INFLUENCE OF HAEMOGLOBIN CONFORMATION, NITRITE AND EICOSANOIDS ON K⁺ TRANSPORT ACROSS THE CARP RED BLOOD CELL MEMBRANE

By FRANK B. JENSEN

Institute of Biology, Odense University, DK-5230 Odense M, Denmark

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Summary

The regulation of K⁺ transport across the red blood cell (RBC) membrane by haemoglobin (Hb) conformation was studied in carp, and the K⁺ transport mechanisms were identified. When a large proportion of Hb in the R quaternary structure was secured by oxygenation of blood at pH 8.14, a net RBC K⁺ efflux was induced, which was accompanied by RBC shrinkage. This K⁺ efflux was resistant to ouabain and inhibited by furosemide and DIDS and by substitution of NO₃⁻ for Cl⁻, showing it to result from a K⁺/Cl⁻ cotransport mechanism. Deoxygenation of the RBCs (Hb in T structure) eliminated the CP-dependent K⁺ efflux and resulted in a net K⁺ uptake via the Na⁺/K⁺ pump. These changes were fully reversible. Nitrite-induced methaemoglobin formation in deoxygenated blood, which converts a large fraction of the T structure Hb into an R-like conformation, shifted the K⁺ uptake to a Cl⁻-dependent K⁺ efflux similar to that seen in oxygenated cells. When the allosteric equilibrium between the R and T structures of Hb was gradually shifted towards the T state by decreases in pH, the Cl⁻-dependent K⁺ efflux from oxygenated cells decreased. At pH 7.52, where the Root effect caused a potent stabilisation of the T state, the K⁺ efflux was reversed to a net K⁺ uptake. A similar change was induced in methaemoglobin-containing deoxygenated blood, since low pH also favours a T-like conformation of metHb. The variable K⁺ fluxes could not be related to changes in membrane potential or pH but were always directly related to the experimental modulation of the relative proportions of R- and T-structure Hb. It is proposed that Hb conformation governs K⁺ movements via a different binding of T and R structures to integral membrane proteins, and that a large fraction of R-structure Hb triggers the Cl⁻-dependent K⁺ efflux mechanism. Application of inhibitors and a substrate of prostaglandin and leukotriene synthesis did not influence the K⁺ efflux from oxygenated erythrocytes. However, a fraction of the K⁺ efflux from nitrite-treated deoxygenated cells was inhibited by nordihydroguaiaretic acid, suggesting that a slightly larger K⁺ efflux from these RBCs than from oxygenated RBCs was related to leukotriene production caused by nitrite entry. A much larger influx of nitrite to deoxygenated than to oxygenated RBCs was positively correlated with the

Key words: K⁺ transport, red blood cells, haemoglobin conformation, oxygenation-dependent ion transport, leukotrienes and prostaglandins.
distribution ratio of $H^+$ and the membrane potential, supporting the view that nitrite primarily enters the cells via conductive transport. The physiological implications of the results are discussed.

Introduction

Recent research has shown that haemoglobin (Hb), in addition to its fundamental role in blood $O_2$, $CO_2$ and $H^+$ transport, may serve important control functions in ion transport across the red blood cell (RBC) membrane. The adrenergic $Na^+/H^+$ exchange in nucleated fish RBCs depends on the degree of oxygenation of Hb, being larger in deoxygenated than in oxygenated RBCs (Motais et al. 1987; Salama and Nikinmaa, 1988; Nikinmaa and Jensen, 1992). $K^+$ fluxes similarly respond to changes in the degrees of oxygenation of Hb: oxygenation induces a net RBC $K^+$ release in carp (Jensen, 1990a) and rainbow trout (Borgese et al. 1991), whereas a net $K^+$ uptake occurs in deoxygenated cells (Jensen, 1990a). The $K^+$ release of oxygenated RBCs can be mimicked by CO binding to the Hb (Borgese et al. 1991) and by nitrite-induced methaemoglobin formation in deoxygenated cells (Jensen, 1990a). These findings suggest that it is Hb conformation rather than oxygenation conditions per se that influences $K^+$ fluxes across the RBC membrane. It was originally suggested that the variable $K^+$ fluxes could be related to the relative proportions of R- and T-structure Hb inside the RBCs, with a 4,4'-dianisostilbene-2,2'-disulphonic acid (DIDS)-sensitive $K^+$ efflux being induced whenever a large fraction of the Hb molecules assumes the R quaternary structure (Jensen, 1990a). The implication of this hypothesis is that changes in the allosteric equilibrium between the R and T structure of Hb should produce predictable changes in $K^+$ movements. The purpose of the present study was to verify and examine in detail this hypothesis and to identify the mechanisms by which $K^+$ permeates the membrane in carp RBCs. Since leukotrienes have recently been shown to stimulate the $K^+$ permeability of mammalian cells during regulatory volume decrease (Lambert et al. 1987; Hoffmann et al. 1988), a further aim was to examine whether eicosanoids (prostaglandins and leukotrienes) are involved in the RBC $K^+$ efflux that is induced by oxygenation and by nitrite-induced methaemoglobinaemia.

Materials and methods

Experimental animals

During the experimental period (March–December, 1991), carp (Cyprinus carpio, mass 1–2 kg, $N=35$) of the same stock were maintained at 15°C under normoxic conditions ($P_{O_2}>18$ kPa=135 mmHg) in 5001 holding tanks supplied with a constant inflow of fresh aerated tap water. The animals were subjected to a 12 h light:12 h dark rhythm and fed with commercial fish food pellets.
**Haemoglobin conformation and RBC K⁺ fluxes**

