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Effect of Intracellular Magnesium and Oxygen Tension on K^+-Cl^- Cotransport in Normal and Sickle Human Red Cells

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Abstract

In red cells from normal individuals (HbA cells), the K^+-Cl^- cotransporter (KCC) is inactivated by low O_2 tension whilst in those from sickle cell patients (HbS cells), it remains fully active. Changes in free intracellular [Mg^{2+}] have been proposed as a mechanism. In HbA cells, KCC activity was stimulated by Mg^{2+} depletion and inhibited by Mg^{2+} loading but the effect of O_2 was independent of Mg^{2+}. At all $[Mg^{2+}]_i$s, the transporter was stimulated in oxygenated cells, minimally active in deoxygenated ones. By contrast, the stimulatory effects of O_2 was abolished by inhibitors of protein (de)phosphorylation. HbS cells had elevated KCC activity, which was of similar magnitude in oxygenated and deoxygenated cells, regardless of Mg^{2+} clamping. In deoxygenated cells, the antisickling agent dimethyl adipimidate inhibited sickling, P_{sickle} and KCC. Results indicate a role for protein phosphorylation in O_2 dependence of KCC, with different activities of the relevant enzymes in HbA and HbS cells, probably dependent on Hb polymerisation, but not on $[Mg^{2+}]_i$.

Keywords

KCl cotransport; Oxygen; Magnesium; Sickle cell

Introduction

The K^+-Cl^- cotransporter (probably the KCC1 isoform, and ascribed KCC in the following) represents one of the major passive K^+ transport pathways in human red blood cells, mediating solute efflux and volume decrease [1–4]. Importantly, its behaviour differs between red cells from normal individuals containing haemoglobin A (HbA; and here termed HbA cells) and those from sickle cell patients (containing HbS, and termed HbS cells). HbS cells have an elevated capacity for KCC, and also show abnormalities in regulation [2, 5–9]. KCC is thought to contribute to dehydration of HbS cells, which encourages sickling through raising [HbS], since the lag time to polymerisation following deoxygenation is inversely proportional to a very high power of HbS concentration (15–30^{th}: [10]). KCC in sickle cells has lost its usual dependence on oxygen tension (PO_2) [7, 11, 12]. In HbA cells, deoxygenation inactivates KCC; by contrast, KCC activity in fully deoxygenated HbS cells is similar in magnitude to that in fully oxygenated cells. This behaviour is important because it would allow KCC in HbS cells to respond to stimuli such as elevated concentration of urea or reduction in extracellular pH (from 7.4 to about 7), which usually occur in relatively hypoxic regions of the circulation (like the renal medulla or active muscles). KCC could thereby contribute more to HbS cell shrinkage than would

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otherwise be the case. This study was designed to examine the Mg^{2+} dependence of KCC in oxygenated and deoxygenated human red cells, directly comparing HbA and HbS cells, together with the effects of *N*-ethylmaleimide and calyculin A (inhibitors of some of the protein kinases and phosphatases thought to regulate KCC). In addition, the effect of the antisickling agent dimethyl adipimidate was investigated.

Materials and Methods

Chemicals

Calyculin A was purchased from Calbiochem (Nottingham, UK), and ^{86}Rb from NEN Du Pont (Stevenage, UK). All other reagents were purchased from Sigma Chemical Co. (Poole, UK).

Blood

Heparinised samples from normal (HbAA) individuals and sickle cell patients (HbSS) were obtained with ethical consent and stored at 4°C until required. Red cells were washed 3x by centrifugation (2,000 g; 5 min) and aspiration of the supernatant in saline to remove buffy coat, platelets and plasma.

Solutions

The standard medium was MOPS-buffered saline (MBS) comprising (mM); NaCl 145, MOPS 10, glucose 5, pH 7.4 at 37°C, with an osmolality of 290 ± 5 $mOsm.kg^{-1}$. In some experiments NaCl was replaced by equimolar $NaNO_3$. In the experiments on HbS cells described in Figure 3, a high K^+-containing medium was used, with composition (mM): NaCl 70, KCl 80, MOPS 10, glucose 5, pH 7.4.

Tonometry

Oxygen tension (PO_2) was controlled by incubating cells in Eschweiler tonometers coupled to a Wösthoff gas mixing pump as described previously [13].

