Effect of dimethyl adipimidate on K^+ transport and shape change in red blood cells from sickle cell patients

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Abstract Dimethyl adipimidate (DMA) reduces K^+ loss from, and dehydration of, red cells containing haemoglobin S (HbS cells). Three membrane transporters may contribute to these processes: the deoxygenation-induced cation-selective channel (P_{sickle}), the Ca^{2+}-activated K^+ channel (or Gardos channel) and the K^+-Cl^- cotransporter (KCC). We show that DMA inhibited all three pathways in deoxygenated HbS cells. The Gardos channel could be activated following Ca^{2+} loading. Considerable KCC activity was present in oxygenated HbS cells, showing a selective action of DMA on the transporter in deoxygenated cells. Inhibition of sickling correlated strongly with that of P_{sickle} and moderately with that of KCC activity. We conclude that DMA does not inhibit the K^+ pathways directly, but acts mainly by preventing HbS polymerisation and sickling. These findings are relevant to the development of novel chemotherapeutic agents for amelioration of sickle cell disease.

Key words: Sickle; Potassium; Oxygen; Dehydration

1. Introduction

Sickle cell disease (SCD) is characterised by anaemia and vaso-occlusion (reviewed in [1]). These features result from the presence of haemoglobin S (HbS) rather than the normal HbA. Abnormal cation transport in red blood cells from sickle cell patients (termed HbS cells) (reviewed in [2]) is of particular importance because it leads to solute loss and cell shrinkage which, in turn, greatly increases the probability of HbS polymerisation and the sickling shape change [3]. Sickled HbS cells have a greater rigidity and cytoplasmic viscosity, resulting in increased microvascular resistance, features which are central to the pathophysiology of SCD. Three membrane transporters, which are quiescent in, or absent from, normal HbA-containing red blood cells (HbA cells), are important in mediating K^+ loss and cell shrinkage: a deoxygenation-induced non-specific, though cation-selective, channel (termed P_{sickle} [4]) whose molecular identity remains obscure [5–8]; the Gardos channel, a Ca^{2+}-activated K^+ channel (probably IK1) [9–12]; and the K^+-Cl^- cotransporter or KCC (probably KCC1) [13–16].

Prevention of cell sickling remains the major ambition of SCD therapy. A number of reagents have been identified which inhibit HbS polymerisation, cell sickling and dehydration. One such antisickling agent is dimethyl adipimidate (DMA) [17]. This compound functions as a crosslinking agent, reacting covalently with available amino groups, particularly those on lysine residues. It reduces sickling, K^+ loss and dehydration in deoxygenated HbS cells [17,18] but its effects on the specific membrane transporters have not been elucidated.

In this report, we investigated the effects of DMA on the major passive cation pathways in HbS cells. Results show that DMA has modest effects on transport in oxygenated HbS cells. By contrast, in deoxygenated cells, DMA inhibits sickling, P_{sickle}, the Gardos channel and K^+-Cl^- cotransport, thereby accounting for its beneficial effects on HbS cell volume. The mechanism of these effects and their significance are discussed.

2. Materials and methods

2.1. Chemicals

A23187, clotrimazole and staurosporine were purchased from Calbiochem (Nottingham, UK); ^{86}Rb from NEN Du Pont, Stevenage, UK; and N_2 was obtained from BOC (Guildford, UK). All other reagents came from Sigma Chemical Co., Poole, UK.

2.2. Solutions

The standard MOPS-buffered saline (MBS) comprised (in mM): 145 NaCl, 5 glucose and 10 MOPS (pH 7.4 at 37°C; 290 ± 5 mOsm/kg). For experiments in which Cl^- dependence of K^+ influx was examined, Cl^- was substituted with NO_3^-. To investigate the effects of hypotonic saline, osmolality was adjusted by addition of distilled water; where required, pH was altered by addition of HNO_3 or NaOH. Stock solutions of ouabain (10 mM) were prepared in distilled water. Stock solutions of bumetanide (10 mM) and *N*-ethylmaleimide (NEM; 100 mM) were made daily in 100 mM Tris base and distilled water, respectively; those of clotrimazole and staurosporine were prepared in dimethyl sulphoxide and frozen until required. In all cases, controls and cells treated with inhibitors or other reagents were exposed to the same concentrations of solvent, whose final concentrations did not exceed 0.5%.