**Experimental protocol**

**Series I**

This series studied the influence of oxygenation degree, nitrite-induced methaemoglobinaemia and pH on K⁺ movements across the carp red blood cell membrane, as well as the influence of oxygenation degree and pH on nitrite entry into the red cells. Whole blood drawn from individual carp was divided into two identical 5 ml subsamples that were equilibrated at 15°C in an Eschweiler (Kiel, FRG) tonometer system with gas mixtures supplied from cascaded Wösthoff (Bochum, FRG) gas-mixing pumps. The two tonometers received humidified gases with the same CO₂ content, but with different oxygen content. One tonometer received a gas with 30% O₂ to oxygenate the red cells, and the other tonometer received an oxygen-free gas (a mixture of CO₂ and pure analyzed N₂) to deoxygenate the cells. The blood was pre-equilibrated for 35 min to guarantee equilibrium with respect to oxygenation degree and P_CO₂. At the end of this pre-equilibration period the sampling regime was initiated. This was defined as time zero in the experiments. The equilibration was continued for an additional 240 min either in the absence or in the presence of nitrite. Nitrite was added to the blood at time zero to produce a nominal plasma nitrite concentration of approximately 3 mmol l⁻¹. Nitrite was added as microlitre samples from a 140 mmol l⁻¹ NaNO₂ stock solution, which has an osmolality similar to that of carp plasma. Blood samples were removed from each tonometer every 10 min and immediately centrifuged to obtain plasma for later potassium measurements. At 20 min intervals, further blood samples were taken for measurements of plasma nitrite, blood methaemoglobin content and haematocrit. Blood pH and total haemoglobin concentration were measured at the beginning (time zero) and end (time 240 min) of the individual experiments. Experiments on oxygenated and deoxygenated blood in the absence and presence of nitrite were conducted at three CO₂ levels: 0.4% CO₂ (P_CO₂=0.4 kPa=3 mmHg), 1.2% CO₂ (P_CO₂=1.2 kPa=9 mmHg) and 3% CO₂ (P_CO₂=2.99 kPa=22.4 mmHg). The oxygen saturation of the haemoglobin in oxygenated blood at variable P_CO₂/pH (i.e. the Root effect) was evaluated in separate experiments from measurements of O₂ content, pH, total [Hb] and metHb content in blood equilibrated to 30% O₂ at variable P_CO₂ (0.1<P_CO₂<5 kPa).

**Series II**

To study the reversibility of oxygenation-dependent K⁺ movements and the effect of ouabain, whole blood was deoxygenated in two tonometers with a 0.4% CO₂/99.6% N₂ gas mixture. At time zero, ouabain (Sigma Chemical Co.) was added to one of the tonometers to a final concentration of 2 mmol l⁻¹. The other tonometer received a 'sham' injection of physiological saline. Sampling of blood was then initiated. After 100 min, the gas mixture supplying the two tonometers was shifted to 0.4% CO₂/30% O₂/69.6% N₂ to oxygenate the red cells. After a further 100 min, the gas mixture was returned to 0.4% CO₂/99.6% N₂ for an
additional 100 min to re-deoxygenate the cells. Blood samples were removed from both tonometers at 10 min intervals and analyzed for plasma [K⁺].

Series III

The effect of furosemide on the potassium efflux from oxygenated red cells, and from nitrite-treated (3 mmol l⁻¹ nominal plasma nitrite concentration) deoxygenated cells, was studied by comparing the change in plasma K⁺ concentration in blood equilibrated at 0.4% CO₂ in the absence and presence of furosemide. Furosemide (Aldrich Chemical Co.) was added to a final concentration of 2 mmol l⁻¹ at time zero. Blood samples were taken after a 30 s mixing period and after an additional 180 min of incubation. Plasma [K⁺], haematocrit and plasma nitrite concentration were measured.

Series IV

This series studied the influence of substitution of nitrate for chloride on potassium fluxes and red cell volume. Freshly drawn blood was divided into two subsamples. After centrifugation and removal of plasma, the cells were washed twice either in a chloride-containing physiological saline or in a saline in which NO₃⁻ had been substituted for Cl⁻. The physiological saline was based on natural plasma ion levels (Jensen, 1990a) and had the following composition: 118 mmol l⁻¹ NaCl, 2.8 mmol l⁻¹ KH₂PO₄, 1 mmol l⁻¹ MgSO₄·7H₂O, 10.7 mmol l⁻¹ NaHCO₃, 2 mmol l⁻¹ CaCl₂·2H₂O, 3.9 mmol l⁻¹ glucose. The nitrate saline had the same composition except that NaNO₃ (118 mmol l⁻¹) replaced NaCl and Ca(NO₃)₂·4H₂O (2 mmol l⁻¹) replaced CaCl₂·2H₂O. The washed cells were left overnight at 5°C to achieve equilibrium with their respective salines. The cells were then centrifuged and resuspended in fresh saline to a natural haematocrit of approximately 24% and transferred to the tonometer system for the 35 min pre-equilibration. Four experimental tonometers were run in parallel: oxygenated red cells in (i) Cl⁻ saline and (ii) NO₃⁻ saline, and nitrite-treated (time zero) deoxygenated red cells in (iii) Cl⁻ and (iv) NO₃⁻ salines. The equilibration gases all contained 0.4% CO₂. Blood samples were removed from each tonometer at 0, 30, 60, 120, 180 and 240 min for measurements of plasma K⁺, NO₂⁻, haematocrit and methaemoglobin content. Blood pH was measured at 0 and 240 min.

Series V

The possible involvement of eicosanoids in potassium movements across the red cell membrane was studied by application of inhibitors and a substrate of eicosanoid synthesis. In each experiment, blood from individual carp was divided among five tonometers receiving the same gas supply (0.4% CO₂/30% O₂/69.6% N₂ in experiments on oxygenated blood, and 0.4% CO₂/99.6% N₂ in experiments on deoxygenated blood). Microlitre samples of chemicals were then added from stock solutions that were freshly prepared with dimethylsulphoxide as solvent. The additions to the five tonometers were: tonometer 1 (control), solvent only; tonometer 2, nordihydroguaiaretic acid (NDGA, Sigma Chemical Co.) to a final
concentration of 500 μmol l⁻¹; tonometer 3, arachidonic acid (AA, Sigma Chemical Co.) to a final concentration of 80 μmol l⁻¹; tonometer 4, indomethacin (Sigma Chemical Co.) to a final concentration of 200 μmol l⁻¹; tonometer 5, arachidonic acid plus nordihydroguaiaretic acid to final concentrations of 80 μmol l⁻¹ and 500 μmol l⁻¹, respectively. The substrate and inhibitors were administered at concentration/cell-number levels found to be effective on eicosanoid production in various cell types. Following the 35 min pre-equilibration, a blood sample was withdrawn (time zero). In experiments on deoxygenated blood, nitrite was added to a concentration of 3 mmol l⁻¹ 0.5 min prior to this initial sampling. A final blood sample was taken from each tonometer at 180 min.

