Two different oxygen sensors regulate oxygen-sensitive K⁺ transport in crucian carp red blood cells

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The O₂ dependence of ouabain-independent K⁺ transport mechanisms has been studied by unidirectional Rb⁺ flux analysis in crucian carp red blood cells (RBCs). The following observations suggest that O₂ activates K⁺–Cl⁻ cotransport (KCC) and deactivates Na⁺–K⁺–2Cl⁻ cotransport (NKCC) in these cells via separate O₂ sensors that differ in their O₂ affinity. When O₂ tension (Pₐₐ) at physiological pH 7.9 was increased from 0 to 1, 4, 21 or 100 kPa, K⁺ (Rb⁺) influx was increasingly inhibited, and at 100 kPa amounted to about 30% of the value at 0 kPa. This influx was almost completely Cl⁻ dependent at high and low Pₐₐ, as shown by substituting Cl⁻ with nitrate or methanesulphonate. K⁺ (Rb⁺) efflux showed a similar Pₐₐ dependence as K⁺ (Rb⁺) influx, but was about 4–5 times higher over the whole Pₐₐ range. The combined net free energy of transmembrane ion gradients favoured net efflux of ions for both KCC and NKCC mechanisms. The KCC inhibitor dihydroindenyloxyalkanoic acid (DIOA, 0.1 mM) abolished Cl⁻-dependent K⁺ (Rb⁺) influx at a Pₐₐ of 100 kPa, but was only partially effective at low Pₐₐ (0–1 kPa). At Pₐₐ values between 0 and 4 kPa, K⁺ (Rb⁺) influx was further unaffected by variations in pH between 8.4 and 6.9, whereas the flux at 21 and 100 kPa was strongly reduced by pH values below 8.4. At pH 8.4, where K⁺ (Rb⁺) influx was maximal at high and low Pₐₐ, titration of K⁺ (Rb⁺) influx with the NKCC inhibitor bumetanide (1, 10 and 100 μM) revealed a highly bumetanide-sensitive K⁺ (Rb⁺) flux pathway at low Pₐₐ, and a relative bumetanide-insensitive pathway at high Pₐₐ. The bumetanide-sensitive K⁺ (Rb⁺) influx pathway was activated by decreasing Pₐₐ, with a Pₐₐ for half-maximal activation (Pₐₐ) not significantly different from the Pₐₐ for haemoglobin O₂ binding. The bumetanide-insensitive K⁺ (Rb⁺) influx pathway was activated by increasing Pₐₐ with a Pₐₐ significantly higher than for haemoglobin O₂ binding. These results are relevant for the pathologically altered O₂ sensitivity of RBC ion transport in certain human haemoglobinopathies.

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In many cell types, volume regulation involves two Cl⁻- and K⁺-dependent ion transport pathways, K⁺–Cl⁻ cotransport (KCC) and Na⁺–K⁺–2Cl⁻ cotransport (NKCC). Hyposmotic cell swelling activates KCC, causing the net efflux of K⁺, Cl⁻ and osmotic water until the original volume is re-established. In contrast, hyperosmotic shrinkage activates NKCC in several cell types, which leads to the net uptake of cotransported ions and osmotic water, thereby restoring original cell volume (Hoffmann & Simonsen, 1989). Both transporters have been cloned and sequenced, and occur in different isoforms (KCC1, 2, 3 and 4; NKCC1 and 2) in a wide range of mammalian tissues with additional, tissue-specific functions such as epithelial Cl⁻ secretion and cellular Cl⁻ and K⁺ homeostasis (Russell, 2000; Adragna et al. 2004).

Red blood cells (RBCs) of certain non-mammalian vertebrates that express high activities of either of the two transporters have become important model systems for studying the functional properties, regulation and pharmacology of these widely expressed ion transport systems (Gibson et al. 1998). Indeed, turkey and rainbow trout RBCs were among the first model systems in which the powerful effects of molecular O₂ on the activity of NKCC and KCC, respectively, were described (Palfrey & Greengard, 1981; Borgese et al. 1991; Nielsen et al. 1991;
1992). In all species studied so far, including man, O₂ has opposing effects on the two RBC transport systems, activating KCC and deactivating NKCC (Gibson et al. 2000). Because the effects of O₂ are rapid, they appear to depend on constitutive signal transduction pathways, rather than on hypoxia-induced changes in gene expression (López-Barneo et al. 2001). However, despite continuous efforts, the nature of the O₂ sensor(s) and the transduction pathway(s) modulating RBC ion transport are still unknown.

Studies on rainbow trout RBCs – which appear to lack the NKCC but express high activities of KCC and a β-adrenergically activated Na⁺–H⁺ exchange system (NHE) – have suggested that haemoglobin (Hb) is a component of the O₂ sensor transducing the effects of molecular O₂ on NHE and KCC activity (Motais et al. 1987; Borgese et al. 1991; Nielsen et al. 1992). Subsequently, Hb has also been discussed as the O₂ sensor modulating NKCC and KCC in mammalian and avian RBCs, where the O₂ affinities for KCC activation or NKCC deactivation approximately match the O₂ affinity of Hb inside RBCs (Speake et al. 1997; Muzyamba et al. 1999; Flatman, 2005). However, O₂ affinity of KCC in rainbow trout RBCs is much lower than that of Hb, casting doubt on a direct role of bulk Hb oxygenation in modulating KCC in this species (Berenbrink et al. 1994). As no differences in K⁺ (Rb⁺) influx were apparent between the RBCs of the two crucian carp stocks, despite marked difference in their size, results were combined.