2.3. Sample collection and handling

Blood samples were obtained with consent and ethical permission from homozygous HbSS patients, using heparin as anticoagulant. Cells were washed three times in MBS by centrifugation (2500 × *g*, 5 min, 4°C), and the buffy coat removed by aspiration. HbS cell suspensions were then stored on ice until use, within 36 h of collection.

2.4. Treatment with DMA

DMA solutions (1–10 mM) were made freshly in MBS and titrated to pH 8. HbS cell samples were suspended in DMA solutions or control MBS (also adjusted to pH 8 and the same osmolality) at

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4% haematocrit for 30 min at 37°C, after which they were washed twice in ice-cold MBS (pH 7.4, 20 times volume of cells) to remove unreacted DMA.

2.5. Tonometry, O_2 saturation and cell shape

After treatment with DMA (or control MBS), red cell suspensions were incubated at about 40% haematocrit in glass tonometers (Eschweiler, Kiel, Germany) flushed with gas mixtures of the appropriate O_2 tension (air replaced with N_2 using a Wösthoff gas mixing pump), warmed to 37°C and fully humidified prior to delivery [19]. Unless indicated otherwise, oxygenated cells refer to those equilibrated at a P_{O_2} of 150 mm Hg, deoxygenated ones with 0 mm Hg (i.e. pure N_2). Where required, aliquots were removed using a Hamilton syringe for measurement of O_2 saturation (following the method of Tucker [20]), or fixation in glutaraldehyde (MBS plus 1% glutaraldehyde, taken to an osmolality of 300 mOsm/kg using distilled water) for analysis of sickling (using light microscopy and a haemocytometer).

2.6. K^+ influx

For K^+ influx measurements, HbS cell samples were removed from the tonometers and diluted 10-fold into flux tubes (final haematocrit about 4%) also at 37°C. For measurement of fluxes in experiments shown in Figs. 2 and 3, cells were exposed to pH 7 and osmolality of 270 mOsm/kg to stimulate the activity of KCC. Ouabain (100 μM) and bumetanide (10 μM) were always present; A23187 (10 μM), different $[Ca^{2+}]_o$s (10 μM or 2.5 mM), clotrimazole (10 μM) or EGTA (100 μM) were added as required. To investigate the effects of NEM (1 mM) or staurosporine (2 μM), samples were incubated for a further 15 min with these inhibitors before flux measurement, otherwise it was determined immediately. $^{86}Rb^+$ was used as a tracer for K^+, added in 150 mM KNO_3 solution to give a final K^+ of 7.5 mM. K^+ influx was measured over a 10 min period after which unincorporated radioisotope was removed by washing in ice-cold isotonic buffered $MgCl_2$ saline (107 mM, pH 7.4). Haematocrit was measured either by the cyanomethaemoglobin method or by microhaematocrit determination. Influxes are expressed as mmol K^+ per litre of cells per hour. Although this technique measures K^+ influx [21], because of the outwardly facing electrochemical gradients, the transport pathways studied will mediate a net K^+ efflux. For this reason, the term K^+ transport (rather than influx) is used in much of the following.

2.7. Statistics

Data are presented as mean ± S.D. for n replicates for single experiments representative of at least two others, or as mean ± S.E.M. for n experiments on samples from different individuals. Linear regression (Fig. 3B,C) was calculated using a Sigmaplot program (Jandel Scientific).