Measurements

Plasma potassium was measured by atomic absorption spectrophotometry (Perkin-Elmer 2380). Plasma nitrite was measured spectrophotometrically at 540 nm after reaction of the sample nitrite with sulphanilamide to yield a diazo compound which couples with N-1-naphthyl-ethylendiamide-dihydrochloride to give a red azo dye. For methaemoglobin determinations, blood was lysed in a weak phosphate buffer at pH 7.3. After centrifugation, the absorbance of the supernatant was measured at 560 nm, 576 nm and 630 nm using a Milton Roy Spectronic 1201 spectrophotometer. The methaemoglobin content was calculated using the equations of Benesch et al. (1973). The total haemoglobin concentration of the blood was assessed following conversion of all haemoglobin to cyanmethaemoglobin, using a millimolar extinction coefficient of 11 at 540 nm. Haematocrit was determined by centrifugation (2 min at 12,000 revs min⁻¹) in glass capillaries. Blood pH was measured with a Radiometer (Copenhagen, Denmark) BMS3 electrode system thermostatted at 15 °C and connected to a PHM73 monitor and REC80 recorder. The distribution ratio of H⁺ across the red cell membrane (rH = [H⁺]c/[H⁺]i) was calculated as 10^(pHi - pHc), using the measured extracellular pH (pHe) and the corresponding red cell pH (pHi) given by the relationships between pHi and pHe in oxygenated and deoxygenated carp blood (Albers et al. 1983). The oxygen content (C_{O_2}) of blood equilibrated to 30 % O₂ at variable P_{CO_2}, was measured by the method of Tucker (1967), and the oxygen saturation of the functional haemoglobin was calculated from the equation (Jensen et al. 1987):

\[
%\text{oxyHb} = \frac{100(C_{O_2} - aO_2 \times P_{O_2})}{(C_{O_2}^{Hb} - C_{O_2}^{Hb} \times f_{metHb})},
\]

where \(aO_2\) is the solubility coefficient of O₂ (Boutilier et al. 1984), \(C_{O_2}^{Hb}\) is the O₂ capacity calculated from the total Hb concentration and \(f_{metHb}\) is the fractional metHb content.

Results

Experimental modulation of haemoglobin conformation

Ion movements across the red cell membrane were studied when the confor-
mation of the red cell haemoglobin was modulated by (i) oxygenation and deoxygenation of the blood, (ii) nitrite-induced methaemoglobin formation and (iii) changes in pH. Using the conventional terminology regarding Hb structures (e.g. Perutz, 1983), oxygenation and deoxygenation allowed comparison of blood with the red cell haemoglobin predominantly in the oxygenated R and deoxygenated T structures, respectively. Nitrite-induced methaemoglobin formation in deoxygenated blood allowed examination of the effects of gradually changing the Hb structure from the T conformation towards the R-like conformation of methaemoglobin. Finally, by changing pH, the allostery equilibrium between the R and T structures could be gradually shifted. Lowering of pH stabilises the T structure. This effect is especially large in Root-effect fish haemoglobins. As pH is lowered, not only oxygen affinity (the Bohr effect) but also cooperativity is lowered, and the T state of Hb is extended to very high O₂ saturations (cf. Perutz, 1983; Brittain, 1987; Jensen, 1991). In whole blood, the Root effect is seen as a decrease in the oxygen saturation of the Hb when pH is lowered in spite of a high equilibration $P_{O_2}$ (Fig. 1). The three CO₂ levels used in the main experiments produced pH values of 8.14 (at 0.4 % CO₂), 7.84 (at 1.2 % CO₂) and 7.52 (at 3.0 % CO₂) in blood equilibrated with a $P_{O_2}$ of 30 kPa (225 mmHg). The designation 'oxy' used in the figures therefore means 100 % O₂ saturation at 0.4 % CO₂, 92 % O₂ saturation at 1.2 % CO₂ and 83 % O₂ saturation at 3.0 % CO₂ (as inferred from Fig. 1).

Fig. 1. Oxygen saturation of carp blood at $P_{O_2}$ 30 kPa as function of pH. Arrows highlight the mean pH values of blood at the three CO₂ equilibration levels used in the main experiments.
Red cell nitrite entry and methaemoglobin formation

The entry of nitrite into carp red cells depended on oxygenation degree and $P_{CO_2}$ (Fig. 2). Following addition of nitrite under physiological $P_{CO_2}$ (0.4 kPa) and pH conditions, the plasma nitrite concentration remained almost unchanged in oxygenated blood, whereas it decreased by about 70% in deoxygenated blood during the 240 min experimental period (Fig. 2A). Thus, nitrite did not enter oxygenated red cells to any significant extent, whereas it rapidly entered deoxygenated cells.

When pH was lowered by elevating $P_{CO_2}$, nitrite started to enter oxygenated cells. The influx increased gradually with increments in $P_{CO_2}$ (Fig. 2) but the RBC nitrite influx was always lower in ‘oxy’ than in ‘deoxy’ blood.

The decrease in plasma nitrite with time was satisfactorily described by non-linear curve fits of the data to a biexponential equation (Fig. 2) from which the initial RBC nitrite influx ($J_{in}$ when $t\to0$) was calculated (Fig. 3). The initial nitrite influx was positively correlated with the elevation of the distribution ratio of H+ across the red cell membrane ($r_{H^+}$) that resulted from pH decrease and deoxygenation (Fig. 3).

