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K^+ transport in red blood cells from human umbilical cord

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Abstract

The current study was designed to characterise K^+ transport in human fetal red blood cells, containing mainly haemoglobin F (HbF, and termed HbF cells), isolated from umbilical cords following normal parturition. Na^+/K^+ pump activity was comparable to that in normal adult human red cells (which contain HbA, and are termed HbA cells). Passive (ouabain-resistant) K^+ transport was dominated by a bumetanide (10 μM)-resistant component, inhibited by [(dihydroxyindenyl)oxy]alkanoic acid (100 μM), calyculin A (100 nM) and Cl^- removal, and stimulated by *N*-ethylmaleimide (1 mM) and staurosporine (2 μM) – all consistent with mediation via the K^+-Cl^- cotransporter (KCC). KCC activity in HbF cells was also O_2-dependent and stimulated by swelling and urea, and showed a biphasic response to changes in external pH. Peak activity of KCC in HbF cells was about 3-fold that in HbA cells. These characteristics are qualitatively similar to those observed in HbA cells, notwithstanding the different conditions experienced by HbF cells in vivo, and the presence of HbF rather than HbA. KCC in HbF cells has a higher total capacity, but when measured at the ambient PO_2 of fetal blood it would be similar in magnitude to that in fully oxygenated HbA cells, and about that required to balance K^+ accumulation via the Na^+/K^+ pump. These findings are relevant to the mechanism by which O_2 regulates membrane transporters in red blood cells, and to the strategy of promoting HbF synthesis as a therapy for patients with sickle cell disease.

Keywords: Hemoglobin F; Potassium-chloride cotransport; Regulation; Oxygen

1. Introduction

Over the last 50 years, cation (Na^+, K^+, Ca^{2+}) transport in red blood cells has been the subject of extensive research. Red blood cells have been used as model systems with which to investigate the nature of ion permeability across biological membranes, for example the Na^+/K^+ pump [1]; they have been valuable in the elucidation of new transport pathways such as the cation-chloride cotransporters [2,3]; and abnormal cation transport has been associated with certain disease states, notably sickle cell disease (SCD) and the hereditary stomatocytoses [4]. The majority of this work has involved cells taken from adults. By contrast, very little is known about the

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membrane transport properties of red blood cells in the fetus.

There are several reasons why there may be differences in cation transport, and especially K^+ transport, in fetal red blood cells compared to adult ones. First, they represent a younger cell population. Young normal adult human red blood cells contain haemoglobin A (HbA; and are here termed HbA cells), have differences in transport compared to older ones, particularly as regards activity of K^+-Cl^- cotransporter (KCC) [5,6]. Second, in some species, fetal red blood cells have different transport characteristics to those of postnatal animals, for example higher rates of glucose, amino acid and nucleoside transport [7–9] and differences in Ca^{2+}-activated K^+ transport [10]. Third, they experience a different environment from adult cells. They are less likely to encounter changes in body fluid composition, for example the anisotonic conditions in renal or gastrointestinal capillaries. They will, however, experience relatively low O_2 tensions (PO_2s), since arterial O_2 tensions in the fetus are more similar to the venous maternal circulation [11]. Fourth, due to the presence of γ chains of Hb rather than β ones, they contain mainly HbF (and are here termed HbF cells) rather than the normal adult haemoglobin, HbA [12]. Hb has been implicated in control of membrane transporters [13–15], possibly through interactions with the negatively charged cytoplasmic tail of band 3 (termed cdb3 [16]). It is possible HbF could differ in this respect to HbA. The influence of HbF (compared to HbA) on red blood cell cation transport may also be relevant to clinical situations in the adult. In some individuals, red blood cells continue to express high levels of HbF. In addition, HbF interferes with the propensity of HbS to polymerise, and high levels of HbF have been correlated, at least in some cases, with less severe disease in sickle cell patients [12]. One possible therapy for SCD, which has received considerable attention, is elevation of HbF expression (for example using hydroxyurea). For these reasons, it is pertinent to investigate the properties of K^+ transport in HbF cells.