3. Results

3.1. Effect of DMA on HbS cell shape, O_2 saturation and K^+ transport

The relationship between O_2 saturation and O_2 tension (P_{O_2}) is shown in Fig. 1. DMA (5 mM) caused a leftward shift in the curve with P_{O_2} required for half-maximal O_2 saturation being reduced from about 30 to 10 mm Hg, consistent with a marked increase in O_2 affinity. Nevertheless, O_2 saturation fell to 0% when cells were incubated in N_2 (0 mm Hg O_2) showing that O_2 binding was fully reversible. The effect of DMA (5 mM) on the shape of HbS cells was also assessed after 15 min incubation in N_2. Percentage sickling in fully deoxygenated control cells was 76 ± 5% (mean ± S.E.M., $n = 6$), declining to 7 ± 3% in DMA-treated cells (91% inhibition of sickling).

The effect of DMA (5 mM) on K^+ transport was determined on oxygenated and deoxygenated HbS cells, in the absence and presence of Cl^- (Fig. 2). DMA had little effect in oxygenated HbS cells, whilst K^+ transport was markedly reduced in deoxygenated cells (Fig. 2A). Results are analysed

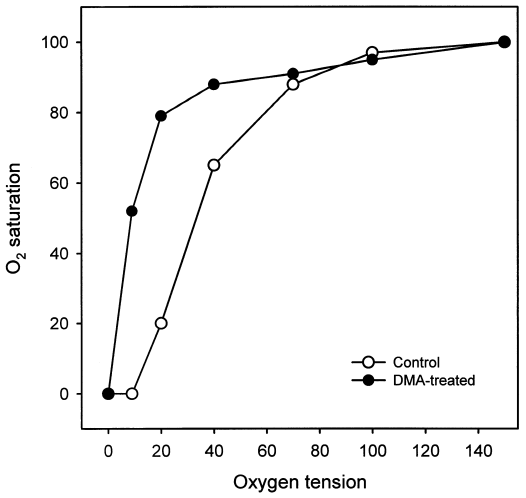

Fig. 1. Effect of DMA on O_2 saturation of HbS cells. HbS cells (about 40% haematocrit), either untreated or following exposure to 5 mM DMA, were equilibrated for 15 min in tonometers at the O_2 tension indicated before aliquots were withdrawn for measurement of their O_2 saturation following the method of Tucker [20]. Single experiment representative of two others.

in more detail in Fig. 2B. The first two histograms of Fig. 2B (i and ii) show that DMA inhibited the deoxygenation-induced Cl^--independent K^+ transport (K^+ transport in Cl^--free medium measured at 150 mm Hg O_2 subtracted from that measured in N_2) by 86 ± 4% (mean ± S.E.M., $n = 10$). Cl^--independent K^+ flux could be via either P_{sickle} or the Gardos channel, activated by Ca^{2+} entry through P_{sickle}. Separate experiments showed that deoxygenation-induced Cl^--independent K^+ transport was inhibited even in the complete absence of extracellular Ca^{2+} (nominally Ca^{2+}-free plus 100 μM EGTA) or after the addition of 10 μM clotrimazole (mean inhibition of 81% by 5 mM DMA). In deoxygenated HbS cells exposed to high $[Ca^{2+}]_o$ (2.5 mM) to activate Ca^{2+}-dependent K^+ transport, DMA also inhibited the clotrimazole-sensitive K^+ flux (mean inhibition of 88% by 5 mM DMA). These results imply inhibition of both P_{sickle} and the Gardos channel. The other four histograms (Fig. 2B, iii–vi) show changes in the Cl^--dependent K^+ transport (K^+ fluxes in the absence of Cl^- subtracted from those measured in its presence) in oxygenated and deoxygenated HbS cells, usually taken as an indication of the activity of KCC. In air, Cl^--dependent K^+ transport was similar in magnitude in the presence and absence of DMA, implying only a modest change in KCC activity (16 ± 11% stimulation, mean ± S.E.M., $n = 7$). Although

Table 1
Effect of DMA on the response of KCC in HbS cells to protein kinase (PK) inhibitors

	Untreated cells	DMA-treated cells
No PK inhibitor	0.19 ± 0.18	0.49 ± 0.11
N-Ethylmaleimide	3.95 ± 0.48	0.95 ± 0.18
Staurosporine	2.02 ± 0.29	1.10 ± 0.16

HbS cells were first treated with 5 mM DMA. They were then exposed to *N*-ethylmaleimide (1 mM), staurosporine (2 μM) or no PK inhibitor for 15 min before measuring Cl^--dependent K^+ fluxes (mmol/(l cells h)). These were calculated as the difference in K^+ transport measured in the presence and absence of Cl^-, substituted with NO_3^-, and were taken as a measure of KCC activity. Data represent means ± S.E.M. ($n = 6$).