**Methods**

**Animals**

Crucian carp (Carassius carassius) were obtained either from a pond in the area of Turku, Finland (mass 0.7–1.8 kg, total length 31–40 cm, n = 20), or from a pond near Ipswich, UK (20–123 g, 12.1–18.3 cm, n = 25). Rainbow trout (Oncorhynchus mykiss), 130–214 g, 24.5–28.0 cm, n = 4) were purchased from a commercial fish farm near Turku, Finland. Both species were kept indoors at 15°C in running, dechlorinated tap water for at least 1 week prior to experimentation at the fish holding facilities of Åbo Akademi University, Turku, Finland, or of the School of Biological Sciences, University of Liverpool, UK. Experiments were performed from July to early December to minimize seasonal variations in the magnitude of RBC ion-transport pathways (Berenbrink & Bridges, 1994).

**Chemicals and solutions**

Inorganic salts, and dimethyl sulfoxide (DMSO), d-glucose, imidazole, ouabain and perchloric acid (PCA) were obtained from Merck, Darmstadt, Germany. Bumetanide, ethyl-m-aminobenzoate (MS 222), Hapes, methanesulphonic acid, N-methyl-d-glucamine (NMDG), sodium heparin and Tris were purchased from Sigma-Aldrich Chemical Company, while Triton X-100 was from Serva, Heidelberg, Germany. The radioactive tracer ⁸⁶Rb⁺ (as RbCl) was obtained from NEN Life Science Products, Belgium, and the KCC inhibitor DIOA (dihydroindenyloxyalkanoic acid) was from Research Biochemicals, Natick, MA, USA. Stock solutions (10 mM) of DIOA and ouabain were prepared in ethanol and DMSO, respectively, and the chemicals were used at a final concentration of 0.1 mM. Bumetanide (10 mM) was prepared in ethanol and used at final concentrations of 1, 10 and 100 μM. The final volume of the respective solvents did not exceed 1% of the volume of RBC suspensions.

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Standard fish saline for RBCs of crucian carp and rainbow trout consisted of (mM): 125.5 NaCl, 3 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 5 d-glucose and 20 Hapes, adjusted with NaOH to pH 7.97 at 15°C (Berenbrink et al. 2000; Völkel et al. 2001). The pH of standard saline was varied between 6.9 and 8.4 by adding NaOH or HCl. In Cl⁻-free salines, Cl⁻ salts were replaced either by the respective nitrates salts or, alternatively, by the respective cation hydroxides. In the latter case, pH was adjusted using methanesulphonic acid, creating a Cl⁻-free saline with methanesulphonate as the principal anion. Na⁺-free saline was prepared by equimolar replacement of NaCl with NMDG. In this case pH was adjusted with HCl.
Blood sampling and preparation of RBCs

Fishes were normally killed by a sharp blow on the head and immediate exsanguination by caudal venipuncture using heparinized hypodermic syringes. Large crucian carp were immersed in an overdose of anaesthetic (1 g l\(^{-1}\) MS 222, neutralized with Tris salt) until all movement ceased, before exsanguination. Procedures were carried out in accordance with national ethical committee guidelines. RBCs were then resuspended in three times with a 10-fold excess of ice-cold standard saline, each time removing the buffy coat. The resulting RBC suspensions were adjusted to half the original blood haematocrit value, oxygenated by contact with air and stored at 5°C for at least 16 h to allow for stabilization of cell volume and cellular ion content.

Experimental procedure

Immediately before experimentation, RBCs were washed in ice-cold standard saline. In the case of small crucian carp, RBCs from three or four animals were pooled to obtain a sufficiently large volume. For ion replacements, RBCs were washed three times with a 10-fold excess of Cl\(^{-}\)- or Na\(^{+}\)-free saline, allowing 5 min for ion equilibration at room temperature after each wash. RBCs were then resuspended at the original blood haematocrit value in the respective saline, and subjected to 45 min standard pre-equilibration at 15°C in shaking glass tonometers (Eschweiler, Kiel, Germany) with a water-vapour-saturated gas mixture of 5% air–95% N\(_2\), provided by mass flow controllers (Gf-3MP Cameron Instruments, Port Aransas, TX, USA) or a precision gas-mixing pump (Wösthoff KG, Bochum, Germany). Standard pre-equilibration at the resulting P\(_{O_2}\) of 1 kPa is frequently used to deactivate O\(_2\)-dependent KCC in fish RBCs with minimal effects on cellular ATP levels (Nielsen et al. 1992; Berenbrink et al. 1997, 2000; Völkel et al. 2001). After 45 min, the haematocrit value was determined by microcentrifugation (Micro-Compur M110, Compur Elektronik, München, Germany), and equilibration continued in humidified gases at P\(_{O_2}\) values of 0, 1, 4, 21 or 100 kPa. After 10 min of experimental equilibration, RBC suspensions were diluted with 9 vols pre-equilibrated standard saline adjusted to the desired pH range, for determination of unidirectional K\(^{+}\) (\(^{86}\)Rb\(^{+}\)) fluxes, Hb O\(_2\) saturation and final pH.

