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Regulation of Cl-dependent K transport by oxy-deoxyhemoglobin transitions in trout red cells

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The oxygenation of trout red cells opens a Cl-dependent K pathway inhibited by furosemide, and by inhibitors of the erythrocyte anion exchanger such as DIDS and niflumic acid. The trigger is the deoxy-oxy conformational change of hemoglobin. The binding of carbon monoxide to heme, which induces a similar conformational change, mimics the effect of oxygen. The possible mechanisms enabling molecular oxygen to control the transport protein are discussed. This oxygenation-activated K transport appears to play a regulatory role in the control of the extracellular K concentration.

Introduction

In the course of experiments performed to analyze the nature of the oxygenation dependence of the Na^+/H^+ exchange [1], two types of unexpected observations were made. When hypoxic trout erythrocytes were oxygenated a cell shrinkage occurred which was greater than could be predicted from Gibbs-Donnan considerations, and one hour at least was necessary for the cells to reach the new steady state volume. Moreover, after stabilization of the volume, the K content of the cells was found to be significantly lower than that of hypoxic erythrocytes. It is thus possible that oxygenation of red blood cells promotes a K leak, leading to a cell volume decrease far greater than that expected from the redistribution of anions induced by hemoglobin ionization.

In this paper we show that oxygenation activates a K pathway. We then analyze the characteristics of this K transport system, the mechanisms involved in its activation and its physiological significance.

Materials and Methods

Blood was removed from caudal vein of a trout, the red cells were washed in isotonic saline solution and then incubated overnight at 4°C to ensure that they had reached a steady state with respect to ion and water content before experimental treatment [2]. The pH of all media used was 7.9 (physiological value for the trout) and the experiments were performed at 15°C. The experimental normal saline contained (in mM): 145 NaCl, 5 $CaCl_2$, 1 $MgSO_4$, 4 KCl, 5 glucose, 15 Hepps. In some experiments Cl^- was totally replaced with NO_3^-. Establishment of anoxia was obtained by washing the cells in saline flushed with nitrogen. To oxygenate anoxic cells at time zero, the suspension was placed in a tonometer under an oxygen atmosphere, and kept there during the one hour sampling period.

The techniques used for measuring cell volume and intracellular ionic concentrations have been previously described [2].

Results

As illustrated in Fig. 1A, oxygenation of erythrocytes induced a cell volume decrease involving two phases: the rate of water loss was initially very fast (45% of the total volume decrease occurred in the first 5 min); water loss then continued but very slowly until the volume reached a new steady state value after 1.5 h. The experiment shown inFig. 1 was performed in the presence of ouabain (10^{-4} M) but the time course and magnitude of these volume changes were strictly similar with or without ouabain. During the 2-h sampling period the oxygenated cells lost 37.6 μmol K/gram dry cell solid (g dcs) (Fig. 1C) and 55.4 μmol Cl/g dcs (Fig. 1B) whereas the K and Cl contents of control cells,

