on right in the shaded grey box. (c) Oxygenated (open circles) and deoxygenated (closed circles) HbS cells incubated in isosmotic solutions of different ionic strength (saline replaced with mannitol). Ouabain (100 μ M), bumetanide (10 μ M) and clotrimazole (10 μ M) were present throughout. Data are given as means $\pm$ S.E.M., $n = 3$. * denotes a significant difference between samples by paired 2-tailed Student's t test.

to haemolysis during the first episode of deoxygenation, underwent haemolysis in responsive to the second exposure to deoxygenation (Figure 3).

We went on to investigate the O_2 dependence of lysis of HbS cells (Figure 4a).

For this experiment, tonometers were flushed with gas from a Wösthoff gas mixing pump. Minimal lysis (%) was observed at O_2 tensions above 40 mmHg. As O_2 tension was reduced, lysis became apparent. Half maximal haemolysis occurred at about 20 mmHg O_2 . For comparison, K^+ influx, in $\text{mmol} \cdot (\text{l cells} \cdot \text{h})^{-1}$, is also presented (Figure 4b). The profile of O_2 dependence of lysis in isosmotic sucrose was similar to deoxygenation-induced K^+ influx.

To confirm entry of non-electrolytes into deoxygenated HbS cells, influx of [^{14}C]sucrose was also measured. In MOPS-buffered saline (145 mM NaCl, 5 mM KCl), at 5 mM extracellular sucrose, sucrose influx was negligible (Figure 5a). In ionic solutions, therefore, sucrose influx was minimal and independent of O_2 tension. Measurement of sucrose influx was repeated in red cells suspended in non-electrolyte solutions (either 300 mM mannitol or 300 mM taurine, with 5 mM sucrose). In this case, a substantial deoxygenation-induced component of sucrose influx was apparent in HbS cells, but was absent in HbA cells regardless of the O_2 tension (Figure 5b). At 5 mM, influx was approximately $2 \text{ mmol} \cdot (\text{l cells} \cdot \text{h})^{-1}$. As it was evident that reduced ionic strength was required to elicit sucrose influx, this was investigated in more detail (Figure 5c). With progressive reduction in ionic strength (by replacing NaCl with mannitol), sucrose influx was apparent as Cl^- was reduced below 40 mM (i.e. at a total ionic strength c. 80 mM).

DIDS and dipyrindamole are well established inhibitors of $P_{\text{sickle}}^{\text{c}}$, the deoxygenation-induced cation conductance, in HbS cells [24, 25].

In the final series of experiments, therefore, these

Fig. 6. Effect of inhibitors of P_{sickle} on haemolysis and sucrose influx into HbS cells. (a) Haemolysis in deoxygenated HbS cells in the absence (open circles) and presence (closed circles) of DIDS (50 μM). HbS cell samples were suspended in isosmotic sucrose solutions and incubated in tonometers under N_2 . Haemolysis was determined spectrophotometrically and shown plotted against time. Data represent means $\pm$ S.E.M., $n = 3$. (b) Sucrose influx into HbS red cells in the absence (white bars) or presence (grey bars) of DIDS or dipyridamole. HbS cells were suspended in taurine saline and gassed for 15 minutes with fully humidified air or N_2 with DIDS (100 μM) or dipyridamole (10 μM). ^{14}C -sucrose was added to the cells and influx then measured over a further 30 min at the same O_2 tension. Values are shown as mean $\pm$ SEM for oxygenated (Oxy) and deoxygenated (Deoxy) samples, in the absence of inhibitor (Control) or in the presence of DIDS or dipyridamole, as indicated. Ouabain (100 μM), bumetanide (10 μM) and clotrimazole (10 μM) were present throughout. For all conditions $n = 2$. * denotes a significant difference between 2 samples determined by Student's paired t test.

reagents were tested for their effects on the apparent deoxygenation-induced non-electrolyte permeability. DIDS (50 μM) markedly inhibited haemolysis in isosmotic taurine solution (Figure 6a), whilst both DIDS (100 μM) and dipyridamole (10 μM) also markedly inhibited the deoxygenation-induced sucrose influx (Figure 6b). The effect of dipyridamole on haemolysis was not tested.