Determination of K\(^{+}\) fluxes

Unidirectional K\(^{+}\) fluxes were determined in the presence of 0.1 mm ouabain using \(^{86}\)Rb\(^{+}\) as a tracer, substituting for K\(^{+}\), as previously described (Berenbrink et al. 2000). Briefly, for influx measurements, 11.1–18.5 kBq ml\(^{-1}\) \(^{86}\)Rb\(^{+}\) was added to the diluted RBC suspensions (standard salines with or without ion replacements, 2–3% haematocrit) and after predefined time points 200 μl aliquots of the suspension were removed. RBCs were immediately washed three times by centrifugation and resuspension in ice-cold wash solution (100 mm MgCl\(_2\), 10 mm imidazole or Hepes, pH 7.97 at 15°C). After final centrifugation, the supernatant was removed and the RBC pellet lysed (0.5 ml 0.05 vol% Triton X-100) and deproteinized (0.5 ml 0.6 M PCA). Cellular \(^{86}\)Rb\(^{+}\) activity was determined by Cerenkov radiation. K\(^{+}\) (Rb\(^{+}\)) influx was calculated by linear regression from the rate of increase in cellular \(^{86}\)Rb\(^{+}\) activity with time and the extracellular \(^{86}\)Rb\(^{+}\) activity per extracellular K\(^{+}\) concentration (3 mm). Influx is expressed in millimoles ofK\(^{+}\) (Rb\(^{+}\)) per hour and per litre of cells. RBC volume was determined from haematocrit measurements at the end of the pre-equilibration period. For efflux measurements, RBCs at high haematocrit (30–40%) were loaded with \(^{86}\)Rb\(^{+}\) for ≥3.5 h at 20°C (37 kBq ml\(^{-1}\) suspension). After standard pre-equilibration, P\(_{O_2}\) was changed to the experimental value and 10 min later RBCs were diluted 20-fold in saline pre-equilibrated with the same gas mixture. The accumulation of \(^{86}\)Rb\(^{+}\) activity in the extracellular medium (standard saline) was followed for 15 min by centrifugation of aliquots at predefined time points and processing the supernatant as described above. As in our previous study (Berenbrink et al. 2000), \(^{86}\)Rb\(^{+}\) release appeared linear with time and K\(^{+}\) (Rb\(^{+}\)) efflux was determined from the initial \(^{86}\)Rb\(^{+}\) release rate and the initial cellular \(^{86}\)Rb\(^{+}\) activity per cellular K\(^{+}\) concentration. The latter was 102.2 ± 1.3 nmol per litre of RBCs (mean value ± s.e.m., n = 6 animals) in cells processed the same way as for K\(^{+}\) (Rb\(^{+}\)) efflux determinations and measured by atomic absorption spectrometry (Perkin-Elmer 2380). Efflux is expressed in mmol K\(^{+}\) (Rb\(^{+}\)) per hour and per litre of cells.

Hb O\(_2\) saturation and pH measurements

Hb O\(_2\) saturation was determined according to the method of Tucker (1967) in dilute RBC suspensions under the same experimental conditions as for K\(^{+}\) (Rb\(^{+}\)) flux measurements. Experimental procedures and calculations were identical to those used in our previous study on rainbow trout RBCs (Berenbrink et al. 2000). The pH of final RBC suspensions used for Hb O\(_2\) binding studies and K\(^{+}\) (Rb\(^{+}\)) flux measurements was checked using a thermostatted (14.9–15.1°C) capillary glass electrode with calomel reference (Radiometer BMS 3 Mk 2) and a pH meter (Radiometer PHM 72). The pH electrode assembly was calibrated at the experimental temperature with precision buffers (Radiometer). The final pH of RBC suspensions diluted with four different pH-adjusted salines was 6.942 ± 0.014, 7.387 ± 0.005, 7.931 ± 0.017 and 8.375 ± 0.036 (means ± s.e.m., n = 3). For readability, experiments at these pH values are referred to as pH 6.9, 7.4, 7.9 and 8.4 experiments. Oxygenation-induced
pH changes of the extracellular medium amounted to maximally 0.07 pH units, as estimated by comparing the pH difference between O$_2$- and N$_2$-equilibrated RBCs in the four different salines.

**Intracellular ion concentrations and net free energy for cotransport**

Intracellular Na$^+$, K$^+$ and Cl$^-$ concentrations ([Na$^+]_i$, [K$^+]_i$ and [Cl$^-$]$_i$) were measured in RBCs that had been pre-equilibrated for 45 min under standard conditions and subsequently exposed to a $P_O_2$ of 21 kPa for 10 min. Hence values refer to the time point where K$^+$ ($Rb^+$) flux determinations were started. RBC ion and water content were determined as described before (Berenbrink & Bridges, 1994). Briefly, RBCs were separated from the extracellular medium by rapid centrifugation in narrow 400 μl capacity Eppendorf tubes and quickly frozen in liquid nitrogen. Frozen tubes were cut with a razor blade 2 mm below the boundary between supernatant and pellet. The remaining pellet was weighed and deproteinized by addition of 200 μl 0.6 m PCA. After centrifugation (5 min, 10 000 g), ion concentrations in the supernatant were determined, Na$^+$ and K$^+$ by atomic absorption spectrometry (Perkin-Elmer 2380), and Cl$^-$ coulometrically using a chloride titrator (CMT 10, Radiometer, Copenhagen, Denmark). In parallel samples, pellets were preweighed and then dried to constant weight (≥ 40 h at 80°C) for determination of cellular water content. This allowed the intracellular ion concentrations to be expressed in millimoles per litre cell water for calculation of the net free energy in transmembrane ion gradients.