In these experiments, we have characterised the properties of K^+ transport in red blood cells taken from human umbilical cord. Results show that K^+ transport in HbF red cells is very similar to that in HbA cells. The predominant passive K^+ pathway is the K^+-Cl^- cotransporter and its properties were investigated in some detail. The significance of this to control of K^+ transport is discussed.

Some of these findings have been presented previously in abstract form [17,18].

2. Materials and methods

2.1. Chemicals

Bumetanide, [(dihydroxyindenyl)oxy]alkanoic acid (DIOA), 3-[*N*-morpholino]propanesulphonic acid (MOPS), *N*-ethylmaleimide (NEM), ouabain, salts and staurosporine were purchased from Sigma (Poole, Dorset, UK). Calyculin A was purchased from Calbiochem (Nottingham, UK), ^{86}Rb from NEN Du Pont (Stevenage, UK), and N_2 was obtained from BOC (Guildford, UK).

2.2. Solutions

The standard saline comprised (in mM): 145 NaCl, 5 glucose and 10 MOPS, (pH 7.4 at 37°C; 290 ± 5 mOsm kg^{-1}). For experiments in which Cl^- dependence of K^+ influx was examined, Cl^- was substituted with NO_3^-. To investigate the effects of anisotonic saline, isotonic sucrose was added to the saline and osmolality was adjusted by replacing it with distilled water or hypertonic sucrose, thereby keeping ionic strength constant. Where required, pH was altered by addition of HNO_3 or NaOH. Stock solutions of ouabain (10 mM) were prepared in distilled water and used at a final concentration of 100 μM. Bumetanide stocks (1 mM) were made daily in 100 mM Tris base and used at a final concentration of 10 μM. Stock solutions of NEM (100 mM) were prepared daily in distilled water; those of calyculin A (0.1 μM), DIOA (10 mM) and staurosporine (2 mM) were prepared in DMSO and frozen until required. In all cases, controls and cells treated with inhibitors or other reagents were exposed to the same concentrations of DMSO (whose final concentrations did not exceed 0.5%).

2.3. Sample collection and handling

Blood samples were obtained with permission

from human umbilical cords from newborn fetuses using heparin as anticoagulant. Red blood cells were washed three times in MOPS-buffered saline (MBS) by centrifugation, and the buffy coat removed by aspiration. They were then filtered through glass wool to remove nucleated cells. Cells were then stored on ice until use, within 36 h of collection. Mean HbF content (measured by HPLC) was 84 ± 3% (mean ± S.E.M., $n = 7$). Cell water content, determined by the wet weight/dry weight method of Borgese et al. [14] and expressed as ml g^{-1} of dry cell solids (d.c.s.), was typically 2.12 ± 0.01 ml g^{-1} (d.c.s.; mean ± S.E.M., $n = 3$), which is high relative to HbA cells but typical for fetal cells [19].

2.4. Tonometry and O_2 saturation

Before influx or O_2 saturation measurements, red blood 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 through three humidifiers prior to delivery. Samples for O_2 saturation were taken directly from the tonometers and processed following the method of Tucker [20].

2.5. K^+ influx

K^+ influx was measured at 37°C using $^{86}Rb^+$ (stock dissolved in 150 mM KNO_3) as a tracer for K^+. $^{86}Rb^+/K^+$ solution was added to give a final $[K^+]$ of 7.5 mM during measurement of influxes. Unincorporated radioisotope was removed by washing in isotonic buffered $MgCl_2$ medium. For experiments involving urea, an equivalent concentration of urea was added to this wash medium to prevent cell lysis. Haematocrit was measured either by the cyanomethaemoglobin method [21] or by microhaematocrit determination. Although the procedure measures tracer influx, passive or secondarily active transporters with an outwardly directed electrochemical gradient (such as KCC) will mediate a net efflux of solute [2]. All fluxes are expressed as millimoles of K^+ per litre of cells per hour (mmol (l cells·h)$^{-1}$].

2.6. Statistics

Data are presented as means ± S.D. for n replicates for single experiments representative of at least two others on samples from different cords, or as means ± S.E.M. for n experiments.

