

Reactive Oxygen Species Regulate Oxygen-sensitive Potassium Flux in Rainbow Trout Erythrocytes

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ABSTRACT In the present study, we have investigated if reactive oxygen species are involved in the oxygen-dependent regulation of potassium-chloride cotransport activity in trout erythrocyte membrane. An increase in the oxygen level caused an increase in chloride-sensitive potassium transport (K^+Cl^- cotransport). 5 mM hydrogen peroxide caused an increase in K^+Cl^- cotransport at 5% oxygen. The increase in flux could be inhibited by adding extracellular catalase in the incubation. Pretreatment of the cells with mercaptopropionyl glycine (MPG), a scavenger of reactive oxygen species showing preference for hydroxyl radicals, abolished the activation of the K^+Cl^- cotransporter by increased oxygen levels. The inhibition by MPG was reversible, and MPG could not inhibit the activation of transporter by the sulfhydryl reagent, *N*-ethylmaleimide, indicating that the effect of MPG was due to the scavenging of reactive oxygen species and not to the reaction of MPG with the cotransporter. Copper ions, which catalyze the production of hydroxyl radicals in the Fenton reaction, activated K^+Cl^- cotransport significantly at hypoxic conditions (1% O_2). These data suggest that hydroxyl radicals, formed from O_2 in close vicinity to the cell membrane, play an important role in the oxygen-dependent activation of the K^+Cl^- cotransporter.

KEY WORDS: potassium-chloride cotransport • red blood cell • Fenton reaction • *Oncorhynchus mykiss* • oxygen-sensitive ion transport

INTRODUCTION

Studies mainly on excitable cells and on erythrocytes from several species have shown that ion transport across cellular membranes can be affected by oxygen tension. In several excitable cell types, hypoxia causes a pronounced, immediate inhibition of potassium channels (Zhu et al., 1996; Conforti and Millhorn, 1997; Lopez-Barneo et al., 1999). Also, hypoxia inhibits all of the following: the Ba^{2+} -sensitive potassium channel, which is active in isotonic conditions; the swelling-activated chloride channel of lamprey erythrocytes (Virkki et al., 1998); the potassium-chloride cotransporter of teleost erythrocytes (Nielsen et al., 1992); sodium-potassium-chloride cotransporter of alveolar epithelial cells (Mairbäurl et al., 1997); calcium channels of smooth muscle cells (Rekalov et al., 1997); and the sodium channels of pneumocytes (Planes et al., 1997). On the other hand, the sodium/proton exchanger of fish erythrocytes (Motais et al., 1987), the ATP-sensitive potassium channels of smooth muscle (Dart and Standen, 1995), and one type of sodium channels in rat cardiac myocytes (Ju et al., 1996) are activated by low oxygen levels.

The mechanisms by which oxygen rapidly influences membrane transport pathways are poorly known. It is possible that oxygen directly, or via a transducer molecule, influences the conformation of the transporter

(Acker, 1994). If such a transducer exists, it is most likely a heme protein (Bunn and Poyton, 1996). In fish erythrocytes, it has been suggested that hemoglobin itself would be the heme-containing transducer molecule. Oxygen-dependent conformational changes of the hemoglobin molecule would directly or indirectly affect the activity of the sodium/proton exchanger (Motais et al., 1987) and the potassium-chloride cotransporter (Jensen, 1990; Gibson et al., 2000). However, using rainbow trout erythrocytes, Berenbrink et al. (2000) carried out studies designed to investigate the interactions of hemoglobin oxygenation and potassium-chloride cotransporter activity at different pH values and oxygen tensions. After a 45-min hypoxic pre-equilibration at 1 kPa, erythrocytes were equilibrated at oxygen tensions varying from 0 to 100 kPa and at pH values ranging from 7.0 to 8.4 for 10 min, thereafter both potassium fluxes across the erythrocyte membrane and oxygen saturation of hemoglobin were determined. In these experiments, the pH and oxygen tension dependence of hemoglobin oxygen saturation differed markedly from the pH and oxygen tension dependence of potassium fluxes. Thus, the results strongly suggest that transporter activity is independent of the function of the bulk oxygen-carrying hemoglobin (Berenbrink et al., 2000). Consequently, Berenbrink et al. (2000) suggest that the oxygen sensor in erythrocytes could be a hemoprotein distinct from the oxygen-carrying hemoglobin. Gibson et al. (2000), in a review on oxygen-sensitive membrane transporters in vertebrate erythrocytes, similarly concluded that bulk

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hemoglobin was unlikely to be the oxygen sensor influencing erythrocyte transporter activity, but suggested that a fraction of hemoglobin, possibly bound to band 3, could play a role in oxygen sensing.