The combined net free energy change, $\Delta G$, for the transport of 1 mol Na$^+$, 1 mol K$^+$ and 2 mol Cl$^-$ into the cell was calculated according to Russell (2000):

$$\Delta G = RT \ln \left\{ \frac{[Na^+]_i[K^+]_i[Cl^-]_i}{([Na^+]_o[K^+]_o[Cl^-]_o)^2} \right\} \quad (1)$$

where $R$ is the gas constant (8.314 J mol$^{-1}$ K$^{-1}$), $T$ is absolute temperature (288 K) and subscripts ‘i’ and ‘o’ refer to intra- and extracellular ion concentrations, respectively. The net free energy change for the transport of 1 mol K$^+$ and 1 mol Cl$^-$ into the cell was calculated according to Lauf (1985):

$$\Delta G = RT \ln \left\{ \frac{[K^+]_i[Cl^-]_i}{([K^+]_o[Cl^-]_o)} \right\} \quad (2)$$

In both cases, positive values for $\Delta G$ indicate that ion gradients favour a net outward direction of transport.

**Data analysis and representation**

Values are expressed as means ± s.e.m. of $n$ experiments on RBCs of separate animals, or on RBCs pooled from separate groups of animals. In contrast to classical model organisms, animals were not from inbred laboratory lines and K$^+$ ($Rb^+$) influx and efflux values were somewhat variable between individuals. A similar degree of variability in ion transport activity is also evident in RBCs from other species, including humans (Ellory et al. 1985). In yet other studies, interindividual variation may be obscured by the common presentation of mean values and error bars of multiple measurements in a single, representative experiment.

Statistical differences between treatments were assessed using one-way or two-way analyses of variance, as appropriate, followed by Tukey’s test for pairwise comparisons (SigmaStat version 2.03). Data deviating from a normal distribution were transformed before further analysis according to $x' = \log(x + 1)$ or $x' = 1/(x + 1)$ (Sachs, 1988). Statistical significance was accepted at $P < 0.05$.

$P_O_2$ values for half-maximal K$^+$ ($Rb^+$) influx ($P_{50}$) were calculated by non-linear curve fitting (SigmaPlot version 8) using equations for simple saturation curves of the form

$$y = c + (ax)/(b + x) \quad (3)$$

or simple hyperbolic decay curves of the form

$$y = c + (ab)/(b + x) \quad (4)$$

where $y$ is K$^+$ ($Rb^+$) influx, $x$ is $P_O_2$, $a$ is maximal O$_2$-dependent K$^+$ ($Rb^+$) influx, $b$ is $P_{50}$, and $c$ is basal O$_2$-independent K$^+$ ($Rb^+$) influx.

The $P_{50}$ for Hb O$_2$ binding was calculated from non-linear curve fits according to a simple saturation curve

$$y = x/(b + x) \quad (5)$$

where $y$ is fractional Hb O$_2$saturation, $x$ is $P_O_2$, and $b$ is $P_{50}$. Using more complex equations for these fits, e.g. by introducing sigmoidicity constants (Berenbrink et al. 1997, 2000), did not improve the curve fits. The fractional amount of deoxygenated Hb was calculated as $1 - (\text{fractional amount of oxygenated Hb})$.

**Results**

K$^+$ transport pathways were characterized using unidirectional $^{86}$Rb$^+$ tracer fluxes. All K$^+$ ($Rb^+$) fluxes were measured in the presence of 100 μM ouabain to inhibit the Na$^+$–K$^+$–ATPase, and thus refer to ouabain-insensitive fluxes. Figure 1A shows the O$_2$ sensitivity of K$^+$ ($Rb^+$) influx at pH 7.9, which is close to arterial blood pH in normoxic, resting crucian carp at 15°C (Van den Thillart & Van Waarde, 1990). K$^+$ ($Rb^+$) influx was moderate at $P_O_2$ values between 4 and 100 kPa, but increased by about 150% at lower O$_2$ tensions of 1 and 0 kPa. These fluxes were Cl$^-$ dependent over the whole $P_O_2$ range studied, as K$^+$ ($Rb^+$) influx was nearly completely abolished when Cl$^-$ was replaced by...
either nitrate or methanesulphonate in the incubation medium.

The major Cl\textsuperscript{−}-dependent K\textsuperscript{+} transport pathways in RBCs of higher vertebrates are NKCC and KCC (Lauf et al. 1992). In human RBCs, 100 μM DIOA inhibits KCC without affecting NKCC (Garay et al. 1988). In crucian carp RBCs at high \(P_{O2}\), 100 μM DIOA inhibited K\textsuperscript{+} (Rb\textsuperscript{+}) influx almost completely and to a similar extent as Cl\textsuperscript{−} replacement (Fig. 1A). At lower \(P_{O2}\) values DIOA inhibition became progressively less complete, revealing a low-\(P_{O2}\)-activated, relatively DIOA-insensitive K\textsuperscript{+} (Rb\textsuperscript{+}) influx pathway, which showed a significantly higher activity than the small basal flux observed in the absence of Cl\textsuperscript{−}.

Figure 1. Effect of \(O_2\) tension, DIOA and ion replacements on K\textsuperscript{+} (Rb\textsuperscript{+}) fluxes