3. Results

3.1. Active and passive K^+ transport in HbF cells

Table 1 presents data on K^+ transport via the major active and passive pathways in fully oxygenated or deoxygenated HbF cells which were otherwise unstimulated (isotonic, pH 7.4). The largest component of K^+ influx was ouabain (100 μM)-sensitive, compatible with mediation via the Na^+/K^+ pump. Transport via this component was slightly, but not significantly, increased by deoxygenation. The bumetanide (10 μM)-sensitive component of K^+ influx, representing transport via the Na^+-K^+-$2Cl^-$ cotransporter (NKCC; probably the NKCC1 isoform [3]), was modest in oxygenated HbF cells but was stimulated by 4 ± 1-fold (mean ± S.E.M., $n = 4$) by deoxygenation. In the presence of ouabain and bumetanide, much of the remaining K^+ influx was sensitive to Cl^- substitution (with NO_3^-) and we show later that it was also inhibited by calyculin A and deoxygenation, and stimulated by protein kinase inhibitors (see below). These characteristics are consistent with mediation via the K^+-Cl^- cotransporter (probably KCC1 [22]). Residual influx (both ouabain- and bumetanide-resistant) in the absence of

Table 1
Major K^+ transport pathways in oxygenated and deoxygenated HbF cells

	Oxygenated	Deoxygenated
Na^+/K^+ pump	1.91 ± 0.09	2.08 ± 0.23
NKCC	0.16 ± 0.04	0.54 ± 0.09
KCC	0.37 ± 0.09	N.D.

Na^+/K^+ pump was defined as ouabain-sensitive K^+ transport, NKCC as bumetanide-sensitive, and KCC as Cl^--dependent, ouabain- and bumetanide-insensitive. Ouabain was used at 100 μM and bumetanide at 10 μM. Values represent influxes in mmol (l cells·h)$^{-1}$ and are given as means ± S.E.M. ($n = 4$). N.D., not determined in this experiment, but see Figs. 4 and 5.

Cl^- was small (<0.2 mmol (l cells.h)$^{-1}$) and insensitive to changes in PO_2. Under normal conditions in human red blood cells, NKCC is poised close to electrochemical equilibrium and unable to mediate sizeable net fluxes [23], whilst KCC has a large outwardly directed chemical gradient and would therefore dominate passive K^+ transport. This component of K^+ transport was therefore examined in more detail.

3.2. Characteristics of K^+-Cl^- cotransport in oxygenated HbF cells

In this section, all cells were fully oxygenated and treated with ouabain (100 μM) and bumetanide (10 μM) to obviate flux via the Na^+/K^+ pump and NKCC, respectively. Under these conditions, the residual K^+ transport is almost entirely via KCC. The volume dependence of K^+ transport, in the presence and absence of Cl^-, is shown in Fig. 1. The Cl^--dependent K^+ transport was stimulated markedly by swelling, whilst transport in the absence of Cl^- was largely volume-insensitive. These results are compatible with volume-stimulated KCC, thought to carry out regulatory volume decrease (RVD) in a number of tissues including red blood cells [24].

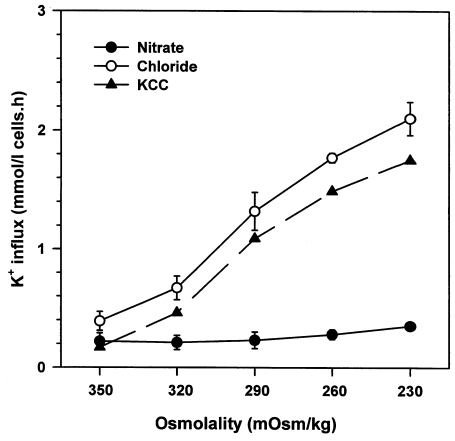

Fig. 1. Effect of osmolality on K^+ transport in HbF cells. K^+ influx (mmol (l cells·h)$^{-1}$) was determined in the presence (○) and absence (●) of Cl^- (substituted with NO_3^-) in oxygenated HbF cells. Cells were shrunken and swollen anisotonically by adding hypertonic sucrose or distilled water to the saline, whilst keeping ionic strength constant. Cl^--dependent K^+ transport, taken as representing transport by the KCC, was calculated as the difference in influx with and without Cl^-. Ouabain (100 μM) and bumetanide (10 μM) were present in all cases. Symbols represent means ± S.D., $n=3$.