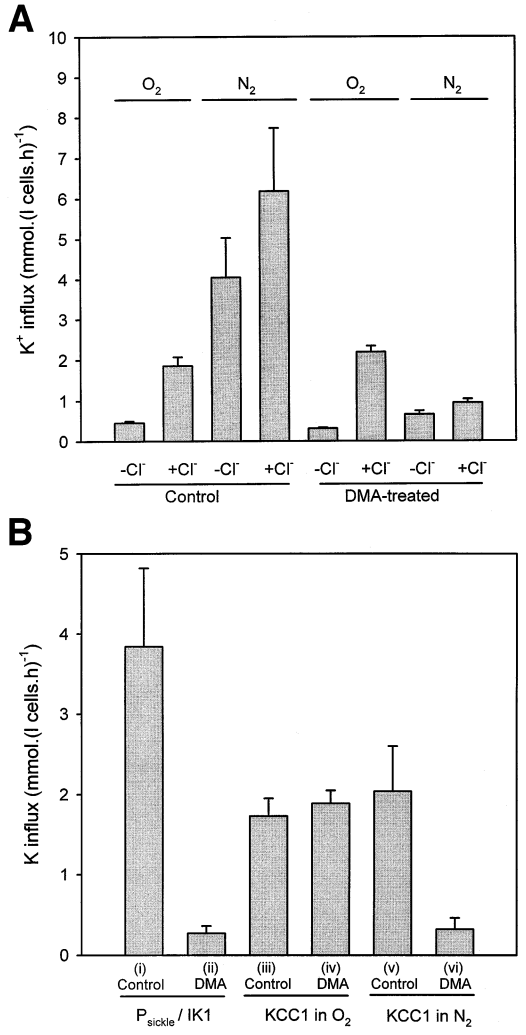

Fig. 2. Effect of DMA on K^+ transport in HbS cells. HbS cells (about 40% haematocrit), either untreated or following exposure to 5 mM DMA, were equilibrated for 15 min in tonometers at 150 or 0 mm Hg O_2 tension (labelled O_2 or N_2, respectively) before dilution 10-fold into flux tubes for measurement of K^+ influx (mmol/(l cells h)) at this same O_2 tension. A: Influxes measured in the presence or absence of Cl^- (substituted with NO_3^-). B: K^+ influx through the different K^+ pathways: (i) and (ii) show P_{sickle}/IK1 (deoxygenation-induced transport in the absence of Cl^-, calculated as the difference in influx in deoxygenated and oxygenated samples in Cl^--free medium); (iii)–(vi) show KCC activity (Cl^--dependent K^+ transport, calculated as the difference in influx ± Cl^-) in oxygenated (iii and iv) or deoxygenated (v and vi) cells. Histograms represent means ± S.E.M. for 8 (KCC) or 10 (P_{sickle}) experiments on cells from different patients.

the mean change in KCC activity was small, the effect of DMA was noticeably variable (ranging from a 23% inhibition to a 64% stimulation). In N_2, by contrast, Cl^--dependent K^+ transport was consistently and markedly inhibited (by 77 ± 8%, mean ± S.E.M., $n = 10$), indicating substantial inhibition of KCC in deoxygenated HbS cells (Fig. 2B, v and vi).