On the other hand, it has been shown that factors influencing the cellular redox levels also influence the potassium-chloride cotransport activity in erythrocytes. Low reduced glutathione levels in the erythrocytes are associated with a high activity of the cotransporter (Lauf et al., 1992). As suggested by Gibson et al. (2000), changes in oxygen tension could act on the ion transporters by causing changes in red cell redox status. One possible way by which changes in oxygen tension could alter the cellular redox state is via generation of reactive oxygen species during reduction of molecular oxygen within the cell. In addition to influencing the redox state of the cells, it is possible that reactive oxygen species directly influence the transporter or its regulatory mechanism. Notably, hydrogen peroxide influences potassium transport in excitable cells (Acker, 1994; Wang et al., 1996) and affects the potassium-chloride cotransporter activity in mammalian erythrocytes (Bize and Dunham, 1995; Bize et al., 1998). However, the role of reactive oxygen species in causing the oxygen sensitivity of the potassium transport pathways in erythrocytes has not been previously studied in detail. Thus, in the present studies, we have investigated the role of the intermediate products of O_2 reduction including H_2O_2 , superoxide ions, and hydroxyl radicals in the regulation of potassium-chloride cotransporter activity.

MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) weighing 600–900 g were obtained from Parainen Fishery School and maintained in the aquaria of Department of Biology at 15°C for at least 2 wk. The fish were anesthetized with MS 222 (3-aminobenzoic acid, ethyl ester, 0.1 g/l) buffered with 0.1 g/liter $NaHCO_3$, and the blood was taken from the caudal vein into a heparinized syringe. Erythrocytes were isolated by centrifugation, washed three times with the standard incubation medium, and left overnight at 4°C to reach a steady state in salt and water composition (Bourne and Cossins, 1984).

Solutions and Chemicals

The standard incubation saline solution contained (in mM): 125.5 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 20 mM HEPES-NaOH, and 5 glucose. The pH of the solution was adjusted to 7.9. In chloride-free medium, chloride salts were replaced by corresponding nitrate salts. All experiments were conducted at room temperature. Hydrogen peroxide stock solution (100 mM) was prepared in water from 30% concentrate immediately before addition to the suspension. Stock solutions of 3-amino-1,2,4-triazol (5 M) and *N*-ethylmaleimide (100 mM) were prepared in DMSO, and *N*-(2-mercaptopropionyl)glycine (1 M–100 mM) in ethanol;

¹Abbreviations used in this paper: MPG, mercaptopropionyl glycine; NEM, *N*-ethylmaleimide.

these solutions were stored at 4°C. 100 U/mg catalase was dissolved in 10 mg/ml water and stored at –20°C. Catalase, 3-amino-1,2,4-triazol, *N*-(2-mercaptopropionyl) glycine (MPG),¹ ouabain, and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich; inorganic salts were of analytical grade.

K^+ Flux Measurements

For the evaluation of oxygen-induced activation, the cells were washed twice in the standard incubation medium and resuspended in the medium (final hematocrit value 5–7%) containing 100 μ M ouabain. Erythrocyte suspension was equilibrated for 1 h at 1% O_2 , 99% N_2 (gas mixture was obtained using a Cameron Instruments GF-3/MP gas mixing flowmeter, and humidified before use), thereafter the percentage of oxygen was increased to either 5 or 21%. After a 10-min equilibration at the increased oxygen tension, ⁸⁶Rb was added to the suspension and