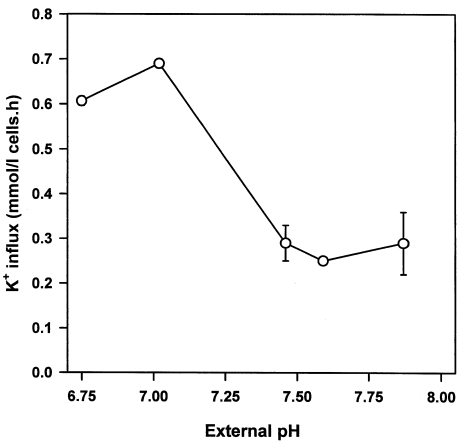

Fig. 2. Effect of extracellular pH on K^+ transport in HbF cells. K^+ influx (mmol (l cells·h)$^{-1}$) was determined in the presence of Cl^- in oxygenated HbF cells. Extracellular pH was adjusted using HNO_3 or NaOH before adding cells. It was allowed to stabilise and measured before addition of radioisotope. Ouabain (100 μM) and bumetanide (10 μM) were present in all cases. Symbols represent means ± S.D., $n=3$.

The response of K^+ transport to alteration in external pH is shown in Fig. 2. In all cases, pH was measured in the presence of cells (4% haematocrit) after it had stabilised and before flux measurement. The response of K^+ transport to changes in pH was biphasic. It was stimulated as pH was reduced from 8 to 7, but then inhibited at lower pH values. Again, these characteristics are typical of KCC. By contrast, K^+ transport measured in the absence of Cl^- was insensitive to pH changes (in a typical experiment, K^+ transport (mmol (l cells·h)$^{-1}$) in NO_3^- medium was 0.17 ± 0.01 at pH 7.4 and 0.13 ± 0.01 at pH 7.0 (means ± S.D., $n=3$).

Urea, at physiological concentrations found in the renal medulla during antidiuresis in adult humans, stimulates KCC in red blood cells (see [25] for references). Although high urea concentrations are not found in the fetus or neonate, and hence HbF cells would not normally be exposed to appreciable concentrations of urea, we tested its effect on HbF cells. The effect of different concentrations of urea on K^+ transport is shown in Fig. 3. K^+ transport in Cl^--containing medium was stimulated by urea at concentrations of 0.25–1.00 M. Higher concentrations

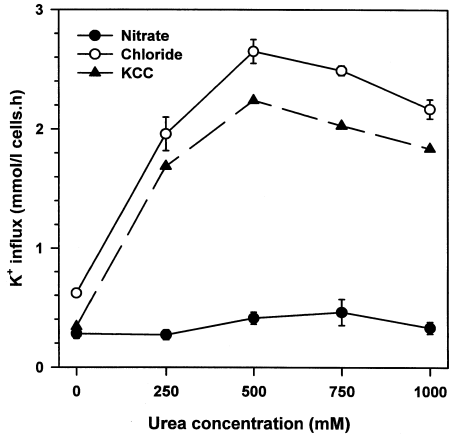

Fig. 3. Effect of urea on K^+ transport in HbF cells. K^+ influx (mmol (l cells·h)$^{-1}$ was determined as described in the legend to Fig. 1, but in standard MBS with urea (0–1 M) added as indicated. Ouabain (100 μM) and bumetanide (10 μM) were present in all cases. Symbols represent means ± S.D., $n = 3$.

were not tested because of their potential effects on protein denaturation. As for the effects of hydrogen ions and volume, K^+ transport in HbF cells suspended in Cl^--free media was not affected by urea.