Transport measurements

K^+ influxes were determined at 37°C using ^{86}Rb as a congener, see [14] for details. Ouabain (0.1 mM) and bumetanide (10 μM) were present in all experiments to inhibit the Na^+/K^+ pump and Na^+-K^+-$2Cl^-$ cotransporter. Packed cell volume was determined by microhaematocrit centrifugation. K^+ influxes are expressed in standard units of mmol K^+ (l cells.h)$^{-1}$. Activity of KCC was defined as the Cl^--dependent or dihydroindenyloxy alkanoic acid (DIOA; 100 μM)-sensitive component of K^+ influx.

Magnesium clamping

Intracellular $[Mg^{2+}]$ ($[Mg^{2+}]_i$) was altered following the method of Flatman & Lew [15]. Cells were incubated at 4% Hct with the divalent cation ionophore A23187 (10 μM) for 30 min in the presence of 0.09, 0.19 or 0.34 mM extracellular $[Mg^{2+}]$ ($[Mg^{2+}]_o$). 50 ìM EGTA was also present to chelate contaminant Ca^{2+}. In the presence of ionophore, $[Mg^{2+}]_i = [Mg^{2+}]_o \times r^2$, where r is the Donnan ratio ($[H^+]_i / [H^+]_o$). r was determined by measuring intra- and extracellular pH in cell aliquots treated in the same way as used for flux measurements.

Statistics

Data are given as means ± S.E.M. for n determinations on samples from different individuals, or ± S.D. for triplicate determinations on a single sample, representative of at

least 3 others. Where appropriate, comparisons between groups were made using student's paired t test and a value of $p < 0.05$ considered significant.

Results

Effect of Mg^{2+} clamping on KCC activity of HbA cells

KCC activity, was low in deoxygenated control HbA cells (in the absence of A23187), < 0.02 mmol.(l cells.h)$^{-1}$, increasing to about 0.3 on oxygenation. Depletion of $[Mg^{2+}]_i$ caused a progressive stimulation of KCC activity in oxygenated cells (Figure 1a). Over the physiological range of $[Mg^{2+}]_i$ (about 0.5 to 0.2 mM), however, there was only a modest change in activity. In deoxygenated cells, by contrast, KCC activity remained minimal irrespective of $[Mg^{2+}]_i$. Oxygenation of HbA cells therefore caused substantial activation of KCC, and this was not prevented by clamping $[Mg^{2+}]_i$. These data can also be used to estimate free $[Mg^{2+}]_i$ in control cells, by estimating the free $[Mg^{2+}]_i$ in the presence of A23187 required to maintain KCC activity at the same level as that in cells untreated with ionophore. We obtain an estimate of 0.29 ± 0.01 mM (mean ± S.E.M., n = 3) for oxygenated HbA cells. KCC activity was also measured at PO_2s intermediate between those required for total saturation and desaturation of Hb with O_2 (Figure 1b), both in control cells (no ionophore) and in cells whose free $[Mg^{2+}]_i$ was clamped at 0.3 mM (ie close to the value in control oxygenated cells). The relationship between KCC activity and PO_2 was similar in the two sets of cells. Thus HbA cells, whose free $[Mg^{2+}]_i$ could not change with PO_2, nevertheless showed a normal O_2 dependence of KCC activity.

Effect of inhibitors of phosphorylation / dephosphorylation in HbA cells

In this set of experiments, cells were treated with *N*-ethylmaleimide (NEM; 1 mM) and/or calyculin A (calyculin; 100 nM). The aim was to prevent normal regulation of KCC, through inhibiting changes in serine-threonine phosphorylation of the transporter or a regulatory protein, and to examine effects on O_2 dependence of KCC.

Samples were first deoxygenated in tonometers at about 40% Hct and then diluted 10-fold into deoxygenated salines under three different conditions: (i) controls, with no inhibitors added (filled circles); (ii) addition of NEM only (filled triangles); and, (iii) addition of NEM followed by calyculin 6 min later (filled squares). KCC activity was measured in the three groups of deoxygenated cells at 0, 10, 20 and 30 min following addition of NEM (Figure 2). There was minimal KCC activity in control cells, intermediate activity in cells treated with NEM/calyculin and highest activity in cells treated with NEM only. Transporter activity remained level for the duration of the experiment. After 20 min, whilst transporter activity in the three groups of deoxygenated cells was stable, aliquots from the three sets of cells were removed and fully oxygenated. KCC activity was again measured and values are depicted in open symbols in Figure 2. On oxygenation, control cells showed activation of KCC to about 0.3 mmol.(l cells.h)$^{-1}$, the normal O_2 response. Cells treated with NEM only or NEM/calyculin had KCC activities not significantly different from those in deoxygenated cells (about 1.0 and 0.3 mmol.(l cells.h)$^{-1}$, respectively). This experiment shows that treatment with the protein kinase / protein phosphatase inhibitors NEM and calyculin prevented KCC in HbA cells from responding to changes in O_2 tension. Similar findings were observed when HbS cells were treated with NEM and calyculin A (data not shown).