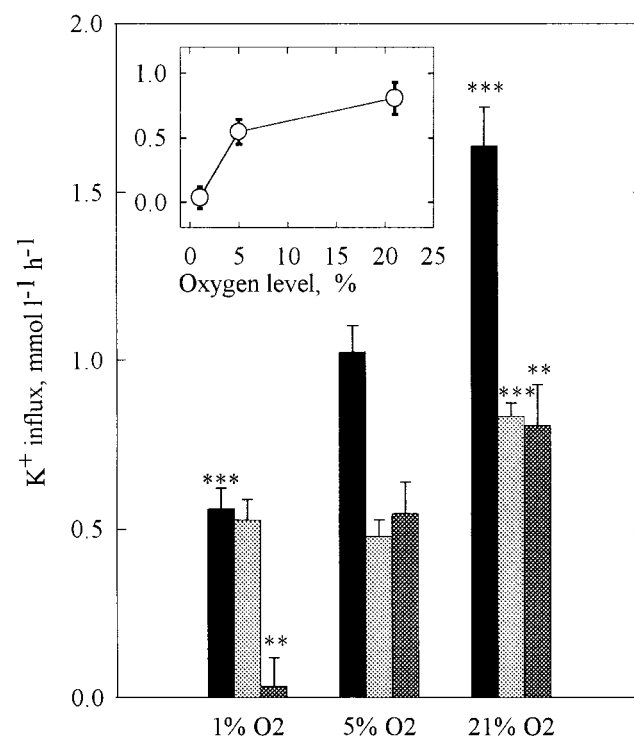


FIGURE 1. The effect of oxygen on ouabain-resistant potassium influx into rainbow trout erythrocytes. The cells were incubated for 60 min at 1% O_2 in the presence of 100 μ M ouabain, thereafter a further 10-min equilibration at 1, 5, or 21% oxygen was carried out. After this equilibration, ⁸⁶Rb was added to the suspensions, its uptake was measured at 5 and 15 min, and the rate of influx was calculated. Experiments were made on parallel samples in chloride-containing and chloride-free media to enable estimations of chloride-independent and chloride-dependent K^+ influx. Black bars indicate total ouabain-resistant flux; light gray bars chloride-independent flux; and dark-gray bars indicate chloride-dependent flux (difference between total and chloride-independent flux). Error bars indicate SEM; $n = 5$. Asterisks indicate that the mean flux is significantly different from that at 5% oxygen (**, $P < 0.01$; ***, $P < 0.001$). The statistical significance of the difference between means at the different oxygen levels was tested using ANOVA followed by the LSD test. Inset shows the change in chloride-dependent K^+ influx as a function of oxygen level in the medium. Bars indicate \pm SEM.

0.8-ml aliquots were taken after 5, 10, and/or 20 min of treatment to assay the tracer uptake by the cells. During this period of time, the fluxes remained linear. The 0.8-ml sample of cell suspension was centrifuged (at 2,700 *g* for 1 min), 0.2 ml of the supernatant was removed (extracellular medium), and the cell pellet was washed twice with 10 ml ice-cold washing solution (100 mM MgSO₄, 10 mM imidazole, pH 7.9 at 4°C) and lysed in 0.3 ml distilled water. Proteins were removed from the lysate by adding 0.4 ml 0.6 M HClO₄ and subsequent centrifugation (at 2,700 *g* for 1 min). Finally, 0.2 ml of the resultant supernatant (diluted intracellular medium) was taken to determine the uptake of ⁸⁶Rb by the cells. The radioactivity of the extracellular and intracellular medium was measured using a liquid scintillation counter (model Wallac 1450 Microbeta Plus; Perkin Elmer Biosystems). K⁺ influx (J_K) was calculated using the following equation:

$$J_K = [K^+]_o \times (A_{ml\ cells} / A_{ml\ med}) \times (1/t),$$

where [K⁺]_o is the extracellular potassium concentration, *A*_{ml cells}/*A*_{ml med} is the activity ratio of ⁸⁶Rb in 1 ml of packed cells and 1 ml medium, and *t* is time. In the text and figures, the units for influx are mmol/liter of packed cells/h. When required, the cellular water content was determined by weighing a red cell pellet, drying it to a constant weight at 80°C, and reweighing as described previously (Nikinmaa and Huestis, 1984).