3.2. Effect of DMA on P_{sickle} and Ca^{2+}-dependent K^+ transport

Activation of the deoxygenation-induced pathway, P_{sickle}, is associated with HbS polymerisation and cell sickling. The Gardos channel is activated stochastically at low P_{O_2}s following Ca^{2+} entry via P_{sickle} [4]. The inhibitory action of DMA on Cl^--independent K^+ transport could therefore be explained either through effects on HbS polymerisation and sickling, or through a direct inhibitory effect on the transport pathways per se. These two possibilities were addressed in the following experiments.

Application of A23187 (10 μM) and 10 μM $[Ca^{2+}]_o$ was used to elevate intracellular Ca^{2+} in deoxygenated HbS cells treated with DMA (5 mM). They resulted in a pronounced activation of K^+ transport (from 1.03 ± 0.10 to 40.28 ± 0.15 mmol/(l cells h)) which was inhibited almost completely (96%) by addition of clotrimazole (10 μM), an inhibitor of Ca^{2+}-dependent K^+ channels [9,22]. Considerable Gardos channel activity can therefore be induced in the presence of DMA if Ca^{2+} entry into the cell is sufficient. Fig. 3 shows the effect of various concentrations of DMA (1–10 mM) on sickling and Cl^--independent K^+ transport. Increasing [DMA] progressively inhibited both sickling and Cl^--independent K^+ transport by a similar magnitude (Fig. 3A). Sickling and Cl^--independent K^+ transport were strongly correlated ($r^2 = 0.92$; Fig. 3B). It is unlikely that a separate action of DMA would inhibit P_{sickle} directly by the same amount as it reduces HbS polymerisation. Taken together, these findings imply that DMA inhibits polymerisation and shape change, that this inhibits the development of P_{sickle} and that decreased Ca^{2+} entry via a reduced P_{sickle} activity, prevents activation of the Gardos channel.

3.3. Effect of DMA on K^+-Cl^- cotransport

Fig. 2 shows that treating cells with 5 mM DMA inhibited KCC activity only in deoxygenated HbS cells, but not in oxygenated cells, which suggests an inhibitory effect on a control pathway rather directly on the cotransporter per se. Regulation of KCC is complicated, with many stimuli acting via a regulatory phosphorylation cascade involving both serine/threonine and tyrosine kinases and phosphatases. The combined effect of DMA with modulators of the regulatory phosphorylation cascade was therefore examined.

The effect of two protein kinase inhibitors, NEM (1 mM) and staurosporine (2 μM), is shown in Table 1. These reagents stimulated KCC activity in both untreated and DMA-treated deoxygenated HbS cells, showing that the cotransporter could be activated pharmacologically in both cases. Stimulation in the absence of DMA was consistently higher, being about two-fold greater for staurosporine, and four-fold for NEM, compared with DMA-treated cells. Conversely, KCC activity was modestly increased by DMA treatment in unstimulated deoxygenated HbS cells (pH 7.4, isotonic MBS, in the absence of kinase inhibitors, Table 1). Finally, we investigated the effects of various concentrations of DMA (0–5 mM) on Cl^--dependent K^+ transport in deoxygenated HbS cells (Fig. 3A,C). As for Cl^--independent K^+ transport, increasing [DMA] progressively inhibited KCC. Again, however, effects varied between experiments and the correlation between cell sickling and KCC activity was weaker ($r^2 = 0.64$; Fig. 3C) than that observed for P_{sickle}.

4. Discussion

In this study, we have investigated the mechanism by which DMA protects HbS cells against K^+ loss and sickling. We show that DMA inhibited all three pathways for K^+ loss in deoxygenated cells: P_{sickle}, the Gardos channel and KCC. Findings are consistent with the main effect of DMA being