Effect of Mg^{2+} clamping on HbS cells

The effect of $[Mg^{2+}]_i$ clamping was also investigated in HbS cells, following the same protocol as that used for HbA cells (see Figure 1a). KCC activity against $[Mg^{2+}]_o$ is shown in Figure 3a, and KCC activity against $[Mg^{2+}]_i$ in Figure 3b. The absolute magnitude of KCC in oxygenated control HbS cells (without ionophore) was about 10-fold higher than in

HbA cells (2.3 vs 0.3 mmol. (l cells.h)$^{-1}$), and deoxygenation stimulated KCC activity by about 10% (to 2.5 mmol.(l cells.h)$^{-1}$). As for HbA cells, reduction in [Mg^{2+}] caused progressive increase in KCC. In this case, however, both oxygenated and deoxygenated HbS cells behaved like oxygenated HbA ones. In marked contrast to HbA cells, at each $[Mg^{2+}]_o$, KCC activity was about 10% higher in deoxygenated sickle cells ($p < 0.05$; was consistent for all samples, the size of the error bars in Figure 3a reflecting heterogeneity in KCC activity in HbS cells from different patients). When these data are replotted using measured r values to calculate $[Mg^{2+}]_i$ KCC activity in oxygenated and deoxygenated Mg^{2+}-clamped HbS cells was found to be similar. In contrast to HbA cells, therefore, if secondary effects of PO_2 are removed (ie the expected slight decrease in r on deoxygenation), activity of KCC in Mg^{2+}-clamped HbS cells is unaffected by oxygenation/deoxygenation.

Effect of dimethyl adipimidate and Mg^{2+} clamping on KCC activity in HbS cells

Deoxygenation of HbS cells does not affect KCC activity regardless of free $[Mg^{2+}]_i$, but almost completely inhibits it in HbA cells. An obvious difference between HbS and HbA cells on deoxygenation is the formation of HbS polymers and cell sickling. It is possible that HbS polymerisation interferes with normal regulation of KCC by deoxygenation. Dimethyl adipimidate (DMA) is a crosslinking reagent shown to inhibit HbS polymerisation and the deoxygenation-induced sickling shape change. We therefore examined this possibility by treating HbS cells with DMA before clamping free $[Mg^{2+}]_i$ In these experiments, HbS cell samples were reacted with 6 mM DMA at 10% Hct for 30 min at 37°C and pH 8, before washing to remove unreacted reagent. High K^+-containing saline was used to prevent net K^+ transport; free $[Mg^{2+}]_i$ was clamped at about 0.3 mM (A23187 and $[Mg^{2+}]_o$ of 0.19 mM plus 50 μM EGTA). Control cells were similarly handled but without addition of DMA. Thereafter cells were treated as described in the preceding section. Percentage sickling and KCC activity were measured in oxygenated and deoxygenated cells, and the deoxygenation-induced Cl^--independent K^+ influx (ie difference in Cl^--independent K^+ influx in N_2 and that in O_2) was taken as a measure of P_{sickle} (Figure 4). As expected, DMA markedly inhibited cell sickling on deoxygenation, together with development of P_{sickle}. KCC activity was also considerably inhibited in deoxygenated cells. In HbA cells, DMA treatment had little effect on KCC activity (data not shown).

Discussion

This study presents the first complete study on effect of Mg^{2+} on O_2 dependence of KCC in normal human red blood cells (HbA cells) compared directly with those from sickle cell patients (HbS cells). Results suggest that changes in free intracellular [Mg^{2+}] cannot account for differences in KCC activity due to oxygenation/ deoxygenation, or when comparing red cells from normal individuals with those from sickle cell patients. They imply that HbS polymerization is important and that protein phosphorylation is involved.