When NEM and MPG were used, they were added in the suspension 30 min before the oxygen tension was increased. In the experiments with copper sulfate, CuSO₄ was added in the medium 10 min after the oxygen percentage was increased from 1 to 5% O₂. In this case, ⁸⁶Rb was added in the incubation 5 min after the addition of CuSO₄.

Statistical Treatment

The results presented are the means of at least four independent experiments with SEM. The statistical significance of the differences between the means was estimated using One-Way ANOVA and the appropriate post-hoc tests (SPSS 9.0; SPSS Inc.), and curves were fitted using nonlinear regression options of the SigmaPlot 5.0 (SPSS Inc.).

RESULTS

Effects of Oxygen on Chloride-dependent and -independent K⁺ Fluxes

At 1% oxygen, there was virtually no chloride-dependent K⁺ influx (Fig. 1). However, increasing the oxygen level to 5% caused a pronounced increase in the chloride-dependent K⁺ influx to ~50% of the total flux without affecting the chloride-independent flux. A further increase of oxygen level to 21% caused an increase in both chloride-dependent and independent fluxes. Thus, it is apparent that, at intermediate oxygen tensions, only the chloride-dependent ouabain-resistant K⁺ influx pathway is affected by oxygen; but, at high oxygen levels, the chloride-independent flux pathway may respond to changes in oxygen tension.

Effects of H₂O₂ on K⁺ Fluxes

H₂O₂ caused an increase in ouabain-resistant K⁺ influx at pH 7.9 and at 5% O₂, in addition to the increase in flux caused by the increase in oxygen level as such (Fig.