Cells were pre-equilibrated for 45 min at pH 7.9 and a \(P_{O2}\) of 1 kPa and then exposed to the indicated \(O_2\) tensions. After 10 min, subsamples were diluted 10-fold in pre-equilibrated salines of the same \(P_{O2}\) and unidirectional K\textsuperscript{+} (Rb\textsuperscript{+}) influx (A and B) and efflux (C) were measured in the presence of 0.1 mM ouabain. A, •, control (\(n = 8\)); ○, 0.1 mM dihydroindenyloxyalkanoic acid (DIOA) (\(n = 4\)). ▲ and △, medium chloride was replaced by nitrate and methanesulphonate, respectively (both \(n = 3\)). B, ■, control; □, sodium salts replaced by NMDG (both \(n = 3\)). C, \(n = 3\). All values are means ± s.e.m. *Significantly different from corresponding control value at the same \(P_{O2}\) (A and B); † significantly different from the corresponding control value and DIOA value at the same \(P_{O2}\) (A only). Note the logarithmic scale for \(P_{O2}\).
Replacing external Na\(^+\) by NMDG yielded somewhat reduced K\(^+\) (Rb\(^+\)) influx values at low \(P_{O_2}\) values, although the effect was statistically not significant (Fig. 1B). Independence from extracellular Na\(^+\) has frequently been used as supporting evidence for excluding NKCC as the mechanism for K\(^+\) tracer fluxes (e.g. Gillen et al. 1996; Muzymba et al. 1999; Mercado et al. 2000). Importantly, however, this cannot be taken as evidence against the involvement of NKCC in Cl\(^-\)-dependent K\(^+\) (Rb\(^+\)) tracer fluxes because in the absence of external Na\(^+\), the NKCC can perform a partial reaction and operate in a K\(^+\) (Rb\(^+\)) self-exchange mode in many systems, provided some internal Na\(^+\) is present (Lauf et al. 1987; Lylte et al. 1998).

In most cell types, the combined net free energy in the physiological transmembrane ion gradients greatly favours net efflux of cotransported ions via the KCC (Lauf et al. 1992). In contrast, the NKCC in RBCs is close to equilibrium and may be operating in a net inward or outward direction, depending on physiological plasma K\(^+\) concentrations (Duhm & Göbel, 1984). Unidirectional K\(^+\) (Rb\(^+\)) efflux showed a similar \(O_2\) dependence as K\(^+\) (Rb\(^+\)) influx, but was about 4–5 times higher over the whole \(P_{O_2}\) range, predicting a net outward direction of ouabain-insensitive K\(^+\) transport pathways at low and high \(P_{O_2}\) (Fig. 1C). Figure 2 shows that at \(P_{O_2}\) of 21 kPa the net free energy of the measured ion gradients across the crucian carp red cell membrane was compatible with a net efflux via both NKCC and KCC.

Below a \(P_{O_2}\) of 21 kPa, the predicted driving force for net efflux via NKCC is even higher, because as in mammals and birds, teleost fish RBCs possess a powerful anion exchange system (band 3; Jensen & Brahm, 1995). This transporter equilibrates Cl\(^-\) passively across the RBC membrane (e.g. Berenbrink & Bridges, 1994). At low \(P_{O_2}\) values, Hb deoxygenation and the associated neutralization of negative charges on Hb by Bohr protons induce a Cl\(^-\) shift from the external medium into the cytosol (Hladky & Rink, 1977). This effect is expected to be even larger in modern teleost fishes because of their much stronger Bohr effect as compared to mammalian and bird Hbs (Berenbrink et al. 2005). Thus, the increase of [Cl\(^-\)] at low \(P_{O_2}\) (Fuchs & Albers, 1988) will create even more favourable conditions for net ion efflux via both NKCC and KCC.

In mammalian RBCs, NKCC activity is strongly stimulated by alkaline pH (Flatman, 1991), whereas KCC is stimulated by acidification (e.g. Speake et al. 1997). In contrast, KCC in fish RBCs is strongly activated by alkaline pH (Berenbrink et al. 2000; Völkel et al. 2001). K\(^+\) (Rb\(^+\)) influxes in crucian carp RBCs at low and high \(P_{O_2}\) fundamentally differed in their pH dependence (Fig. 3A). Between \(P_{O_2}\) values from 0 to 4 kPa, pH did not significantly affect K\(^+\) (Rb\(^+\)) influx. However, at \(P_{O_2}\) values of 21 and 100 kPa, K\(^+\) (Rb\(^+\)) influx was significantly reduced by acidification below pH 8.4 and reached close to baseline levels at pH 7.4 and 6.9. This was similar to the inhibition of KCC by acidification in rainbow trout RBCs measured under the same experimental conditions (Fig. 3B). In rainbow trout, K\(^+\) (Rb\(^+\)) influx was minimal at \(P_{O_2}\) values of 1 and 0 kPa, independent of pH.

DeoxyHb has been implicated as a transducer for the effects of \(O_2\) on membrane ion transport in RBCs of several species (e.g. Motais et al. 1987; Flatman, 2005). Therefore the fraction of deoxyHb in crucian carp RBCs was measured under the same experimental conditions as in Fig. 3A. Figure 3C shows that between pH 6.9 and 7.9, activation of K\(^+\) (Rb\(^+\)) influx by lowering \(P_{O_2}\) closely matched the increase of deoxyHb. Figure 4 illustrates the respective \(P_{O_2}\) values at which half-maximal K\(^+\) (Rb\(^+\)) influx and half-maximal Hb deoxygenation were achieved (\(P_{50}\) values). At both pH 6.9 and 7.9, \(P_{50}\) for Hb and

![Figure 2. Net free energy for coupled Na\(^+\)-K\(^+\)-2Cl\(^-\) and K\(^+\)-Cl\(^-\) cotransport in crucian carp red blood cells](image-url)
K\(^{+}\) (Rb\(^{+}\)) influx were in the same range and not significantly different. Figure 3D shows that the three-dimensional pH and \(P_{O_2}\) profile of K\(^{+}\) (Rb\(^{+}\)) influx in crucian carp RBCs can, in principle, be explained by the action of two different K\(^{+}\) (Rb\(^{+}\)) influx pathways. One pathway is taken to have an activity which depends directly on the amount of deoxyHb as obtained from Fig. 3C. The other component is taken to have the same \(P_{O_2}\) and pH dependence as the KCC of trout (Fig. 3B). The diagram shows for each

\[\text{K}^{+} (\text{Rb}^{+}) \text{ influx model}\]