In the absence of nitrite there was no methaemoglobin formation in either oxygenated or deoxygenated blood (Fig. 4). In the presence of nitrite, the degree of methaemoglobin formation paralleled the velocity of nitrite entry into the red cells. In deoxygenated blood, the rapid nitrite entry elevated the metHb level to 40–50% of the total Hb within 60–90 min, whereafter the metHb content remained practically constant up to 230 min (Fig. 4). This suggests that a balance was established between nitrite-induced oxidation of haem groups and reduction via the metHb reductase system. In oxygenated blood, metHb formation gradually increased when $P_{CO_2}$ was elevated (Fig. 4) in parallel with the increase in nitrite influx (Fig. 2). The metHb levels increased from below 2% to about 6%, 10% and 27% following 230 min of nitrite treatment at $CO_2$ levels of 0.4%, 1.2% and 3.0%, respectively (Fig. 4A).

Potassium movements across the red cell membrane

At physiological pH, the plasma $K^+$ concentration increased with time in oxygenated blood, whereas it decreased in deoxygenated blood (Fig. 5). Thus, oxygenation per se induced a net potassium release from the red cells, whereas deoxygenation caused a net potassium uptake. When pH was lowered from 8.14 to 7.84 in oxygenated blood, the rise in plasma $[K^+]$ with time was strongly reduced, and at pH 7.52 the potassium concentration decreased with time (Fig. 5A). This demonstrated that the net potassium release from oxygenated red cells was reduced by lowering pH, and that it even shifted to a net potassium uptake at pH 7.52. In deoxygenated blood, plasma $[K^+]$ decreased with time at all three pH levels tested (Fig. 5B).

The presence of nitrite (Fig. 5) did not change the time course and pH-dependence of plasma $[K^+]$ changes in oxygenated blood to any appreciable
Fig. 2. Time-dependent changes in plasma nitrite concentration in oxygenated and deoxygenated carp blood at 0.4% CO₂ (A), 1.2% CO₂ (B) and 3.0% CO₂ (C). Nitrite was added to the blood at time zero. Means ± s.e.m. are shown for three individual carp in each panel. Haematocrit (range at time zero) 20–25%. The curves represent non-linear curve fits of the data to the biexponential equation: plasma [NO₂⁻] = Pe⁻αt + Qe⁻βt.
Haemoglobin conformation and RBC K⁺ fluxes

Fig. 3. Correlation between the initial rate of RBC nitrite influx and the distribution ratio of H⁺ across the RBC membrane (rH). The initial nitrite influx was calculated from the curve fits shown in Fig. 2 and the fractional haematocrit (F_HCT) according to:

\[ J_{i} = \lim_{\varepsilon \to 0} (-d[N_{2}O^{-}] / dt) \times (1 - F_{HCT})/F_{HCT} = (P_{a} + Q_{b})(1 - F_{HCT})/F_{HCT}. \]

extent (compare open and closed symbols in the left-hand panels of Fig. 5). In deoxygenated blood, however, adding nitrite had a profound effect on changes in [K⁺]. At pH 8.14, the net red cell K⁺ uptake seen in the absence of nitrite was shifted to a considerable net K⁺ release (increase in plasma [K⁺]) after some 60 min of nitrite treatment (Fig. 5B). The onset of this K⁺ release coincided with the development of a high and stable metHb content in the blood (cf. Fig. 4). Plasma [K⁺] rose to values similar to those observed in oxygenated blood after 240 min, despite the later onset of the K⁺ release in nitrite-treated deoxygenated blood.

At pH 7.84, nitrite-treated deoxygenated red cells also released potassium after some 60–90 min, when high metHb levels had been attained. A clear difference compared to deoxygenated blood in the absence of nitrite therefore persisted (Fig. 5B). The increase in plasma [K⁺] with time (i.e. the rate of potassium release) was, however, smaller than at pH 8.14. This trend was accentuated by lowering pH further to 7.52. At this pH, nitrite-induced methaemoglobinemia was not associated with potassium release from the deoxygenated RBCs (Fig. 5B).

The net K⁺ flux across the red cell membrane (in mmol⁻¹ RBC min⁻¹) is given by:

\[ J_{K_{net}} = - (d[K_{+}]_{plasma} / dt) \times (1 - F_{HCT})/F_{HCT}. \]

In oxygenated and deoxygenated blood the values were calculated for the time interval 0<t<100 min, in which [K⁺] changes were largest and linear with time (Fig. 5), and in methaemoglobin-containing deoxygenated blood (met-deoxy) the fluxes were calculated for the period 90<t<150 min (i.e. the period where the
Fig. 4. Methaemoglobin content as function of time in oxygenated (A) and deoxygenated (B) blood in the absence (O) and presence (●) of nitrite at three different PCO2/pH levels. Means±s.e.m. are shown for three individual carp in each panel.

nitrite-induced K⁺ release was largest and linear with time). The values (Fig. 6A) illustrate the critical dependence of the net K⁺ flux upon oxygenation degree, pH/PCO2, and nitrite-induced methaemoglobin formation in deoxygenated blood. For each type of red cell Hb conformation, the flux values increased with elevation of PCO2 (decrease in pH). In oxygenated red cells a gradual shift from negative (efflux) to positive (influx) values occurred with elevation of PCO2. The same trend
was seen in met-deoxy cells, but the efflux rate was larger than in oxy cells at 0.4% and 1.2% CO₂ and the influx at 3% CO₂ was lower (Fig. 6). In deoxy RBCs, the magnitude of the initial net influx gradually increased with elevation of PCO₂ (Fig. 6). In nitrite-treated oxygenated cells (not shown in Fig. 6) the net K⁺ fluxes were similar to those in oxygenated cells.

The net K⁺ flux was linearly correlated with the distribution ratio of hydrogen
ions across the red cell membrane in both oxygenated and deoxygenated blood, but the regression lines were different (Fig. 6B). Values of \( r_H \) for methaemoglobin-containing blood could be inferred at 0.4% CO\(_2\) from the data of Jensen (1990a). The met-deoxy point lies closer to the extension of the ‘oxy’ line than to that of the ‘deoxy’ line (Fig. 6B).