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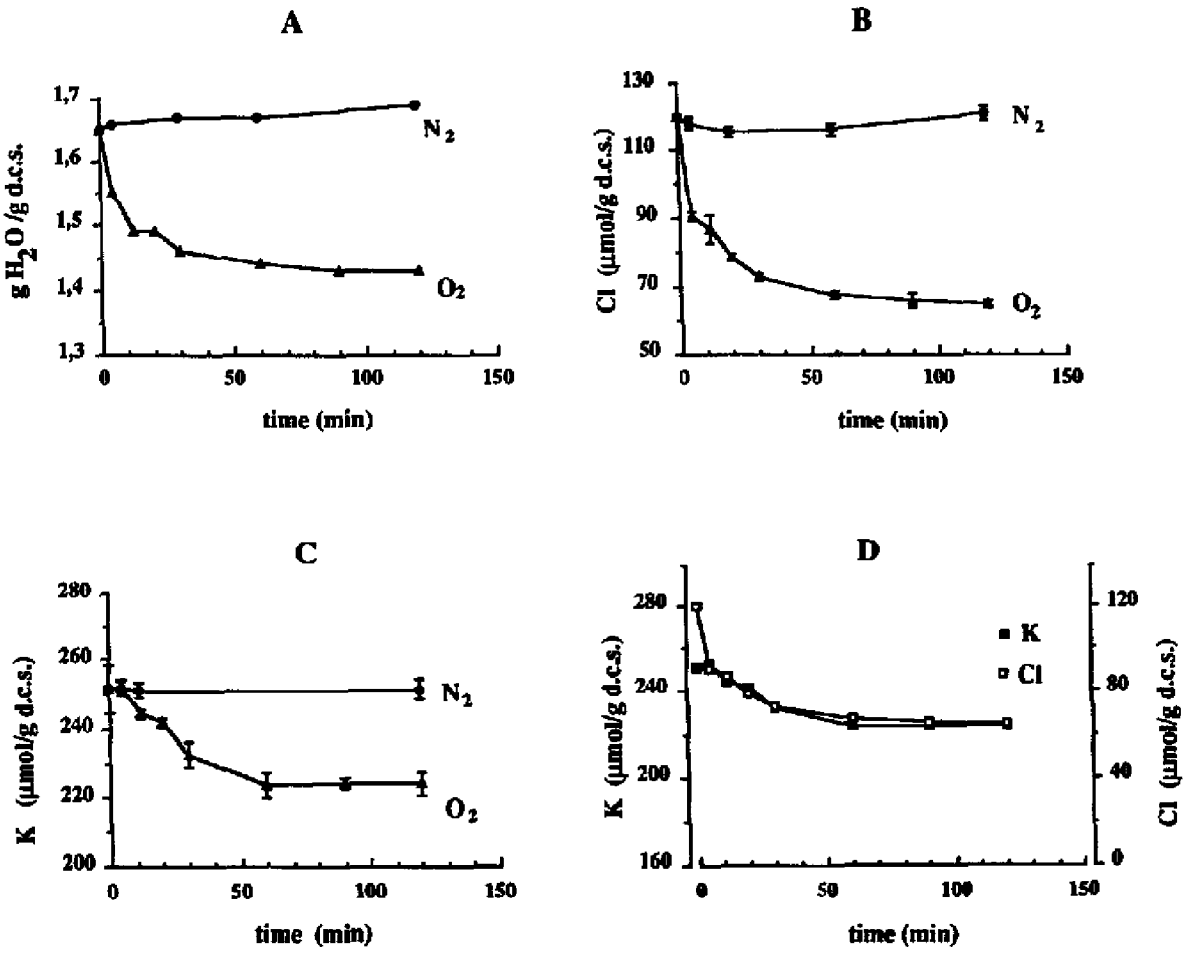

Fig. 1. Water (A), Cl (B) and K (C) contents of trout red cells maintained in anoxic conditions (●) or rapidly oxygenated at time zero by tonometry (△). These experiments were performed in the presence of ouabain (10^{-4} M). (D) Comparison of the time-dependent changes in K (■) and Cl (□) after oxygenation. Mean ± S.E. ($n = 4$). The data are presented as g/gram dry cell solid (g d.c.s.) or μmol/g d.c.s. as a function of time in minutes; temperature = 15 °C.