**Figure 3. Effects of \(O_2\) and pH on \(K^+ (\text{Rb}^+)\) influx and Hb deoxygenation**

After standard pre-equilibration (see Fig. 1), RBCs were exposed to \(P_{O_2}\) values between 0 and 100 kPa. After 10 min they were diluted in salines with pH values between 6.9 and 8.4, and these were pre-equilibrated with the same experimental \(P_{O_2}\), and K\(^{+}\) (Rb\(^{+}\)) influx (A) and fractional Hb deoxygenation (C) were measured. For clarity, mean values are shown with S.E.M. in one direction only (circles and bars, respectively). A, two-way analysis of variance with \(P_{O_2}\) and pH as factors revealed a highly significant interaction between the two factors (\(P \leq 0.001\)). Thus, the effect of \(P_{O_2}\) on K\(^{+}\) (Rb\(^{+}\)) influx (\(n = 3–10\)) depended on pH. *Significantly different from values at 0 kPa at the same pH; †significantly different from values at pH 8.4 at the same \(P_{O_2}\). B, comparable data on rainbow trout RBCs (\(n = 3–4\), partly taken from Berenbrink et al. (2000). C, fractional deoxyHb in crucian carp RBCs, measured under identical experimental conditions as for K\(^{+}\) (Rb\(^{+}\)) influxes (\(n = 3\)). D, reconstruction of the basic shape of \(P_{O_2}\) and pH-dependent K\(^{+}\) (Rb\(^{+}\)) influx as seen in A. At each \(x, y\) coordinate, the fraction of deoxyHb from C was added to the fractional K\(^{+}\) (Rb\(^{+}\)) influx taken from B, and the sum is plotted as the z-value. This demonstrates that fluxes in crucian carp RBCs (A) can be modelled in principle by the sum of a deoxyHb-activated flux and a deoxyHb-independent flux, the latter being similar to KCC in rainbow trout RBCs. See text for further details. Note the logarithmic scale for \(P_{O_2}\).
pair of $x$, $y$ co-ordinates ($P_{O_2}$, pH) the sum of the fractional amounts of these two putative $K^+$ ($Rb^+$) influx components ($z$-axis). This yields the same basic shape as observed in Fig. 3A, and it can be seen that allowing for the differences in pH dependency of $K^+$ ($Rb^+$) influx at high $P_{O_2}$ between crucian carp and rainbow trout RBCs (Fig. 3A and B; Volk et al. 2001) would even further increase the similarity between Fig. 3A and D.

To test the idea of two different $K^+$ ($Rb^+$) transport pathways, which are differentially modulated by $O_2$, further experiments were carried out using bumetanide at pH 8.4, where $K^+$ ($Rb^+$) influx was maximally activated at both high and low $P_{O_2}$. At low concentrations, bumetanide specifically inhibits NKCC in various vertebrate and invertebrate tissues with a 50% inhibitory concentration ($IC_{50}$) of about 0.1 $\mu$m (Russell, 2000). At higher concentrations bumetanide also inhibits KCC, albeit with a more than 1000-fold higher $IC_{50}$ ($\sim 180$ $\mu$m for KCC1 and $\sim 900$ $\mu$m for KCC4; Mercado et al. 2000). Figure 5A shows that $K^+$ ($Rb^+$) influx at low $P_{O_2}$ was already significantly inhibited by more than 50% in the presence of 1 $\mu$m bumetanide. Increasing the concentration to 10 $\mu$m led to significant further reductions, and 100 $\mu$m essentially abolished $K^+$ ($Rb^+$) influx at low $P_{O_2}$. With $IC_{50}$ values greater than 100 $\mu$m, any KCC isoform should be less than 50% inhibited at this bumetanide concentration. Therefore the near elimination of $K^+$ ($Rb^+$) influx by 100 $\mu$m bumetanide suggests that the flux at $P_{O_2}$ 0–1 kPa is almost entirely due to NKCC. At an intermediate $P_{O_2}$ of 4 kPa, significant inhibition of $K^+$ ($Rb^+$) influx required 10 $\mu$m bumetanide. At 21 and 100 kPa $P_{O_2}$, only 100 $\mu$m bumetanide caused a significant reduction of $K^+$ ($Rb^+$) influx. Thus, at higher $P_{O_2}$, an increasing fraction of $K^+$ ($Rb^+$) influx was carried by a more bumetanide-resistant pathway such as KCC. However, the moderate, but consistent reductions of $K^+$ ($Rb^+$) influx by low bumetanide concentrations (1 and 10 $\mu$m) even at high $P_{O_2}$ values suggest that NKCC was still partially active at 21 and 100 kPa.