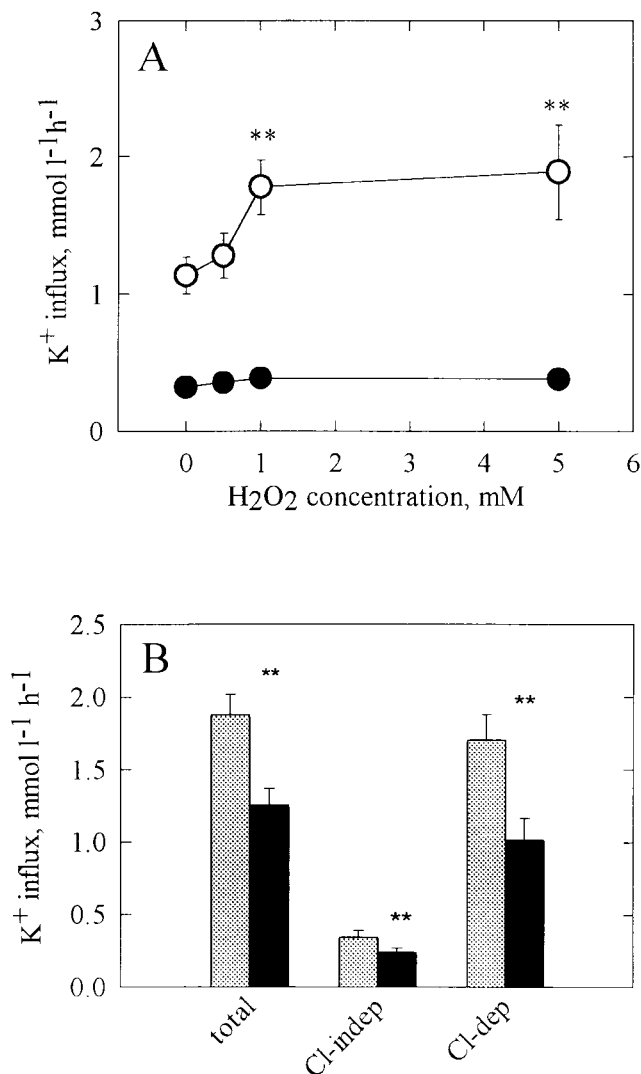


FIGURE 2. (A) The effect of H₂O₂ on K⁺ influx into trout erythrocytes in the presence of 1% (closed circles) or 5% O₂ (open circles). The cells were incubated for 50 min at 1% O₂ in the presence of 100 μM ouabain, thereafter H₂O₂ was added. 10 min after the addition of H₂O₂, the oxygen level was increased to 5% in one set of samples but remained at 1% in another set of samples. After 10 min further of equilibration, ⁸⁶Rb was added to the suspensions, its uptake was measured at 5 and 15 min, and the rate of influx was calculated. Means ± SEM are given; *n* = 5. The statistical significance of the difference between means at the different hydrogen peroxide concentrations was tested using ANOVA followed by the LSD test. Double asterisk indicates that the flux differed at the *P* < 0.01 level from the control (in the absence of hydrogen peroxide). (B) At 1 mM H₂O₂ and 5% oxygen, in which conditions the H₂O₂ effect had reached maximum, the flux was divided to total chloride-independent and chloride-dependent flux and compared with the same fluxes in the absence of H₂O₂. Black bars indicate fluxes in the absence of H₂O₂; and gray bars indicate flux in the presence of H₂O₂. Means and SEM are given; *n* = 5. The statistical significance of the difference between means in the absence and in the presence of hydrogen peroxide concentrations was tested using ANOVA followed by the LSD test, double asterisk indicates that the fluxes differed at the *P* < 0.01 level.

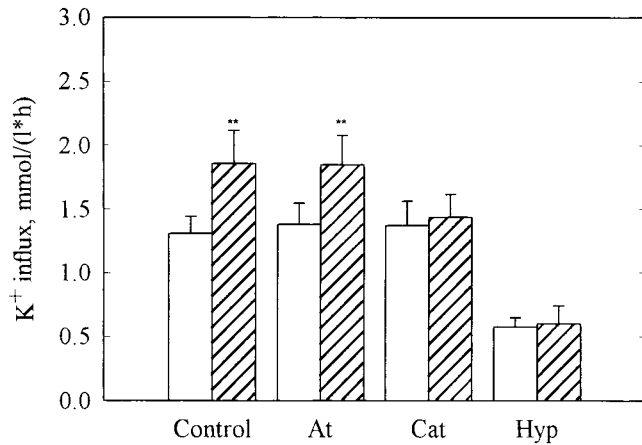


FIGURE 3. The effect of H₂O₂ on the oxygen-stimulated, ouabain-resistant K⁺ influx into trout erythrocytes in control conditions (Control, 5% O₂), in the presence of aminotriazol (At, 50 mM), in the presence of catalase (Cat, 0.1 g l⁻¹), and in hypoxic conditions (Hyp, incubation at 1% O₂). Aminotriazol and catalase were added after a 30-min incubation in the presence of 100 μM ouabain at 1% O₂. Hydrogen peroxide (1 mM) was added after 50 min of incubation, 10 min before the oxygen level was increased to 5%. (Open bars) Incubation in the absence of H₂O₂; (hatched bars) incubation in the presence of H₂O₂ (means and SEM are given; n = 6). The statistical significance of the difference between means in the presence and absence of hydrogen peroxide was tested using ANOVA followed by the LSD test; double asterisk indicates that the flux in the presence of hydrogen peroxide differed at the P < 0.01 level from that in the absence of hydrogen peroxide.