**Reversibility of K\(^+\) movements and effects of ouabain**

The influence on RBC K\(^+\) fluxes of varying the haemoglobin oxygenation degree was fully reversible. When the equilibration gas was shifted from 0.4% CO\(_2\)/99.6% N\(_2\) (deoxygenated blood) to 0.4% CO\(_2\)/30% O\(_2\)/69.6% N\(_2\) (to oxygenate the blood) the K\(^+\) uptake of deoxygenated RBCs shifted to the net oxy-RBC K\(^+\) efflux (Fig. 7), and when the equilibration gas was returned to 0.4% CO\(_2\)/99.6% N\(_2\) the K\(^+\) influx to deoxygenated RBCs was re-established (after a short time lag, which is related to the longer time required to deoxygenate than to oxygenate blood in tonometers).

Addition of the Na\(^+\)/K\(^+\)-ATPase inhibitor ouabain completely abolished the net K\(^+\) uptake of deoxygenated RBCs (Fig. 7). Only a small residual ouabain-resistant K\(^+\) efflux was present in deoxygenated cells, as shown by a limited increase in plasma [K\(^+\)] with time. Upon oxygenation, the K\(^+\) efflux from ouabain-treated cells increased rapidly, and the rise in plasma [K\(^+\)] was slightly larger than in untreated blood due to the absence of counteracting K\(^+\) uptake via the Na\(^+\)/K\(^+\) pump (Fig. 7). Re-deoxygenation of ouabain-treated RBCs strongly reduced the RBC K\(^+\) efflux.
Haemoglobin conformation and RBC K⁺ fluxes

Fig. 7. Effects of changes in oxygenation conditions on time-dependent changes in plasma K⁺ concentration in the absence (○) and presence (●) of ouabain (2 mmol l⁻¹). The blood was in a deoxygenated state at time zero. At 100 min and 200 min the equilibration gas was changed in order to oxygenate and then re-deoxygenate the blood (indicated by arrows). Haematocrit at time zero 19.2%; P<sub>CO₂</sub>=0.4 kPa. See text for further details.

Inhibition of the RBC K⁺ release by furosemide

The rise in plasma [K⁺] with time in oxygenated blood and in nitrite-treated deoxygenated blood at a P<sub>CO₂</sub> of 0.4 kPa was effectively reduced by addition of furosemide (Fig. 8). The red cell K⁺ release was, however, not totally inhibited by furosemide as it is by DIDS (Jensen, 1990a).

Effects of substitution of nitrate for chloride on RBC K⁺ release and volume

Net K⁺ movements across the RBC membrane of washed red cells in equilibrium with a chloride-containing physiological saline were the same as those observed in whole blood. When equilibrated at a P<sub>CO₂</sub> of 0.4 kPa in the oxygenated state, the extracellular [K⁺] rose to the same extent as it did in whole blood. Similarly, nitrite-treated deoxygenated cells first took up K⁺, and after 60 min this reversed to a net K⁺ release (rise in extracellular [K⁺]) (Fig. 9A). The net efflux of K⁺ from both oxygenated cells and nitrite-treated deoxygenated cells was accompanied by red cell shrinkage, as illustrated by the gradual decrease in haematocrit (Fig. 9B) that occurred in parallel with the rise in extracellular [K⁺].

Substitution of nitrate for chloride completely abolished the net K⁺ release from both oxygenated cells and nitrite-treated deoxygenated cells, as shown by the constant extracellular [K⁺] with time (Fig. 9A). This demonstrated a clear
chloride dependence of the K⁺ efflux mechanism. In the absence of the RBC K⁺ release, the red cell shrinkage did not occur (Fig. 9B).

Whereas substitution of nitrate for chloride abolished both RBC K⁺ efflux and RBC shrinkage, it had no effect on either nitrite entry or methaemoglobin formation in deoxygenated cells (Fig. 9C,D). Both nitrite entry and methaemoglobin formation were similar to those observed in whole blood at a $P_{\text{CO}_2}$ of 0.4 kPa.

**Effects of inhibitors and a substrate of eicosanoid production**

The possible involvement of eicosanoids in the control mechanisms underlying the K⁺ release from oxygenated and nitrite-treated deoxygenated red cells was examined by adding inhibitors or a substrate of eicosanoid production. Addition of NDGA (an inhibitor of leukotriene formation), arachidonic acid (AA, a substrate for prostaglandin and leukotriene formation), indomethacine (an inhibitor of prostaglandin synthetase) or AA and NDGA in combination did not change the K⁺ release from oxygenated red cells significantly compared to controls (Fig. 10A). In nitrite-treated deoxygenated RBCs, arachidonic acid, indomethacine and AA+NDGA again did not alter the K⁺ efflux. Administration of NDGA alone, however, reduced the K⁺ release significantly ($P<0.05$, Student’s $t$-test) compared to control cells (Fig. 10B), suggesting that part of the K⁺ release in nitrite-treated deoxygenated red cells was mediated via leukotrien production.

**Discussion**

The present study demonstrates that haemoglobin conformation has a profound
influence on potassium movements across the carp RBC membrane and identifies the underlying mechanisms of K⁺ permeation. The Hb conformation was varied by oxygenation (R conformation at natural pH) and deoxygenation (T conformation), by pH changes (gradually shifting the R–T allosteric equilibrium) and by nitrite-induced formation of methaemoglobin (R-like conformation at natural pH). Since the nitrite influx to carp RBCs is itself dependent on Hb conformation, this topic will be dealt with first.