O_2 tension and KCC activity in HbA cells

KCC activity in normal human red cells, and in red cells from a number of other species (including trout -[16]; horse - [17]; sheep - [18]) is dependent on PO_2, in that activity is minimal at low PO_2s, increasing progressively with PO_2, becoming maximal at around 100 mmHg. The relationship is sigmoidal, similar to O_2 saturation of Hb. We have reviewed elsewhere evidence for the participation of Hb in this response [19]. KCC in trout red cells appears to behave somewhat differently and there are reports that activity increases with PO_2 even after fully saturating Hb [20]. In HbS cells, the relationship between KCC activity and PO_2 is abnormal, with minimum activity levels at about 40 mmHg, with activity increasing at lower PO_2s [11]. Mean activity in fully deoxygenated HbS cells is about 10% higher than that in fully oxygenated ones [12]. This is confirmed in the present results.

One possible mechanism coupling the response of KCC to PO_2 is change in free $[Mg^{2+}]_i$. As HbA cells are deoxygenated, free $[Mg^{2+}]_i$ increases from 0.4 to 0.6 mM [21]. Mg^{2+} loading inhibits KCC activity [22]. We show that changes in free $[Mg^{2+}]_i$ over the physiological range have little effect on KCC activity in HbA cells making it unlikely that simple changes in free $[Mg^{2+}]_i$ regulate KCC in human red cells. We have observed previously similar effects in red cells from sheep [18] and horse [23], but similar results from human red cells have not been published previously (except in abstract form). Differences occur in the physiology and metabolism of red cells between different species [24, 25], and also in regulation of KCC [1] (eg concentration of organic phosphates, affinity of Hb [25, 26], volume and O_2 dependence of KCC [1, 13, 20]). Because these factors could alter the relationship between KCC activity, $[Mg^{2+}]_i$ and PO_2, it was important to ascertain the response of human red cells.

There is considerable evidence for regulation of KCC activity by protein phosphorylation [27, 28] and KCC1 has a number of consensus sites for phosphorylation [3, 29]. We show here that the putative protein kinase inhibitor NEM, and the serine-threonine phosphatase inhibitor calyculin A, either separately, or when added together to “clamp” phosphorylation state [30, 31], abrogate the response of KCC activity to changes in PO_2. These findings suggest that protein phosphorylation also controls the response of KCC to O_2.

Comparison of HbA and HbS cells

The effect of Mg^{2+} clamping in HbS cells has been investigated previously [32, 33]. In the earlier report, the response of cells to O_2 or Mg^{2+} was somewhat variable [32]. In a more complete account, Joiner *et al.* [33] used only the least dense fraction of HbS cells (15–25% total population). They show that, under isotonic conditions at pH 7.4, Cl^--dependent K^+ flux was absent from oxygenated HbS cells. Free Mg^{2+} was clamped with an extracellular $[Mg^{2+}]$ of about 0.05 and 1.4 mM ($[Mg^{2+}]_o$s of 0.15 and 1.5 mM but with 0.1 mM EGTA). The Donnan ratio was not measured so effects of oxygenation on this parameter were not included. Given an r^2 of about 2, these conditions would clamp $[Mg^{2+}]_i$ at about 0.1 and 2.8 mM. There was modest stimulation of KCC in Mg^{2+} clamped cells on deoxygenation (but note that $[Mg^{2+}]_i$ can only be assumed in the absence of measurement of r), and this was inhibited at the higher $[Mg^{2+}]$. Joiner hypothesised that deoxygenation-induced changes in protein phosphorylation (probably dephosphorylation of a key membrane protein) would stimulate KCC, but that under normal conditions this is masked by the inhibitory rise in free $[Mg^{2+}]_i$. Clamping free $[Mg^{2+}]_i$ removes this inhibitory effect and exposes the transporter to deoxygenation-induced stimulation. In our study, free $[Mg^{2+}]_i$ was clamped over a greater range and at more physiological concentrations. In agreement with Joiner, we show that KCC activity increased on deoxygenation for each $[Mg^{2+}]_o$. When account is taken of changes in r, however, we found similar activities of KCC in oxygenated and deoxygenated cells. There are a number of methodological differences between the two studies which may be relevant. We used total cell populations, at pH 7 and anisotonically swollen by 10%. The rate and duration of deoxygenation were different and may affect the nature of HbS polymerisation and its consequences. Our tonometry allows relatively rapid deoxygenation (within a few minutes; probably longer for Joiner) and deoxygenation was maintained for 15 min before measurement of transporter activity (cf >2 hours in Joiner’s study). It will be important to establish the precise conditions under which Mg^{2+} clamping is required in order to support substantial KCC activity in deoxygenated HbS cells.