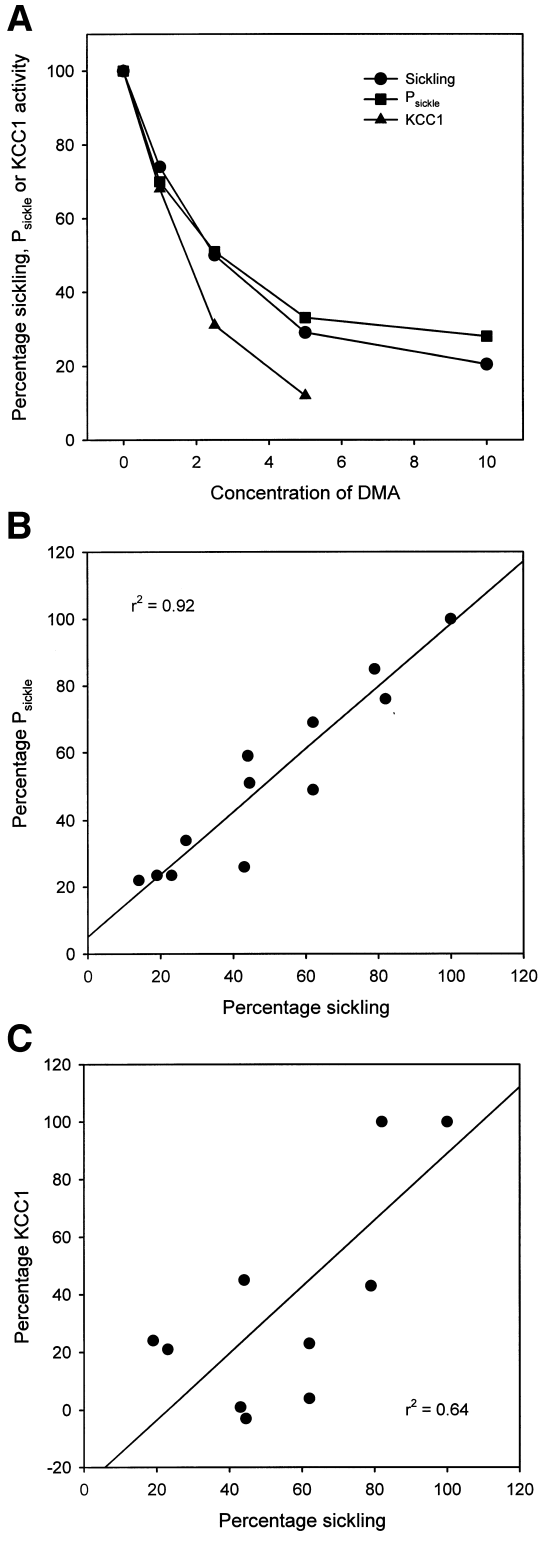

Fig. 3. Effect of different concentrations of DMA on sickling and K^+ transport in HbS cells. HbS cells were exposed to the concentration of DMA indicated (0–10 mM) and then equilibrated for 15 min (about 40% haematocrit) in tonometers at 0 mm Hg O_2 tension. Aliquots were then taken for analysis of cell shape or measurement of K^+ influx. Saline for cell fixation and flux measurement were also equilibrated at 0 mm Hg O_2 tension. A: Percentage sickling or K^+ transport (P_{sickle}/IK1 and KCC calculated as described in legend to Fig. 2) relative to those values observed in the absence of DMA (means for three separate experiments). B: Correlation of percentage sickling and P_{sickle}/IK1, plot calculated by linear regression, $r^2 = 0.92$. C: Correlation of percentage sickling and KCC, plot calculated by linear regression, $r^2 = 0.64$).
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via inhibition of polymerisation and sickling, rather than a direct effect on the transport pathways per se.

In the 1970s and 1980s, a number of crosslinking reagents on HbS cells, including DMA, were shown to decrease sickling and K^+ loss from deoxygenated HbS cells [17,18,23]. We show that DMA had only a modest effect on oxygenated HbS cells. By contrast, it caused a pronounced reduction in K^+ transport in deoxygenated cells through inhibition of both P_{sickle} and the Gardos channel. A substantial clotrimazole-sensitive, Cl^--independent K^+ transport could be activated in DMA-treated HbS cells, loaded with Ca^{2+} using the ionophore A23187. Thus the Gardos channel can function in DMA-treated cells provided Ca^{2+} entry is sufficient, and a direct inhibitory effect of DMA on this transporter is therefore unlikely. We also found that Cl^--independent K^+ transport correlated with sickling ($r^2 = 0.92$). These findings are consistent with a primary action of DMA to inhibit sickling, probably through effects on HbS polymerisation, inhibiting P_{sickle} and Ca^{2+} entry, and hence subsequent activation of the Gardos channel.