maintained in anoxic conditions in a tonometer, remained virtually unchanged. Thus oxygenation increases K permeability of the red cell membrane. No significant change in amino acid content was detected but a very slight Na uptake (about 5 μmol/g dcs) was recorded whereas there was no such uptake in anoxic control cells treated with ouabain (not shown). Thus the cell volume decrease results essentially from a leak of K and Cl. However, a comparison of Figs. 1B and C shows that 5 min after oxygenation a large amount of Cl had already been lost (−32.5 μmol) whereas the K loss was 10-times less (−3.5 μmol). This shows that, as expected, the ionization of hemoglobin due to oxygenation, which corresponds to an increase of the amount of negative fixed charges, promotes a Cl extrusion not accompanied by cations. This 'chloride shift' accounts for the first phase of volume decrease (Fig. 1A). Subsequently during the 5 to 120 min sampling period cells lost K and Cl progressively and virtually at the same rate as illustrated in Fig. 1D. This net loss of K and Cl accounts for the second phase of cell volume decrease. This KCl loss was stopped as soon as cells were deoxygenated (not shown) indicating that the activation of the K pathway by oxygen is a reversible process.

To investigate those properties of the oxygenation-activated K transport system, experiments were performed using inhibitors and anion replacement. Fig. 2A shows that the oxygenation-dependent K loss was completely blocked by the replacement of cell and medium Cl with NO_3. It is noteworthy that, as expected since NO_3 is a permeant anion in trout red cells [3], the first phase of the volume decrease was unaffected by NO_3 substitution. On the other hand the second phase was completely inhibited in the NO_3

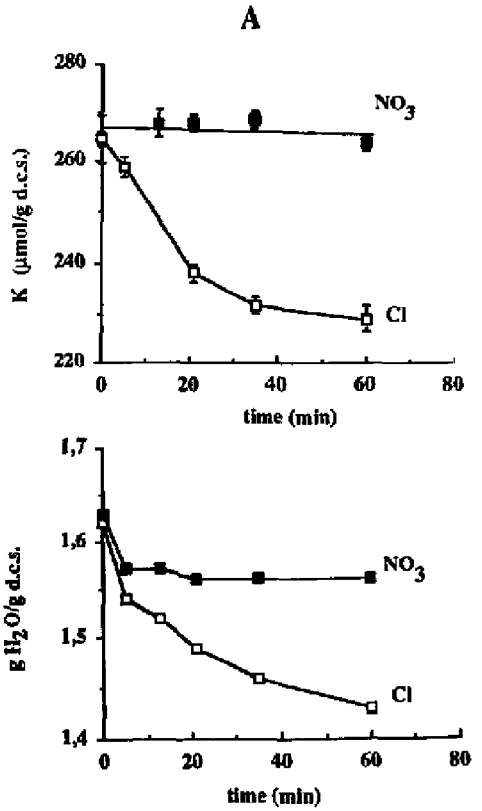

Fig. 2. (A) K contents of trout red cells suspended in a Cl medium (□) or a NO_3 medium (■) and oxygenated at time 0. (B) Volume changes in a Cl or a NO_3 medium. Mean ± S.E. ($n = 3$).

medium (Fig. 2B). We also observed that this Cl-dependent K loss was inhibited by DIDS (10^{-4} M) and niflumic acid ($5 \cdot 10^{-4}$ M), specific inhibitors of the anion exchanger, band 3 [4,5]. It was also blocked by furosemide (10^{-3} M), a loop diuretic known to inhibit KCl cotransport at high concentration but which also inhibits the red cell anion exchanger [6,7]. K loss is, however, unaffected by quinine. Interestingly the K loss induced by cell volume increase has been shown, in fish erythrocytes, to be also inhibable by furosemide, DIDS and niflumic acid [2,8,9].

It appears, therefore, that a Cl-dependent K transport system, which is inactivated when the cells are anoxic, becomes active when the cells are oxygenated. This activation cannot result from the intracellular acidification due to the chloride shift since a similar shift of pH_i (from 7.5 to 7.35) for anoxic cells does not induce a K loss (not shown).