Finally, the effect of different inhibitors of protein kinases and phosphatases was examined. In oxygenated HbF cells swollen anisotonically by 10%, Cl^--dependent K^+ transport (mmol (l cells·h)$^{-1}$) was increased from 0.84 ± 0.05 (all means ± S.D., $n = 3$) to 3.36 ± 0.19 and 2.16 ± 0.02 by the protein kinase inhibitors NEM (1 mM) and staurosporine (2 μM), respectively. The swelling-activated and NEM-activated Cl^--dependent K^+ transport was reduced to 0.02 ± 0.04 and 0.41 ± 0.05, respectively, in cells first treated with the protein phosphatase inhibitor calyculin A (100 nM).

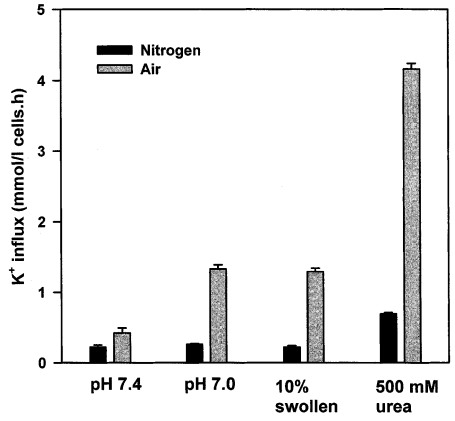

Fig. 4. Effect of deoxygenation on K^+ transport in HbF cells. HbF cells (about 40% haematocrit) were fully oxygenated or deoxygenated in tonometers for 15 min before 10-fold dilution into salines with four different compositions: isotonic pH 7.4, isotonic pH 7.0, 260 mOsm kg^{-1} (for 10% anisotonic swelling) at pH 7.4, and pH 7.4 containing 500 mM urea. K^+ influx (mmol (l cells·h)$^{-1}$) was measured 10 min later. Ouabain (100 μM) and bumetanide (10 μM) were present in all cases. Histograms represent means ± S.D., $n = 3$.

3.3. O_2 dependence of K^+-Cl^- cotransport in HbF cells

In human HbA cells, O_2 has important effects on the activity of KCC and also on its ability to respond to other stimuli, such as volume, hydrogen ions and urea. This was investigated in the results shown in Fig. 4. Stimulation by swelling and hydrogen ions (pH 7.0) was completely inhibited by deoxygenation, and that by urea (500 mM) was markedly and significantly reduced (by almost 90%).

We therefore investigated the effects of physiological PO_2s in more detail. Results (Fig. 5) are shown for cells stimulated by a combination of anisotonic swelling (10%) and hydrogen ions (pH 7). Cl^--inde-

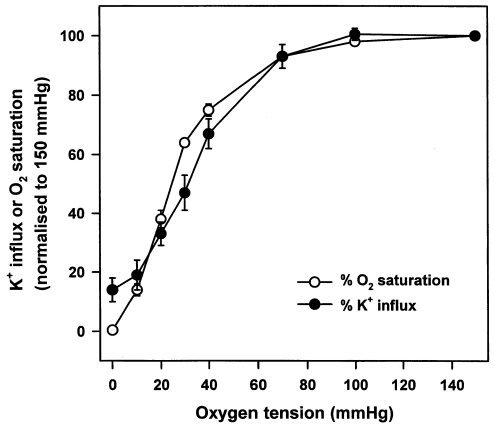

Fig. 5. Effect of physiological oxygen tensions on K^+ transport and O_2 saturation of HbF cells. Cells were handled in tonometers as described in the legend to Fig. 4, except that PO_2 was maintained at a range of PO_2s from 0 to 150 mmHg. Cell aliquots were then removed for measurement of K^+ influx (mmol (l cells·h)$^{-1}$) or O_2 saturation. Influx (●) was measured in saline of pH 7.0 and 260 mOm kg^{-1}, pre-equilibrated to the same PO_2 as the tonometers, with ouabain (100 μM) and bumetanide (10 μM) present in all cases. O_2 saturation (○) was determined using the method of Tucker [20]. Both measurements are expressed as a percentage relative to those found at 150 mmHg. Symbols represent means ± S.D., $n = 3$.