HbS cells show considerable heterogeneity within a single sample (eg [34]). In the present context, there may be differences in concentration of organic phosphates between fractions, which would alter Mg^{2+} buffering, and potentially the free $[Mg^{2+}]_i$ [35]. In addition, the deoxygenation-induced channel P_{sickle} is permeable to Mg^{2+} as well as other cations [6, 35]. Free $[Mg^{2+}]_i$ has been estimated as particularly high in deoxygenated dense cells [35] but

lower in unfractionated samples [36]. These considerations would complicate an estimation of the normal free $[Mg^{2+}]_i$ in control HbS cells (ie without ionophore), using a similar procedure to that undertaken with HbA cells. In our experiments, the constant presence of A23187 coupled with appropriate $[Mg^{2+}]_o$, at low haematocrit (4%), should maintain the requisite clamped $[Mg^{2+}]_i$ regardless of cell fraction or PO_2. In addition, we have shown previously that the abnormal KCC activity in deoxygenated HbS cells is not confined to a single cell fraction, separated by centrifugation through preformed arabinogalactan gradients [12]. Should free $[Mg^{2+}]_i$ be elevated to very high levels in the deoxygenated HbS cells of Joiner, it may explain the stimulation of KCC that was observed on clamping Mg^{2+}, which could then reduce free $[Mg^{2+}]_i$ substantially. A profound depletion of organic phosphate compounds (mainly ATP and DPG) would raise free $[Mg^{2+}]_i$ and may follow prolonged deoxygenation (over 2 hours in his experiments).

Finally, we also examined the effects of deoxygenation in Mg^{2+} clamped HbS cells treated with DMA to prevent HbS polymerisation [37, 38]. In these cells, deoxygenation-incuded sickling and P_{sickle} activation were much reduced. KCC activity in deoxygenated HbS cells was also substantially inhibited compared to that in oxygenated HbS cells. We have observed similar effects of DMA in non-Mg^{2+} clamped HbS cells [39]. In previous studies, we have also examined the effect of the substituted benzaldehyde 12C79, a reagent which increases the O_2 affinity of Hb [40]. This compound activation to occur at lower causes cell sickling and P_{sickle} PO_2s, consistent with polymerisation being responsible for P_{sickle} [12]. It also shifts the activation of the low PO_2 component of KCC to lower PO_2s [7, 12]. Taken together, these experiments all suggest a role for HbS polymerisation in the abnormal activation of KCC in deoxygenated HbS cells.

Differences in total phosphorylation of membrane proteins, and specifically tyrosine phosphorylation of band 3, have been also found between HbA and HbS cells [41]. Tyrosine kinase activity is also different, being elevated in HbS cells [42, 43]. In addition, recent reports show that the expression pattern of KCC (KCC1,3 and 4) varies between normal and sickle erythroid cells, notably the extent to which different splice variants of KCC1 are transcribed [44]. These changes may underlie the different O_2 responses of KCC, with polymerization of HbS on deoxygenation playing a critical intermediary role. Future work should be aimed at identification of the proteins and enzymes involved.

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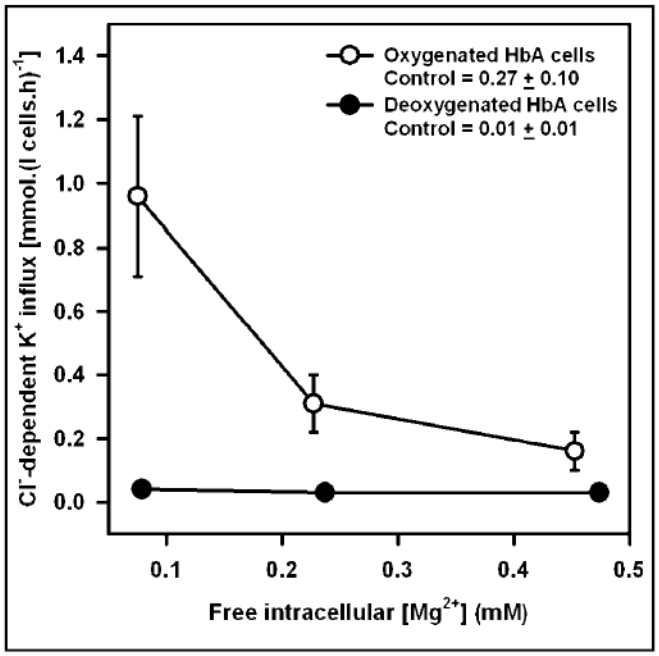

Fig. 1a.