A change in the characteristics of $K^+$ ($Rb^+$) transport between low and high $P_{O_2}$ is also indicated by the biphasic shape of the $O_2$ profile of the control flux. Thus, in the

![Figure 4. $O_2$ affinity of $K^+$ ($Rb^+$) influx and Hb at different pH values](figure)

$O_2$ affinity is expressed as the $P_{O_2}$ at which half-maximal change in $K^+$ ($Rb^+$) influxes or Hb $O_2$ binding occurred ($P_{50}$, means ± s.e.m.). At each pH, $P_{50}$ values for Hb (filled bars) were calculated by fitting hyperbolic saturation curves to data from three independent experiments (same data as in Fig. 3C). $P_{50}$ values for $K^+$ ($Rb^+$) influxes or $Hb$ $O_2$ binding occurred (open bars) or hyperbolic decay curves (hatched bars) to the data, as appropriate. These curve fits allow for a variable, basal $O_2$-independent $K^+$ ($Rb^+$) influx component. At pH 6.9, all flux data from Fig. 3A were used. At pH 7.9, only flux data from animals where $K^+$ ($Rb^+$) influx at $P_{O_2}$ 0 kPa was at least two times higher than at 100 kPa were used (5 out of the 8 independent experiments incorporated into Fig. 3A). At pH 8.4, total $K^+$ ($Rb^+$) influx could be separated into a 1 $\mu$m bumetanide-sensitive component and a 100 $\mu$m bumetanide-resistant component (see Fig. 5). n.s., $P_{50}$ for Hb and total flux did not differ significantly from each other at pH 6.9 and 7.9. At pH 8.4, $P_{50}$ for bumetanide-sensitive $K^+$ ($Rb^+$) influx and Hb also did not differ significantly (n.s.). However, both were significantly smaller than the $P_{50}$ of bumetanide-resistant $K^+$ ($Rb^+$) influx (**).
absence of bumetanide, \( K^+ (Rb^+) \) influx at a \( P_O_2 \) of 4 kPa was significantly lower than at 0 kPa, whereas the fluxes at higher \( P_O_2 \) values did not differ significantly from those at 0 kPa. In contrast, in the presence of bumetanide at 1, 10 and 100 \( \mu M \), fluxes at 100 kPa \( P_O_2 \) were always significantly higher than at 0 kPa (Fig. 5A).

In the presence of 100 \( \mu M \) bumetanide, NKCC is expected to be completely inhibited, and this allowed the determination of the \( P_{50} \) value for the \( O_2 \)-activated, bumetanide-resistant flux mechanism (KCC). In addition, calculation of the 1 \( \mu M \)-bumetanide-sensitive flux in Fig. 5B allowed the determination of the \( P_{50} \) value for the activation of NKCC by low \( P_O_2 \). Figure 4 shows that the \( P_{50} \) for the relative bumetanide-resistant \( K^+ (Rb^+) \) influx pathway (KCC) was significantly higher than the \( P_{50} \) values for NKCC and Hb. In contrast, \( P_{50} \) values for Hb and NKCC were not significantly different.

**Discussion**

Previous studies aimed at unravelling the mechanism of \( O_2 \) sensing in RBCs have tacitly assumed that there is one mechanism by which molecular \( O_2 \) governs the activity of RBC ion transport pathways. Here we suggest that two \( O_2 \) sensors, which differ significantly in their \( O_2 \) affinity, modulate two different \( O_2 \)-dependent \( K^+ (Rb^+) \) transport pathways in crucian carp RBCs.

**Identification of two separate \( O_2 \)-dependent \( K^+ (Rb^+) \) transport pathways**

We propose that the first \( K^+ (Rb^+) \) flux pathway, whose activity positively correlated with the fraction of deoxyHb in crucian carp RBCs, is carried by NKCC. At low \( P_O_2 \), where this mechanism was maximally activated, \( K^+ (Rb^+) \) influx was virtually abolished by replacement of extracellular Cl\(^-\) with nitrate or methanesulphonate. The mechanism was highly sensitive to the specific NKCC inhibitor bumetanide, with 1 \( \mu M \) of the drug causing more than 50% inhibition, and 100 \( \mu M \) bumetanide almost abolishing the flux. The mechanism was progressively activated by decreasing \( P_O_2 \), similar to NKCC in turkey, chicken, human and ferret RBCs (Muzyamba et al. 1999; Drew et al. 2004; Flatman, 2005).

The second \( K^+ (Rb^+) \) influx mechanism was maximally activated by high \( P_O_2 \), and showed the characteristics of KCC. It was abolished by replacement of Cl\(^-\) with nitrate or methanesulphonate, or by 100 \( \mu M \) DIOA. In contrast to NKCC, it showed low bumetanide sensitivity, with 50% inhibition requiring 100 \( \mu M \) bumetanide or more, like the KCC in other systems (Ellory et al. 1985; Mercado et al. 2000). Similar to KCC in normal human, horse and rainbow trout RBCs, the mechanism was virtually silent at a \( P_O_2 \) of 1 kPa or lower (Nielsen et al. 1992; Speake et al. 1997; Berenbrink et al. 1997; Gibson et al. 1998). Contrary to KCC in mammalian RBCs (e.g. Speake et al. 1997), the \( K^+ \) transport mechanism at high \( P_O_2 \) was also strongly activated by alkaline extracellular pH, similar to KCC in
rainbow trout RBCs (Nielsen et al. 1992; Berenbrink et al. 2000).

The partial inhibition of K\(^+\) (Rb\(^+\)) fluxes at low \(P_{O_2}\) by the KCC inhibitor DIOA was unexpected, because the high bumetanide sensitivity of the flux strongly suggests that it is carried by NKCC. However, partial inhibition of the NKCC by 100 \(\mu\)M DIOA has previously been noted in human RBCs (18 \(\pm\) 6\% inhibition, mean value \(\pm\) s.e.m, \(n = 3\); Culliford et al. 2003), calling for a cautionary interpretation of inhibitory effects of DIOA (Berenbrink et al. 2000).