pendent K^+ transport was low and unaffected by O_2 (data not shown). Cl^--dependent K^+ transport increased progressively with PO_2 to a maximum at about 100 mmHg. The curve was approximately sigmoidal with a P_{50} (PO_2 for half-maximal activation) of 32 ± 3 mmHg (mean ± S.E.M., $n = 6$). These experiments showed that KCC in HbF cells had a similar sensitivity to O_2 as in HbA cells (see [26]). Similar results were obtained for cells stimulated by swelling only, hydrogen ions only or urea (data not shown). Finally, we also measured the effect of PO_2 on O_2 saturation of HbF cells, under similar conditions to those used for the transport studies. Results are also shown in Fig. 5. The relationship was again sigmoidal, P_{50} was 25 ± 2 mmHg (mean ± S.E.M., $n = 6$), and maximal saturation was achieved by about 80 mmHg.

4. Discussion

This paper presents the first characterisation of K^+ transport in HbF cells taken from human umbilical cord. We show that passive (ouabain-insensitive) transport is dominated by a Cl^--dependent, bumetanide-insensitive component. This component was stimulated by swelling and urea, and affected biphasically by hydrogen ions. It was also dependent on PO_2. Finally, its activity was modulated by inhibitors of protein kinases and phosphatases, being stimulated by NEM and staurosporine, and inhibited by calyculin A. These features are characteristic of KCC which in red blood cells is probably mediated via KCC1 [22].

Blood samples taken from the cord at parturition will contain a population dominated by cells containing HbF (60–95%), with a small, variable population of new cells containing HbA. Our experiments showed no consistent trends between samples with different fractions of HbA cells, indicating that our results represent the properties of HbF-containing cells. The main components of K^+ transport in HbF cells were similar to those in HbA cells from normal adults [27]. Ouabain-sensitive K^+ transport via the Na^+/K^+ pump was at the upper end of the range found in HbA cells, and represents the only method of active (ATP-driven) accumulation of K^+. Transport via the bumetanide (10 μM)-sensitive system, NKCC, is relatively modest in most HbA cells [27] but was larger in HbF cells. We show that this transport system was stimulated by deoxygenation, like that in avian red cells [28,29]. Residual Cl^--independent and ouabain-insensitive K^+ transport was small (usually < 0.2 mmol (l cells·h)$^{-1}$) and may represent 'leak' not mediated by specific transporters. The largest component of ouabain-insensitive K^+ transport, however, was via a bumetanide-insensitive, Cl^--dependent pathway, consistent with mediation by KCC, which (in high K^+-containing red blood cells) will mediate K^+ loss.

As the most significant component of passive K^+ transport in HbF cells, KCC was studied in more detail. We show that this pathway was stimulated by swelling, hydrogen ions and urea, and that it was sensitive to changes in PO_2. The same physiological stimuli therefore modulate the activity of KCC in both HbF and HbA cells, notwithstanding the fact that the environment encountered by HbF cells will differ from that to which HbA cells are exposed. In particular, the fetal kidney does not produce hypertonic urine and so the high concentrations of urea found in the renal medulla of adults during antidiuresis will not be present; lack of water or solute intake via the gastrointestinal tract precludes anisotonicity in gastrointestinal capillaries. Overall, compared to that in mature HbA cells, the activity of KCC in HbF cells was about 3-fold higher. In fact, activity was more similar to that found in 'young' HbA cells [5], probably due to the younger age of the HbF cells rather than to the presence of HbF per se. Active KCC provides HbF cells with the ability to carry out RVD responses [24]. The presence of NKCC may also permit some measure of RVI. As already discussed, however, because this transporter is close to electrochemical equilibrium, it will not mediate net fluxes of any great magnitude, even though unidirectional fluxes may be large. Furthermore, at least in HbA cells, NKCC is not (or only slightly) volume-sensitive (see [23]), although this aspect was not investigated in our study of HbF cells.