Activity of the K^+-Cl^- cotransporter in Mg^{2+} clamped normal human red cells. Cl^--dependent K^+ influx (given as mmol. (l cells.h)$^{-1}$ and calculated as K^+ influx ± Cl^-) was measured in control (untreated with ionophore) and Mg^{2+} clamped HbA-containing red cells (HbA cells), under fully oxygenated (P = O_2 150 mmHg) or fully deoxygenated (100% N_2) conditions. Cells (40% haematocrit) were first equilibrated for 15 min at the requisite PO_2 in tonometers (pH 7.4, isotonic saline), before diluting 10-fold into hypotonic saline (260 mOsm.kg^{-1}), pre-equilibrated at the same PO_2 and at pH 7. Saline contained A23187 (10 μM) and $[Mg^{2+}]_o$ of 0.09, 0.19 and 0.34, with 50 μM EGTA. The Donnan ratio (r, $[H^+]_i$ / $[H^+]_o$) was measured in separate aliquots under identical conditions and found to be 1.22 and 1.26 in O_2 and N_2, respectively, and independent of $[Mg^{2+}]$. Free $[Mg^{2+}]_i$, given in the Figure, was calculated as the product of $[Mg^{2+}]_o$ and r^2. Ouabain (100 μM) and bumetanide (10 μM) were present to obviate influx through the Na^+/K^+ pump and Na^+-K^+-Cl^- cotransporter, respectively. Control values for KCC activity given in the legend represent fluxes measured in aliquots untreated with A23187. KCC activity in oxygenated samples was significantly ($p < 0.05$) greater than that in deoxygenated ones except at the highest $[Mg^{2+}]$. Symbols represent means ± S.E.M. for 3 determinations.

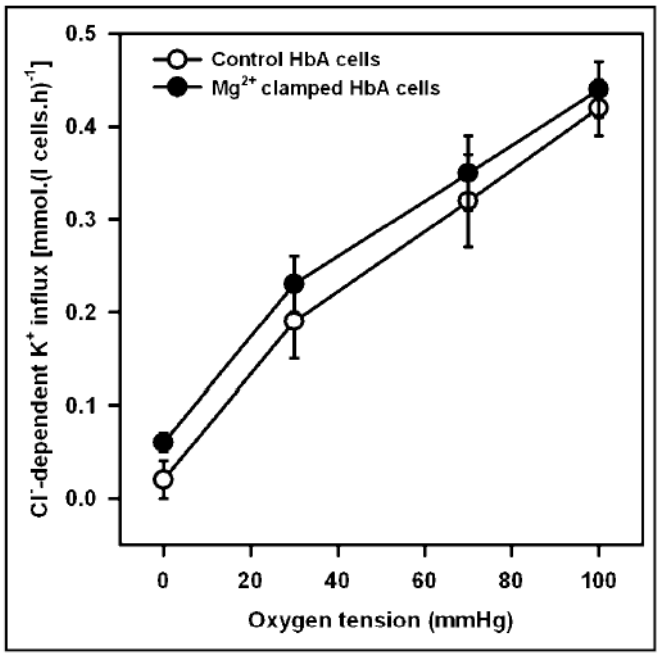

Fig. 1b.

Effect of oxygen tension on activity of the K^+-Cl^-cotransporter in control and Mg^{2+} clamped normal human red cells. Cells (40% haematocrit) were equilibrated for 15 min at the requisite PO_2 in tonometers (pH 7.4, isotonic saline), before diluting 10-fold into hypotonic saline (260 $mOsm.kg^{-1}$), pre-equilibrated at the same PO_2 and at pH 7. For Mg^{2+} clamped cells, saline contained A23187 (10 μM) and $[Mg^{2+}]_o$ of 0.19, with 50 μM EGTA, to give free $[Mg^{2+}]_i$ of between 0.23 and 0.22 mM (at PO_2s of 150 and 0 mmHg). Ouabain (100 μM) and bumetanide (10 μM) were present; KCC activity was taken as K^+ influx ± Cl^-. KCC activity in control and Mg^{2+} clamped cells was not significantly different at any PO_2 ($p > 0.05$). Symbols represent means ± S.E.M. ($n = 3$).