**Oxygenation dependence of nitrite entry and methHb formation**

At physiological $P_{CO_2}$ and pH, nitrite rapidly entered deoxygenated but not oxygenated red cells (Fig. 2; Jensen, 1990a). When pH was lowered, decreasing the oxygen saturation of oxygenated blood via the Root effect (Fig. 1), nitrite began to enter oxygenated cells but at rates lower than in deoxygenated RBCs (Fig. 2). Thus, oxygenation degree and pH are critical determinants of the amount of NO₂⁻ entering the cells. Nitrite does not enter the RBC via the band 3 anion exchange process.
Fig. 10. Change in plasma K\(^+\) concentration during a 180 min equilibration of oxygenated (A) and nitrite-treated deoxygenated (B) blood in the absence (control, C) and presence of nordihydroguaiaretic acid (NDGA), arachidonic acid (AA), indomethacin (Indo) and arachidonic acid+nordihydroguaiaretic acid (AA+NDGA). Means±s.e.m. [N=4 (A) and 3 (B)]. Haematocrit at time zero 22.5±2.5% (mean±s.d. for four carp used in A) and 23.6±2.9% (mean±s.d. for three carp used in B). See text for further details. *Significantly different from the control value, P<0.05.

exchanger to any appreciable extent. This conclusion is based on the fact that DIDS at 10\(^{-4}\)moll\(^{-1}\) does not affect the influx rate (Jensen, 1990a). Also, the initial plasma NO\(_2^-\) concentration (3mmol\(^{-1}\)) is much lower than the plasma concentrations of Cl\(^-\) and HCO\(_3^-\) (118mmol\(^{-1}\) and 12mmol\(^{-1}\), respectively; Jensen et al. 1987; Jensen, 1990a), and therefore competition for the external anion exchange sites does not favour nitrite entry via this route. Nitrite probably enters the RBC via conductive transport (Jensen, 1990a). As nitrite enters the cell it is consumed in the reaction with Hb to form methaemoglobin (Kosaka and Tyuma, 1987), maintaining an inward diffusion gradient for an extended period. Mathematical modelling of the nitrite entry, using the Nernst–Planck expression for a negatively charged monovalent ion and taking into account the removal of nitrite intracellularly (H. Malte and F. B. Jensen, unpublished), suggests that the decrease in extracellular [NO\(_2^-\)] should follow a biexponential decay, as indeed was the case (Fig. 2). With conductive transport as the main route of entry, the influx should increase with elevations in the membrane potential, since a less negative membrane potential reduces the force opposing nitrite entry. This also suggests that the increased nitrite influx upon deoxygenation or pH decrease is mediated via a reduced non-permeable negative charge on the Hb which elevates the distribution ratio of permeable ions (Cl\(^-\), HCO\(_3^-\), H\(^+\)) and the membrane potential. The correlation between the initial rate of influx and r\(_H\) (Fig. 3) seems to support this view. The notable difference between oxygenated and deoxygenated RBCs would then be a consequence of the large Haldane effect in carp Hb (Jensen, 1989). However, the complete absence of NO\(_2^-\) entry in oxygenated
Haemoglobin conformation and RBC K⁺ fluxes

blood at pH 8.14 (Fig. 2) shows that the permeability must also change with the oxygenation degree. In fully oxygenated blood, nitrite is essentially impermeable, whereas the permeability is high in deoxygenated blood.

The metHb formation (Fig. 4) paralleled the degree of nitrite entry to the red cells. In deoxygenated blood, a stable level of 40–50% metHb was developed within 60–90 min, which provided a method for changing the haemoglobin conformation in deoxygenated RBCs from the T structure towards a high proportion of the R-like metHb structure.

Mechanisms of K⁺ permeation of the RBC membrane

The net K⁺ flux across the RBC membrane is the sum of influx and efflux components via different mechanisms. The net influx of K⁺ to deoxygenated RBCs was via the Na⁺/K⁺ pump, as shown by its complete inhibition by ouabain (Fig. 7). The ouabain-resistant K⁺ efflux from deoxygenated cells was small. When deoxygenated RBCs were oxygenated, however, the ouabain-resistant K⁺ efflux increased drastically (Fig. 7). This ouabain-resistant K⁺ efflux effectively dominated K⁺ uptake via the pump and caused the net K⁺ efflux from oxygenated cells at physiological pH.

Both the K⁺ efflux from oxygenated RBCs and that from nitrite-treated deoxygenated cells were inhibited by furosemide (Fig. 8), by substitution of nitrate for chloride (Fig. 9) and by DIDS (Jensen, 1990a), pointing to the same mechanism in both cases. The loop diuretic furosemide is known to inhibit Cl⁻-mediated K⁺ transport (Lauf, 1985). The complete inhibition when nitrate is substituted for chloride confirms that the K⁺ efflux mechanism is Cl⁻-dependent and shows that the mechanism is via an electroneutral K⁺/Cl⁻ cotransport mechanism and not via conductive, separate K⁺ and Cl⁻ channels (Lauf, 1985; Hoffmann and Simonsen, 1989). The complete inhibition of the K⁺ loss by substitution of NO₃⁻ for Cl⁻ argues against K⁺/H⁺ exchange operating in parallel with Cl⁻/HCO₃⁻ exchange, since nitrate should be readily transported by the anion exchanger of fish erythrocytes (Borgese et al. 1987). Also, if coupled K⁺/H⁺ and Cl⁻/HCO₃⁻ exchanges were involved, then DIDS treatment should be expected to produce an RBC K⁺ efflux and extracellular alkalization. DIDS, however, blocks the K⁺ efflux, and extracellular pH stays practically constant (Jensen, 1990a).

The Cl⁻-dependent K⁺ efflux induced by oxygenation and nitrite-induced methaemoglobinemia was accompanied by RBC shrinkage (Fig. 9). Fish RBCs, like other cell types, possess volume regulatory mechanisms. Swelling of the cells in hypotonic media triggers a regulatory volume decrease (RVD), whereby the cells return to their original volume. The RVD mechanism of fish RBCs involves activation of a Cl⁻-dependent K⁺ transport that is inhibited by furosemide, by DIDS (or SITS) and by substitution of nitrate for chloride (Lauf, 1982; Bourne and Cossins, 1984; Borgese et al. 1987). The pharmacological characteristics of the RVD KCl loss accordingly are identical to those of the Cl⁻-dependent K⁺ release examined here. This leads to the hypothesis that oxygenation and nitrite-
induced methaemoglobinaemia trigger the RVD Cl⁻-dependent K⁺ release and cell shrinkage in the absence of the normal triggering mechanism (i.e. cell volume increase).