The activity of KCC in HbF cells also responded to the same pharmacological agents as that in HbA cells. In adult human red blood cells, and those from a number of other vertebrate species, the cotransporter is controlled by protein phosphorylation

[30]. Activity is inhibited by a serine-threonine phosphorylation, either of the transporter or of some regulatory peptide, and stimulated by dephosphorylation. In addition, sequential application of more than one protein phosphatase (PP)/protein kinase (PK) inhibitor has led to the proposal that there is an extended phosphorylation cascade, involving both serine-threonine and tyrosine residues [31,32]. The enzymes responsible, however, remain unidentified. Notwithstanding, these pathways have been implicated in the response of KCC in red blood cells from adult vertebrates to all the normal physiological stimuli, including swelling, hydrogen ions, urea and O_2 [31,33]. The stimulatory effects of NEM and staurosporine, together with the inhibitory action of calyculin A, imply that similar regulation occurs in HbF cells.

In human HbA cells (and mature red cells from many other vertebrates), O_2 tension represents an important physiological modulator [15]. In fact, at high PO_2s, KCC is activated and able to respond to stimuli such as hydrogen ions, swelling and urea, whilst at low PO_2s, the cotransporter is quiescent and refractory to these other modalities of stimulus. Again, this effect of O_2 was also similar in HbF cells. The relationship between PO_2 and activity of KCC was sigmoidal. KCC activity peaked at about 100 mmHg, it was half-maximal at 30 mmHg and minimal at low PO_2s. The normal PO_2 in the umbilical vein, which represents the maximal seen by HbF cells, is about 50 mmHg at which the cotransporter is about 2/3rd activated. We show that in vitro HbF cells can elevate KCC activity in response to higher PO_2s than normally encountered. Although the maximal activity of KCC in fully oxygenated HbF cells is about 3-fold that in HbA cells [27], at the normal levels of PO_2 in the fetus, KCC activity is rather closer to the activity in HbA cells. K^+ balance (through active accumulation by the Na^+/K^+ pump and loss via passive pathways) may therefore be quantitatively similar in HbA and HbF cells. We conclude that K^+ transport in sickle cell patients induced to express high levels of fetal Hb, to prevent Hb polymerisation, would not be adversely affected by the presence of HbF.

Although we have shown that functioning PP/PK enzymes are required for the O_2 response [31,33], the mechanism which couples KCC activity to PO_2 is unknown. Work with red cell ghosts and substituted benzaldehydes implies that bulk Hb is not involved [15,34,35] and a role for membrane-bound Hb has been proposed [13,14]. Much of this associates with the N-terminus of the cytoplasmic domain of the anion exchanger (AE1 or band 3), sometimes termed cdb3, a domain rich in acidic amino acid residues and highly negatively charged [16]. Hb has a higher affinity for this site when deoxygenated, rather than oxygenated [36,37], with cdb3 probably interacting with the 2,3-DPG pocket. Hb may compete for binding with a complex of glycolytic and other enzymes, including phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, catalase and perhaps hexokinase. The terminus also has several tyrosine residues and these can be phosphorylated thereby reducing its affinity for Hb and glycolytic enzymes [38]. This site, therefore, has many attributes associated with modulation of KCC activity. It has been implicated in the regulation of transport by O_2 [4,13,39]. The details, however, and, in particular, how the various proteins are arranged, are critical and remain to be elucidated. Despite HbF having a much lower affinity for 2,3-DPG [12] than bulk HbA, we show that O_2 modulates KCC activity in HbF cells in a similar manner to that in HbA cells. There are a number of possible explanations for this observation: O_2 may regulate the red cell transporters by some other mechanism; membrane-associated Hb, in its unique environment, may differ in its affinity for O_2 and organic phosphates; or the small amount of HbA present in HbF cells may be sufficient to regulate membrane transport. We are currently exploring these possibilities. In any event, the present results indicate that high concentrations of HbF do not markedly affect KCC activity. It is therefore unlikely that complications will arise from changes in red blood cell K^+ balance and hence cell volume following therapeutic manoeuvres to promote HbF levels in patients with SCD.

Acknowledgements

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