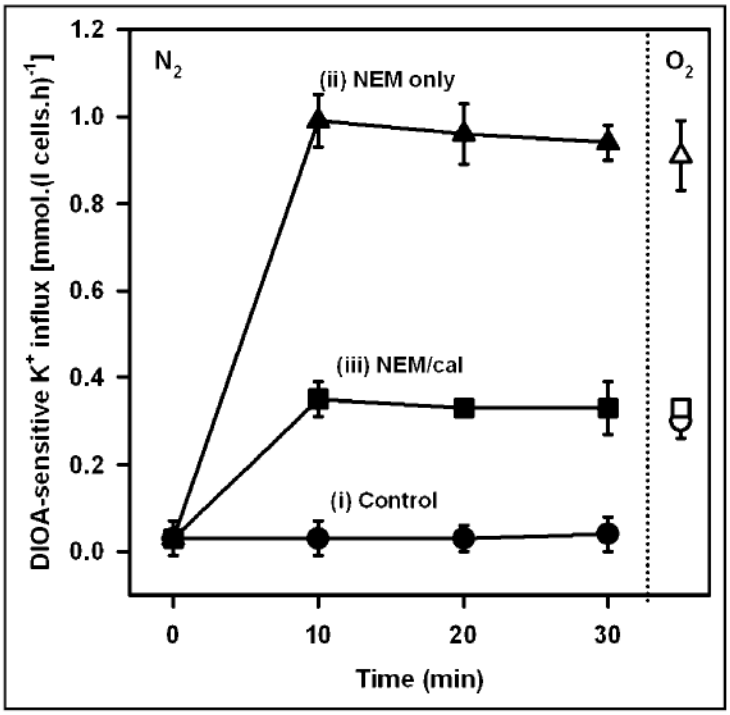

Fig. 2.

Effect of inhibitors of protein phosphorylation on the activity of the K^+-Cl^- cotransporter in normal human red cells. DIOA-sensitive K^+ influx (mmol. (l cells.h)$^{-1}$) was measured in HbA-containing red cells (HbA cells). Cells (40% haematocrit) were first deoxygenated for 15 min in tonometers (100% N_2, pH 7.4, isotonic saline). They were then divided into three aliquots and diluted 10-fold into hypotonic saline (260 mOsm.kg^{-1}), pre-equilibrated with N_2, pH 7. The three conditions were: (i) controls, untreated with kinase / phosphatase inhibitors (filled circles); (ii) *N*-ethylmaleimide (1 mM) only (filled triangles); and, (iii) *N*-ethylmaleimide (1 mM) at time 0, followed by calyculin A (100 nM) after 6 min (filled squares). After 20 min (but placed after 30 min to avoid superimposition on values from deoxygenated cells), red cell samples were taken from each of the three conditions and oxygenated before again measuring K^+ influx (open symbols). KCC activity was significantly different under the three deoxygenated conditions ($p > 0.05$). On oxygenation, activity was significantly changed (ie elevated) only in control cells. Ouabain (100 μM) and bumetanide (10 μM) were present. All symbols represent means ± S.E.M. (n = 3).