Several transport systems can be influenced by cell Mg content [10–13]. For instance, it has been shown that high Mg has an inhitory effect on volume-sensitive KCl cotransport [14–17]. As hypoxia appears to increase free Mg in red cells [15], it is possible that the activation of the K pathway by oxygen is the result of a decrease of cytosolic Mg. Such a transducing effect of Mg appears to explain the deoxygenation-induced inhibition of the volume-stimulated, Cl-dependent K efflux of human red cells in sickle cell anemia [15]. To test this possibility in trout red cells, the total cell Mg was varied. Fig. 3 shows results from experiments in which cells under a nitrogen atmosphere were incubated in a Mg- and Ca-free medium containing EDTA and the divalent cation ionophore A23187. In these conditions hypoxic cells, thought to contain a relatively high concentration of free Mg, were expected to become Mg depleted. The figure shows, however, that the Mg depletion did not activate the K pathway (upper curve). Oxygenated cells, presumed to be Mg depleted, were incubated in the presence of Mg and the divalent cation ionophore. This condition was expected to induce Mg repletion and so shut off the K pathway. However, the K permeability remained unaffected (lower curve). Thus cytosolic Mg does not appear to be involved in the activation of the oxygenation-dependent K pathway. That reminds of the observation of Lauf [18] who showed that Mg and Mn failed to inhibit KCl cotransport in NEM-stimulated LK sheep red cells.

Fig. 4 shows that switching the cells from nitrogen to carbon monoxide in a tonometer activates the K transport as does oxygen, despite the fact that in an atmosphere of carbon monoxide the cells remain anoxic. This result indicates that the activation of the Cl-dependent K pathways does not involve a metabolic role of oxygen. Moreover, cells briefly exposed to carbon monoxide and then put back in a nitrogen atmosphere and stirred under this atmosphere did not show the expected deactivation of the K pathway (not shown).

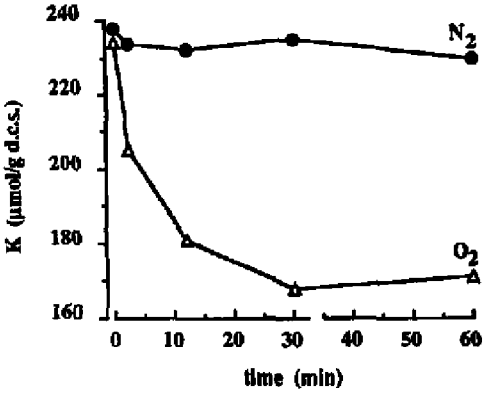

Fig. 3. K contents of red cells suspended under nitrogen in a Ca- and Mg-free saline to which EDTA (1 mM) and ionophore A23187 (10 μM) had been added (●). K contents of red cells placed at time zero under oxygen in a Mg-containing saline (1 mM) in which ionophore A23187 (10 μM) had been added (△). Temperature 15 °C. Hematocrit: 20%.

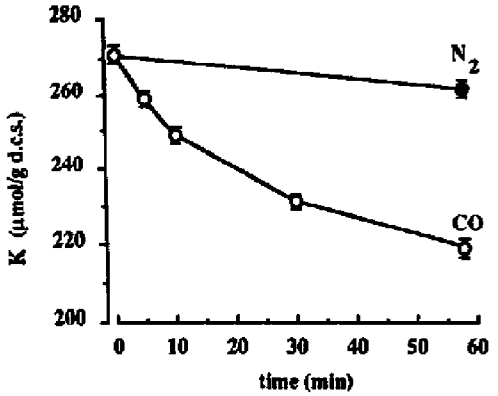

Fig. 4. K contents of red cells maintained in anoxic conditions under nitrogen (●) or shifted at time zero to a carbon monoxide atmosphere (△). Mean ± S.E. ($n = 3$).

This observation indicates that the binding of carbon monoxide to heme, which persists even when nitrogen has flushed out the carbon monoxide in the gas phase and in the suspending medium, is sufficient to activate the K pathway.