Ultimate proof of both KCC and NKCC requires demonstration that concentration changes of each of the respective cotransported ions influence the transport of the other ions in the predicted way. This has been reported only in a few cases, partly because of the high anion exchange activity and anion conductance of RBCs, which presents a difficulty in varying \(Cl^-\) independently on both sides of the membrane (Lauf, 1985; Russell, 2000). In the absence of such evidence, we follow previous studies and use \(Cl^-\) dependence and low bumetanide sensitivity provisionally to ascribe the \(O_2\)-activated \(K^+\) (Rb\(^+\)) flux pathway in crucian carp RBCs to a KCC mechanism.

**Differential \(O_2\) sensitivity of KCC and NKCC**

At pH 8.4, where both NKCC and KCC were maximally activated, titration of \(K^+\) (Rb\(^+\)) influxes with bumetanide allowed dissection of the \(O_2\) affinities of these two transport systems. \(P_{50}\) for NKCC under these conditions was 1.4 kPa. At pH values of 7.9 and 6.9, \(P_{50}\) of total \(K^+\) (Rb\(^+\)) flux was 2.5 and 2.4 kPa, respectively. Figure 3A and comparable data on rainbow trout RBCs (Fig. 3B) suggest that KCC was largely deactivated at these \(P_{O_2}\) and pH values, and hence the majority of the flux was likely to be due to NKCC. These \(P_{50}\) values were not significantly different from the \(P_{50}\) of crucian carp Hb under the same conditions, although they are considerably lower than \(P_{50}\) values for NKCC in turkey and ferret RBCs (5.5 and 3.2 kPa, respectively; Muzyamba et al. 1999; Flatman, 2005).

Few studies have measured the \(O_2\) affinity of Hb and of \(O_2\)-dependent ion transport under strictly comparable experimental conditions. However, the decreasing \(P_{50}\) values of the NKCC from bird to mammalian and fish RBCs are closely matched by the respective \(P_{50}\) values for blood \(O_2\) binding in these groups (Prosser, 1950). These results are thus compatible with a role for Hb as the \(O_2\) sensor affecting NKCC in crucian carp RBCs, as previously suggested for several membrane ion transporters in other RBC systems (Motaix et al. 1987; Gibson et al. 2000). Discussing NKCC in ferret RBCs, Flatman (2005) suggested that competitive binding of deoxyHb to band 3 in the RBC membrane may release a kinase into the cytoplasm, which activates neighbouring NKCC proteins via phosphorylation of the transporter or of another protein, which could then activate NKCC. Indeed, protein kinase inhibitors suppress activation of NKCC by deoxygenation in both turkey and ferret RBCs (Muzyamba et al. 1999; Flatman, 2005). Alternatively, binding of deoxyHb to band 3, which may be modulated by band 3 phosphorylation, could directly activate NKCC via the cytoskeleton or by direct protein–protein interactions (Flatman, 2005).

In contrast, activation of KCC by elevated \(P_{O_2}\) in crucian carp RBCs was associated with a distinctly higher \(P_{50}\) (8.5 kPa at pH 8.4) than displayed by Hb \(O_2\) binding or the NKCC. Therefore in this case the mechanism of \(O_2\) sensing does not involve changes in bulk Hb \(O_2\) saturation, as previously also shown for the KCC in rainbow trout RBCs (Berenbrink et al. 2000).

In human sickle cell disease, and \(\alpha\) and \(\beta\) thalassaemia, the RBC KCC also appears to be at least partially uncoupled from its normal, Hb-like \(O_2\) sensor. These human haemoglobinopathies are all associated with increased production of reactive \(O_2\) species (ROS), and an altered \(O_2\) affinity of KCC in their RBCs (Repka & Hebbel, 1991; Olivieri et al. 1994; Gibson et al. 1998). KCC activity in normal human RBCs is low but can be activated by combined cell swelling and acidification (Gibson et al. 1998). Decreasing \(P_{O_2}\) inhibits the human KCC with a \(P_{50}\) of 4.8 kPa, a value that may be similar to the \(P_{50}\) of Hb under the acidic conditions used to stimulate KCC in that study (pH 7.0; Gibson et al. 1998). However, KCC in RBCs from homozygous sickle cell patients fails to become inactivated at low \(P_{O_2}\), resulting in a biphasic \(O_2\) dependence (Gibson et al. 1998). Conceivably, an increased rate of ROS production in human sickle cells (Repka & Hebbel, 1991) bypasses or overrides an \(O_2\) sensor with Hb-like affinity that regulates KCC in RBCs of healthy subjects. It is tempting to speculate that this prevents KCC deactivation at low \(P_{O_2}\), just as experimentally increased ROS levels prevent deactivation of KCC in rainbow trout RBCs at low \(P_{O_2}\) (Bogdanova & Nikinmaa, 2001). The mechanism behind abnormally elevated KCC activity in human sickle cells at low \(P_{O_2}\) is of considerable clinical importance, since it may contribute to RBC dehydration and thereby increased propensity for sickling (Lew & Bookchin, 2005).

In summary, the present study on crucian carp RBCs provides the first evidence for more than one constitutively expressed \(O_2\) sensor in a single cell type. We confirm the presence of an \(O_2\)-dependent KCC in fish RBCs with significantly lower \(O_2\) affinity than Hb. This has allowed its regulating \(O_2\) sensor to be distinguished from another, high affinity \(O_2\) sensor, which governs the activity of NKCC, described here for the first time in fish RBCs. Similar to NKCC in mammalian and avian RBCs, it is activated by low \(P_{O_2}\), and shows a similar \(O_2\) affinity to that of Hb. These findings are relevant for the understanding
of cellular O$_2$ sensing in general and in particular for the altered O$_2$ dependence of RBC ion transport in certain haemoglobinopathies.

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