Discussion

The present findings demonstrate that, when incubated in isosmotic non-electrolyte solutions, deoxygenated human red cells containing HbS (HbS cells) undergo haemolysis. By contrast, neither oxygenated HbS cells nor normal (HbA cells) were lysed, regardless of the O_2 tension. Haemolysis occurred only in a proportion of HbS cells in response to a single deoxygenation pulse, but unlysed cells showed lysis when exposed to a second episode of deoxygenation. A large sucrose influx into

deoxygenated HbS cells was confirmed radioisotopically, consistent with osmotic lysis subsequent to non-electrolyte entry. DIDS and dipyridamole, inhibitors of P_{sickle} , reduced sucrose influx in HbS cells deoxygenated in isosmotic non-electrolyte solutions, and, DIDS also inhibited haemolysis. These results suggest that, under certain conditions, a non-electrolyte permeability is induced in HbS cells, which shares a number of characteristics in common with P_{sickle} .

The permeability characteristics of the deoxygenation-induced pathway in HbS cells

Our understanding of the deoxygenation-induced permeability pathway in HbS cells dates from the seminal work of Tosteson and colleagues [26, 27]. Subsequently, this pathway was shown to lack selectivity for the alkali cations. It is permeable to divalent cations, Ca^{2+} [28] and Mg^{2+} [29], with Ca^{2+} entry being responsible for Gardos channel activation. The organic cations tetramethylammonium, tetraethylammonium and N-

methylglucamine, however, are excluded [18]. Whether the pathway supports increased anion permeability is controversial ([30] cf [31]). Whilst early reports suggest impermeability to the non-electrolytes, mannitol and erythritol [30], these were carried out in cytochalasin B-treated HbS cells. Ion movements are non-saturable, voltage-dependent and not obligatorily coupled to anions [18, 32]. Taken together, these findings have led to the description of the pathway as a deoxygenation-induced cation channel [9], termed P_{sickle} [33].

We have presented evidence using haemolysis assays that suggests that deoxygenation of HbS cells can, in addition, activate a rather more promiscuous pathway. These assays have been used to assess red cell permeability for many years [34], and latterly have received more attention particularly in assessing the permeability of the NPP of malaria-infected red cells [35]. They are based on the postulate that, if permeable, species with an inwardly directed electrochemical gradient (or chemical gradient only in the case of non-electrolytes) will enter the red cell, water will follow osmotically and the cell will lyse [36]. The haemolysis experiments described here indicate that deoxygenated HbS cells become permeable to sucrose and taurine.

There are a number of similarities between this non-electrolyte pathway and P_{sickle} . First, a percentage of HbS cells suspended in non-electrolyte solutions undergo haemolysis, but only when deoxygenated. Second, haemolysis is "stochastic", in that unlysed cells to one period of deoxygenation, when re-oxygenated, and then deoxygenated again also show a similar percentage of haemolysis as that occurring in response to the first period of deoxygenation. Presumably the event precipitating haemolysis, in this case, but also increase in cation influx in previous work [33], occurs only in a proportion of cells in response to each deoxygenation pulse. In this sense, stochastic activation has been postulated to be an important characteristic of P_{sickle} [33]. Third, the O_2 sensitivity of haemolysis is similar to that for entry of cations (eg K^+) through P_{sickle} [32]. Fourth, haemolysis was inhibited by DIDS, an inhibitor of P_{sickle} . High rates of sucrose influx were confirmed radioisotopically, apparent when deoxygenated HbS cells were incubated in non-electrolyte solutions, but not in saline, or when oxygenated. This sucrose influx was also inhibited by both DIDS and dipyrindamole.

These features imply that haemolysis is due to the entry of non-electrolytes via a novel permeability pathway induced, like P_{sickle} , by deoxygenation. Replacement of saline by non-electrolytes causes intracellular

alkalinisation, as well as a positive membrane potential, in addition to other effects (notably cell shrinkage). How, or whether, these effects precipitate the non-electrolyte permeability are currently under investigation. It is difficult to understand, however, why re-oxygenation and subsequent deoxygenation of HbS cells would elicit haemolysis not apparent during the first exposure to non-electrolytes, unless it requires a specific interaction between HbS polymers and the membrane, or at some other regulatory site involved in modulation of permeability. Given the specificity of its permeability characteristics, however, the possibility that P_{sickle} or non-electrolyte-induced haemolysis, represents simply membrane damage induced by the mechanical effects of HbS polymers seems unlikely.