Modulation of net K⁺ fluxes by changes in Hb conformation

The variable K⁺ fluxes under the different experimental conditions can all be related to concurrent changes in the allosteric equilibrium between the R and T structures of haemoglobin, supporting the idea that the conformation of haemoglobin inside the red cells governs potassium movements across the carp RBC membrane. At physiological pH (Fig. 5, top panels), oxygenated red cells (Hb in R conformation) lose K⁺, whereas deoxygenated (Hb in T conformation) RBCs take up K⁺. Furthermore, nitrite-induced formation of metHb shifts the K⁺ uptake of deoxy cells to a net K⁺ release when high metHb levels (assuming an R-like conformation) are developed. When, by decreasing pH in oxygenated blood, the conformation was gradually changed from a predominantly R structure (high pH) towards a higher proportion of the T structure (lower pH), the net K⁺ efflux from oxygenated cells decreased. At the lowest pH, where the Root effect caused a very potent stabilization of the T structure, the flux even reversed to a net K⁺ uptake. The same effect was seen in deoxygenated cells with elevated metHb content, and the reason appears to be the same. Solutions of methaemoglobin (like ferrous Hb) contain two major allosteric forms in equilibrium. Under natural conditions the R structure is dominant, but conditions known to stabilize the T structure of ferrous Hb (i.e. low pH and addition of organic phosphates) also favour a T-like structure of metHb (Perutz et al. 1974; Marden et al. 1991). The very potent stabilisation of the T structure of fish Hbs at low pH therefore obliterates the transition from the deoxy T structure to the metHb R structure. In oxygenated blood, methaemoglobin formation (Fig. 4A) did not alter K⁺ movements (Fig. 5A), supporting the view that oxyHb and metHb had similar structures under the different conditions.

To conclude firmly that Hb conformation exerts a direct influence on the K⁺ movements it is necessary to consider whether changes in membrane potential and pH, which were inevitably associated with the experimental modulation of the R–T allosteric equilibrium, can explain the K⁺ fluxes. In red cells the membrane potential (V_m) can be estimated from the distribution ratio of permeable ions (Nikinmaa, 1990). The rise in r_H associated with pH decrease and deoxygenation is paralleled by an elevated (less negative) V_m (V_m=RT/zF ln r_H), which increases the potassium current [I_K=G_K(V_m-E_K)] and thereby the driving force for K⁺ efflux. However, the net K⁺ flux did not follow the same relationship with r_H in oxygenated and deoxygenated blood, and an increase in r_H was not associated with an increase in K⁺ efflux, but with the opposite: a decreased K⁺ efflux that even shifted to a net K⁺ influx (Fig. 6). The change in V_m therefore does not explain the K⁺ fluxes. This conclusion is supported by the fact that the K⁺/Cl⁻ cotransport mechanism underlying the K⁺ efflux (see above) is an electrically silent mechanism that is not influenced by the membrane potential (Hoffmann and Simonsen,
Haemoglobin conformation and RBC $K^+$ fluxes

The variable potassium movements also cannot be related to a simple pH dependence of the transport mechanism. The extracellular pH is approximately the same in oxygenated and deoxygenated blood under the different conditions, and intracellular pH values also overlap (oxy pH at 0.4% and 1.2% CO$_2$ being similar to deoxy pH at 1.2 and 3% CO$_2$, respectively). The same pH in oxy, deoxy and met-deoxy blood was, however, associated with very different $K^+$ fluxes.

The data accordingly show a direct relationship between RBC $K^+$ movements and Hb structure with the following characteristics. (i) A high proportion of Hb molecules with an R quaternary structure triggers a Cl$^-$-dependent RBC $K^+$ efflux that overrides $K^+$ uptake via the Na$^+$/K$^+$ pump and causes a net RBC $K^+$ efflux. (ii) A high proportion of T-state haemoglobin eliminates the Cl$^-$-dependent $K^+$ efflux and leads to a net $K^+$ uptake via the Na$^+$/K$^+$ pump. (iii) A gradual shift in the R–T allosteric equilibrium towards the T state gradually shifts RBC $K^+$ movements from a net efflux to a net influx.

This influence of Hb conformation on $K^+$ fluxes may be a general phenomenon in fish red cells. In rainbow trout RBCs, oxygenation as well as carbon monoxide equilibration induce a KCl loss that, as in carp RBCs, is inhibited by furosemide, DIDS and substitution of NO$_3^-$ for Cl$^-$ (Borgese et al. 1991). Similarly, at physiological $P_{CO_2}$, tench RBCs, like carp RBCs, lose $K^+$ when oxygenated and take up $K^+$ when deoxygenated (F. B. Jensen, unpublished observations). The rapidity of the oxygenation-induced $K^+$ loss, however, varies among species. It is very rapid in trout, being fully developed within 1 h, whereas it takes about 3 h to develop fully in carp (Fig. 5, where a 35 min pre-equilibration period precedes time zero).

The mechanism by which Hb conformation exerts its influence upon $K^+$ movements across the RBC membrane may rely on a conformation-dependent binding of the Hb molecule to integral membrane transport proteins. Haemoglobin binds to the anion exchanger (band 3) with a high affinity, but also to other membrane sites (reviewed by Salhany, 1990). The binding to band 3 is electrostatic in nature and involves the cytoplasmic fragment of band 3 and the organic phosphate binding site of deoxyhaemoglobin. DeoxyHb accordingly binds more tightly than oxyHb (Chétrie and Cassoly, 1985; Salhany, 1990). Since the spectrin-based membrane skeleton is also directly linked to band 3 (via ankyrin, which associates with both spectrin and the cytoplasmic domain of band 3; Bennett, 1990), it is possible that the membrane protein conformational change induced by Hb binding and release (Salhany, 1990) will both affect the transport functions of band 3 and be transmitted via the membrane skeleton to other membrane sites. Thus, whether the $K^+$ permeation involves band 3 (as may be suggested by DIDS inhibition; Jensen, 1990a) or not, the differential binding of R- and T-type Hb to the membrane may affect transport proteins involved in $K^+$ permeation. Perhaps the reduced binding of R-type Hb to band 3 (or binding to another integral protein) causes a local mechanical membrane stretch that somehow activates the Cl$^-$-dependent $K^+$ efflux. This could link the $K^+/Cl^-$ cotransport induced by oxygenation to that induced by cell swelling (which stretches the membrane).
The number of RBC Hb molecules greatly exceeds the number of membrane binding sites. With a mean cellular Hb content of \(0.83 \times 10^{-15}\) mol (Jensen, 1990a), the number of tetrameric Hb molecules in an average carp red blood cell is \(5 \times 10^8\), whereas the number of band 3 molecules (the major integral membrane protein) may be about \(10^6\) (as in human RBCs). Thus, only a fraction of the Hb molecules associate with the membrane and only a fraction of the Hb needs to change conformation in order potentially to affect \(K^+\) movements.