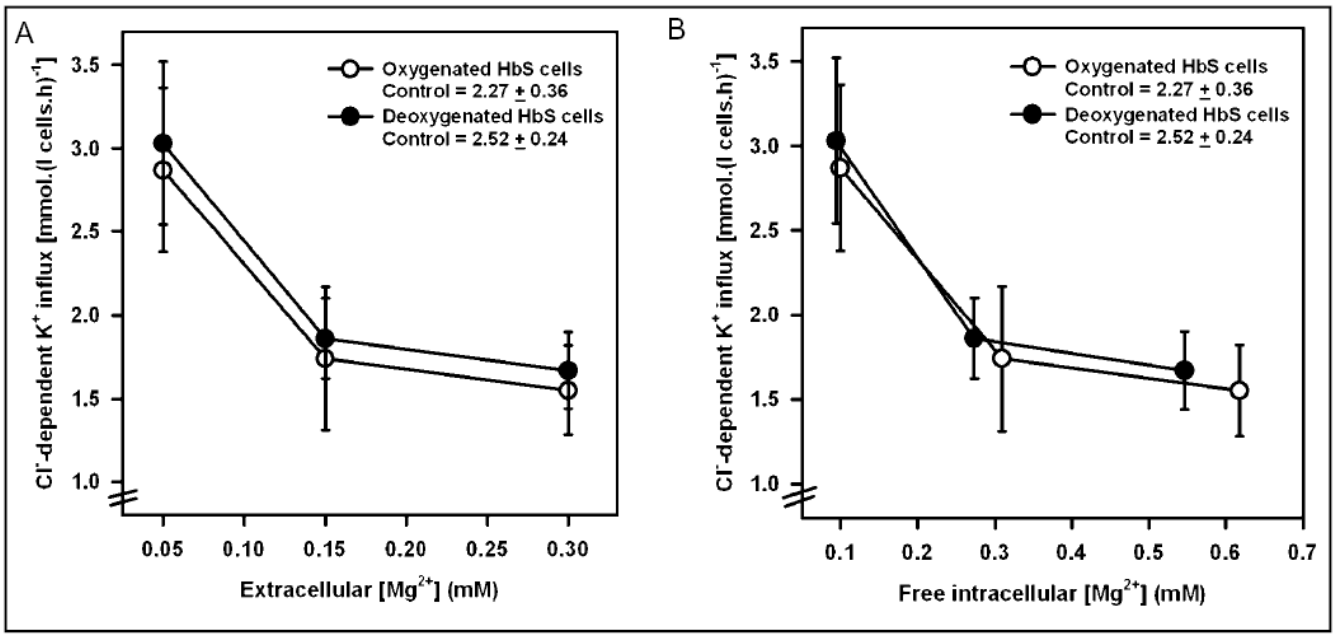

Fig. 3.

Activity of the K^+-Cl^- cotransporter in Mg^{2+} clamped human sickle red cells. Cl^--dependent K^+ influx (calculated as K^+ influx ± Cl^-, given as mmol.(l cells.h)$^{-1}$) was measured in control and Mg^{2+} clamped HbS-containing red cells (HbS cells) following a protocol identical to that described in the legend to Figure 1a. Donnan ratios (r, $[H^+]_i$ / $[H^+]_o$) were 1.43 and 1.35 in O_2 and N_2, respectively. KCC activity in control HbS cells, given in the legend, represents that for cells untreated with ionophore. KCC activity is plotted against $[Mg^{2+}]_o$ in Figure 3a, and against $[Mg^{2+}]_i$ in Figure 3b. Activity was not significantly different between oxygenated and deoxygenated cells. Ouabain (100 μM) and bumetanide (10 μM) were present. Symbols represent means ± S.E.M. for 5 determinations.

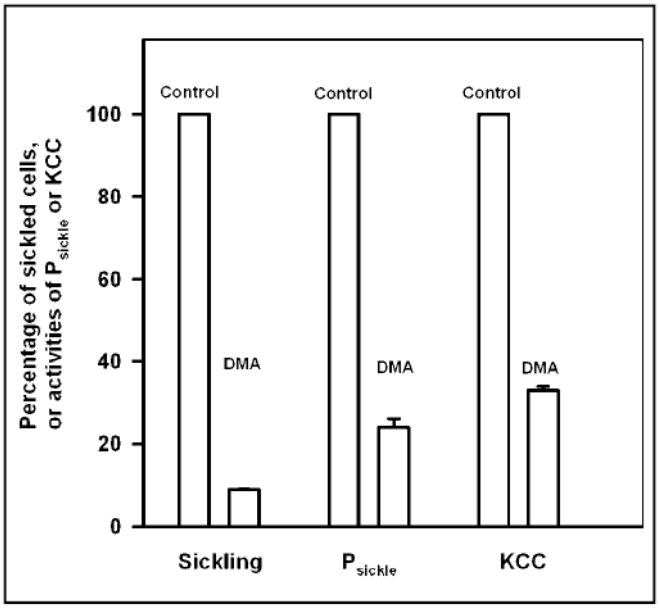

Fig. 4.

Effect of dimethyl adipimidate on sickling and K^+ transport in Mg^{2+} clamped human sickle red cells. HbS cells samples were exposed to dimethyl adipimidate (6 mM) for 30 min at 37°C, pH 8, then washed three times to remove unreacted DMA; control samples were similarly treated but without DMA. Both were then placed in tonometers (40% haematocrit) and equilibrated with O_2 at 150 mmHg or 100% N_2. Cells were subsequently Mg^{2+} clamped, as described in the legend to Figure 1, except that only a single $[Mg^{2+}]_o$, 0.19 mM (plus 50μM EGTA), was used. Aliquots were removed, fixed in saline with 1% glutaraldehyde (pre-equilibrated with air or N_2, as appropriate) for assessment of cell shape using light microscopy. Finally, K^+ influxes were measured: Cl^--dependent K^+ influx was taken as a measure of the activity of K^+-Cl^- cotransporter; the deoxygenation-induced Cl^- independent K^+ influx as a measure of P_{sickle} activity. Ouabain (100 μM) and bumetanide (10 μM) were present. Data for K^+ transport pathways or cell sickling are presented for cells in N_2, normalised to 100% in the absence of DMA. Histograms represent means ± S.E.M. for 3 determinations.