Activation of unusual permeability pathways in normal red cells

Whilst P_{sickle} is described as a permeability pathway found only in deoxygenated HbS cells, the underlying mutation responsible for SCD resides in the gene for Hb rather than that of a transport protein [3]. It follows that any gene encoding for a protein which mediates P_{sickle} activity must also be present in normal individuals. An obvious question is whether a P_{sickle} -like entity can be induced in red cells from normal individuals.

Under certain conditions (exposure to oxidants [37, 38], infection with malaria [36], low ionic strength isosmotic media [39-41], hypertonicity [42, 43], and positive membrane potentials [44-46]), normal red cells do indeed exhibit unusual permeabilities, with increased permeability also described in red cells with band 3 mutations [47, 48]. In most cases, a variety of novel ionic pathways are apparent. These include a non-specific cation channel [42, 45] and a Na^+K^+/H^+ exchanger [49, 50]. There are a number of circumstances, however, in which a significant non-electrolyte permeability is observed. Thus non-electrolyte transport is induced in normal red cells following malaria-infection [35, 51] and also in South-east Asian ovalocytosis, a band 3 mutation [47]. Of particular relevance to the present work, low ionic strength (LIS), but isosmotic, media [39, 41] also induces a pathway in HbA cells which is permeable to a number of organic solutes (including taurine, but not sucrose or sorbitol), in addition to monovalent cations (like Na^+ and K^+). LIS will also cause alkalinisation of red cell interior and imposition of a positive membrane potential. The LIS-induced organic pathway is insensitive to manipulation of pH but affected by manipulating red cell membrane potential [41], and is also DIDS-inhibitable [41].

Two additional novel permeabilities have recently been described in normal red cells in response to hyperosmotic shock. The first pathway has been described as a cation channel [42], proposed as mediating Ca^{2+} influx, thereby activating the scramblase and causing the breakdown of phosphatidylserine asymmetry [52, 53]. In common with the non-electrolyte permeability described here in deoxygenated HbS cells, the hypertonicity-induced cation pathway is inhibited by Cl^- [53]. A second permeability pathway has been recently observed in red cells maintained in saline with the addition of hypertonic trehalose [43]. Under these conditions, trehalose enters the red cell and haemolysis is evident. A link with the abnormal permeability observed in deoxygenated HbS cells is provided by membrane distortion, in the one case elicited by HbS polymers and in the other by hypertonic shrinkage. It may be that omission of Cl^- in our experiments with HbS cells is key to the manifestation of the deoxygenation-induced non-electrolyte permeability which we describe.

The interpretation of these various findings is complicated by the absence, in most cases, of specific inhibitors, and also ignorance of the molecular identities of the pathways involved. The extent to which they represent manifestations of a common pathway, activated by different stimuli, is an intriguing possibility awaiting investigation.

Conclusion

We present evidence which suggests that sucrose and taurine are permeable through a deoxygenation-induced permeability pathway of HbS cells, raising the possibility that P_{sickle} is a more promiscuous pathway than appreciated hitherto. These findings are important for several reasons. First, should non-electrolyte entry be via P_{sickle} , or should non-electrolyte permeability parallel that of P_{sickle} , an haemolysis assay may represent a simple and convenient test to assess the activity of P_{sickle} , which would be very advantageous clinically. Second, it may also be possible to use a non-electrolyte permeability to introduce other organic molecules, such as potential inhibitors against the main dehydration pathways, into HbS cells, and thereby ameliorate the symptoms of SCD. Third, results are relevant to any strategy employed to elucidate the molecular identity of the unusual permeability pathways observed in red cells from SCD patients.

Acknowledgements

We thank the Wellcome Trust and Action Medical Research for financial support. JAB is currently a Foulkes Foundation Fellow. JAB and HCR contributed equally to this work.

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