Hb conformation influences not only \(K^+\) movements but also \(\beta\)-adrenergic \(Na^+\)/\(H^+\) exchange across the fish RBC membrane (Motais et al. 1987; Salama and Nikinmaa, 1988; Nikinmaa and Jensen, 1992). Thus, the important role of haemoglobin in blood \(O_2\), \(CO_2\) and \(H^+\) transport appears to be linked to a role in ion regulation. Whereas the improved capacity for \(\beta\)-adrenergic \(H^+\) extrusion from the RBC when Hb \(O_2\)-saturation and pH decrease protects red cell pH and Hb \(O_2\)-affinity in stress situations (e.g. Nikinmaa, 1990), the physiological role of Hb-conformation-dependent \(K^+\) fluxes may be to buffer plasma \(K^+\) changes in stress situations (Nielsen and Lykkeboe, 1990). Stress in fish \textit{in vivo} is typically associated with a decrease in blood pH and release of \(K^+\) from muscles. Under these conditions (low pH and Hb \(O_2\)-saturation), the red cell will take up some of the \(K^+\) released from the muscles and thereby limit the increase in plasma \(K^+\). Under resting \textit{in vivo} conditions, \(K^+\) may be released from RBCs in arteries and regained in the veins (Jensen, 1990a). The efflux rate from oxygenated RBCs is greater than the influx rate to deoxygenated RBCs (Fig. 6), but this difference could be compensated for by the longer residence time in veins than in arteries, resulting in small fluctuations around a fixed value. Such fluctuations would be larger in rainbow trout than in carp given the larger efflux rate from trout than from carp RBCs (see above). In this respect it is interesting to note that a higher dorsal aortic (arterial blood) than ventral aortic (venous blood) plasma [\(K^+\)] was observed in rainbow trout under resting conditions and that this gradient disappeared in exercise stress (Nikinmaa and Jensen, 1986).

**Involvement of eicosanoids**

Arachidonic acid, which can be mobilized in cells by various stimuli, is enzymatically converted into prostaglandins, leukotrienes and other eicosanoids, which are local hormones with a potent biological function in most tissues. Specifically, it has been shown that prostaglandins and leukotrienes are involved in cell volume regulation of mammalian cells \textit{via} an influence on the permeability of \(Na^+, K^+\) and \(Cl^-\) (Lambert et al. 1987; Hoffmann et al. 1988). The physiological role of eicosanoids in lower vertebrates is not as well known, but various fish tissues (including the RBCs) synthesize eicosanoids, suggesting that these compounds have important control functions (e.g. Mustafa and Srivastava, 1989; Mustafa and Jensen, 1992). Since prostaglandins and leukotrienes regulate ion transport across cell membranes in mammalian cells (Lambert et al. 1987; Hoffmann et al. 1988) it was pertinent to examine whether eicosanoids influenced the \(K^+\) movements examined here.
In oxygenated blood, neither addition of arachidonic acid nor inhibition of leukotriene production (NDGA) or prostaglandin production (indomethacine) affected the net $K^+$ efflux from the RBCs (Fig. 10). This suggests that eicosanoids are not involved in the regulation of the $Cl^-$-dependent $K^+$ efflux mechanism induced by oxygenation. In Ehrlich ascites tumour cells the net KCl loss during regulatory volume decrease is inhibited by NDGA and cell swelling reduces prostaglandin synthesis and increases leukotriene synthesis, the latter accelerating the RVD mechanism through a stimulation of the $K^+$ permeability (Lambert et al. 1987; Hoffmann et al. 1988). The net loss of KCl during RVD in Ehrlich cells is, however, predominantly via separate, conductive $K^+$ and $Cl^-$ transport pathways (Hoffmann et al. 1988) and not via electroneutral $K^+/Cl^-$ cotransport. This difference may underlie the absence of an effect of the inhibitors and substrate of eicosanoid production used in the present case.

Whereas NDGA did not influence the $K^+$ efflux from oxygenated cells it reduced the $K^+$ efflux from nitrite-treated deoxygenated cells (Fig. 10). Furthermore, whereas the $K^+$ efflux rate from nitrite-treated deoxygenated cells was normally higher than that from oxygenated cells, NDGA reduced the $K^+$ efflux to a level comparable to that in oxygenated blood (Fig. 10). This suggests that the $K^+$ efflux from nitrite-treated RBCs may be composed of (i) a $K^+$ efflux related to the formation of methaemoglobin with an R-like conformation, and (ii) an additional $K^+$ efflux related to leukotriene formation. The latter implies that arachidonic acid, somehow mobilized by nitrite-treatment, is directed towards leukotriene production rather than prostaglandin production. Prostaglandin synthetase, which is the first enzyme of prostaglandin synthesis, requires a haem group for activity (Roth et al. 1981). It may thus be hypothesised that nitrite, by analogy with its effects on haemoglobin, inactivates this enzyme, directing mobilized AA towards leukotriene synthesis. Such an effect might also cause $K^+$ loss from other tissues and contribute to the depletion of $K^+$ in muscle tissue of nitrite-exposed animals (Jensen, 1990b). Experiments are in progress to test these possibilities in more detail.

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References


370  F. B. JENSEN


Haemoglobin conformation and RBC K⁺ fluxes