The interaction of DMA with KCC is more complicated. The cotransporter is affected by many stimuli including cell swelling, H^+ ions, urea and P_{O_2} [24,25], involving a cascade of protein kinase and phosphatase enzymes, of both the serine-threonine and tyrosine types [26,27]. In red cells from many vertebrates (including human HbA cells), the cotransporter is O_2-dependent [25,28]. In HbS cells, however, the O_2 dependence is abnormal [28] and the cotransporter remains active even in the absence of O_2. Because many of the stimuli for KCC occur in relatively hypoxic areas of the circulation (H^+ ions in active muscle beds, urea in the renal medulla), the abnormal O_2 dependence may increase the contribution of KCC to cell dehydration in vivo. The most important action of DMA on KCC activity was substantial inhibition, but only in deoxygenated HbS cells, in which KCC was inhibited to about the same extent as Cl^--independent K^+ transport (about 80% at 5 mM). By contrast, DMA had little effect on KCC activity in oxygenated HbS cells. Other inhibitors of KCC (for example the protein phosphatase inhibitor calyculin A [29], or DIOA [30,31]) are not selective for deoxygenated cells and, because the cotransporter is widely expressed in other tissues, their use in vivo is limited. The mechanism by which DMA has this selective effect is uncertain. Compared with P_{sickle}, there was only a moderate correlation between sickling and KCC activity. In addition, the effects of combinations of DMA and protein kinase inhibitors (NEM and staurosporine) showed that KCC activity was stimulated by these reagents in DMA-treated cells, but to a lesser extent than in the absence of DMA. The interpretation of these findings is obfuscated by the complexity of KCC control. They imply an action not solely on HbS polymerisation, and suggest an inhibitory effect of DMA on the regulatory phosphorylation pathway.

Oxidative damage may be important in HbS cells. The relative instability of HbS compared to HbA results in increased generation of free radicals. Levels of both total and reduced glutathione are low, the pentose phosphate pathway is im-

paired, and oxidative damage is present in both proteins and lipids. There is evidence that these changes contribute to the abnormal cation permeability of HbS cells [2,32,33]. Our findings suggest that, if this is the case, these defects must be corrected by DMA treatment, or at least remain cryptic under the conditions of our experiments.

Besides crosslinking reagents like DMA, other approaches have also been used to ameliorate the effects of SCD. Considerable work has been undertaken to identify effective and selective inhibitors of the transport pathways responsible for solute loss. Gardos channel inhibitors include imidazole derivatives such as clotrimazole [9,22,34]. KCC can be inhibited by intracellular Mg^{2+} [35] (in cells loaded either pharmacologically or via long-term oral supplementation with Mg^{2+} [36,37]), and also by bumetanide analogues and alkanoic acid derivatives [30,31]. Less directly, antioxidants (such as *N*-acetylcysteine) have been used to inhibit oxidant damage and, under certain conditions, protect against activation of the Gardos channel (e.g. [38]). Finally, other classes of antisickling agents include substituted benzaldehydes [39], designed to reduce HbS polymerisation through increasing its O_2 affinity. A number of these approaches have proved effective in vitro, and in vivo in transgenic mouse models of SCD [36,40]. Some have also been used in clinical trials [37,41]. To date, however, no method is adequate for clinical use. It is therefore important to understand as fully as possible the pathways which mediate dehydration and how to inhibit them. The unique action of DMA, in inhibiting KCC in deoxygenated cells, may suggest future strategies for treatment of SCD.

In conclusion, we have shown that DMA reduces K^+ transport through all three of the pathways concerned with K loss (P_{sickle}, the Gardos channel and KCC). A major part of its action is due to inhibition of cell sickling. Further work to identify its precise target may be important in the development of future chemotherapy for SCD.

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