Discussion

The binding of either carbon monoxide or oxygen to heme, leading in both cases to a similar quaternary structure in the hemoglobin molecule, appears to be the trigger for activation of the KCl cotransport. This process is reminiscent of the action of hemoglobin in the control of the β-adrenergic-activated Na^+/H^+ exchanger activity in trout red cells. Deoxygenation per se does not activate the Na^+/H^+ antiporter, but we have demonstrated [1] that the magnitude of the activation induced by catecholamines is very high in cells in a nitrogen atmosphere and strongly depressed in oxygenated cells. Moreover, cells in a carbon monoxide atmosphere or cells first exposed to carbon monoxide and then switched to nitrogen, behave as cells in oxygen. These results, again, indicate that the conformation of hemoglobin is a determining factor in the control of Na^+/H^+ exchanges. But how does binding of molecular oxygen on the heme of hemoglobin control the activity of a transport protein? For the Na^+/H^+ exchange system we discarded on experimental evidence [1] the possible transducing role of changes, due to oxy-deoxyhemoglobin transitions, in either the intracellular pH or the concentration of intracellular free allosteric effector of hemoglobin. As hemoglobin specifically binds to the cytoplasmic segment of band 3 protein, but with an affinity which depends on its oxygenation state [19], we proposed [1] that such an oxygenation-dependent interaction may be associated with a functional regulation of the Na^+/H^+ exchanger (either by direct interaction of hemoglobin with presumed specific binding sites located on the antiport or by its interaction with the band 3 protein, transmitter to the antiport via the cytoskeleton on which band 3 is anchored). This hypothesis could also explain the oxygenation-related activation of the Cl-dependent K transport described in this work. In addition to Salama and Nikinmaa [20] who confirmed the oxygenation-dependence of the Na^+/H^+ antiport in trout red cells, several other workers have noted the oxygenation-dependence of other red cell membrane transport proteins. The Na/K/2Cl cotransport of avian red cells is influenced by oxy-deoxy transitions [21], as is the Na permeability of turtle red cells [22]. A K loss is induced in carp red cells not only by oxygenation of the cells [23] but also by the presence of nitrite which induces formation of methemoglobin (which has an oxyhemoglobin-like structure [24]). Curiously it is deoxygenation, not oxygenation, of human erythrocytes in sickle cell anemia which induces a K efflux shown to be Cl-independent [15]. Whether interaction of hemoglobin regulates such varied transport systems (unfortunately possible transducing roles of mediators such as pH, allosteric effectors, or Mg have not been tested in these systems), it would appear unlikely that specific hemoglobin binding sites exist on each of the transport proteins. In other words, we favor the hypothesis that binding of hemoglobin on the cytoplasmic segment of band 3 is associated with some structural modification of the cytoskeleton which regulates at a distance the activities of different types of transport protein such as, for example in trout red cells, the Na^+/H^+ antiporter and the Cl-dependent K pathway. Experiments are in progress to test this possibility.

In addition to considerations concerning the nature of the Cl-dependent K pathway and the mechanism involved in its activation, a further subject of interest is its physiological significance. *In vivo* experiments performed by Nielsen and Lykkeboe [25] with rainbow trout would give an explanation. They submitted fishes to successive periods of supramaximal exercise which promoted hypoxic and hypercapnic states. During exercise they observed an accumulation of K in the plasma. The magnitude of this K accumulation, however, was limited by a simultaneous uptake of K by the red cells. In so far as increased plasma K contributes to fatigue, the red cell K uptake during exercise must have increased the capacity of the trout for performing exercise. Return to resting (normoxic and normocapnic) conditions was accompanied by the release from the red cells of the K accumulated during exercise, probably via the oxygen-related, Cl-dependent K pathway. Thus these results could indicate that trout red cells behave as a transient reservoir buffering the fluctuations of extracellular K. Both the Na/K pump, which permits the active uptake of K when the plasma K

increases, and the oxygenation-dependent K pathway, which drains back this excess of red cell K, are the main elements of this regulatory system.

Acknowledgements

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