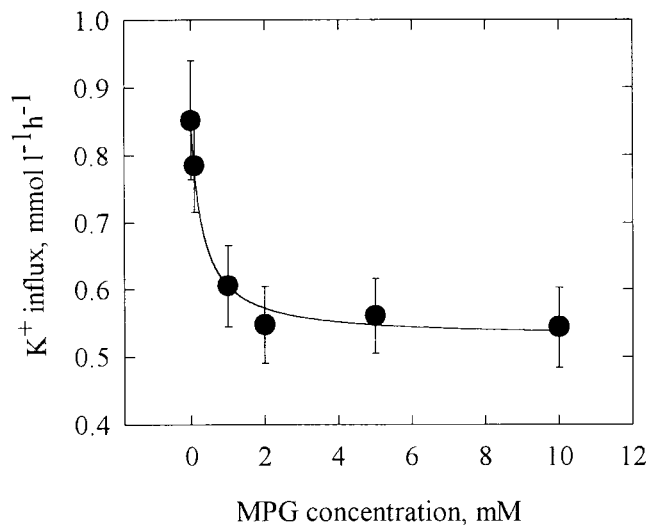


FIGURE 4. The effect of *N*-mercaptopyropionyl glycine (MPG) on oxygen-induced activation of ouabain-resistant K⁺ influx into trout erythrocytes. MPG was added to the incubation medium 10 min before the oxygen level was increased from 1 to 5%. The line in the figure gives the best fit of experimental data, which was obtained using a nonlinear least squares fit of a 3-parameter hyperbolic decay function $y = y_0 + ax/(b + x)$. Symbols indicate means ± SEM at the different MPG concentrations; n = 5. In similar conditions, the potassium flux at 1% oxygen was 0.56 ± 0.06 mmol l⁻¹h⁻¹, which is close to the value obtained with MPG above 2-mM concentration.

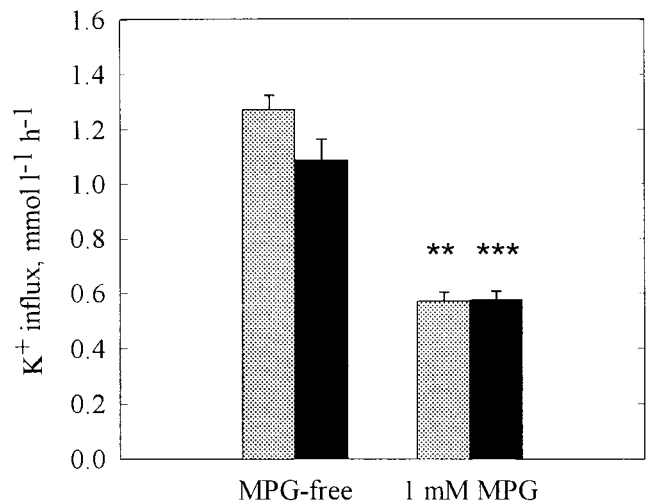


FIGURE 5. Reversibility of the effect of MPG on ouabain-resistant K⁺ influx. The cells were equilibrated in a medium containing 2 mM MPG for 20 min, washed free of the drug, and subjected either to the hypoxia (1% oxygen for 60 min)-re-oxygenation (5% oxygen for 10 min) cycle before measurement of potassium flux (MPG-free) or to the hypoxia-re-oxygenation cycle in which MPG was added to the incubation medium 10 min before the oxygen level was increased (1 mM MPG). Parallel samples, treated identically but without the initial equilibration with 2 mM MPG served as controls. Black bars indicate that the cells were treated with 2 mM MPG and washed free of the drug, gray bars indicate control cells. (Means and SEM are given; n = 5.) The statistical significance of the difference between means in the presence and absence of 1 mM MPG in the final incubation is given by asterisks (**, P < 0.01; ***, P < 0.001); ANOVA followed by the LSD test was used for comparisons. There was no statistically significant difference between control cells and cells treated with 2 mM MPG and subsequently washed free from the drug.

2). Thus, H₂O₂ can influence ouabain-insensitive potassium transport. The activation of potassium fluxes was mainly due to the chloride-dependent component, as indicated by the fact that the flux in chloride-free medium was only slightly affected by hydrogen peroxide; at 1 mM H₂O₂ the chloride-independent potassium flux was 0.35 ± 0.04 mmol l⁻¹cells h⁻¹ as compared with 0.24 ± 0.03 mmol l⁻¹cells h⁻¹ in control cells. Notably, H₂O₂ was without an effect on potassium fluxes in hypoxic conditions (1% O₂), suggesting that hydrogen peroxide alone is not capable of activating potassium-chloride cotransport.

The addition of extracellular catalase (0.1 mg/ml) inhibited the effect of H₂O₂ on the potassium influx (Fig. 3). On the other hand, an inhibitor of catalase, aminotriazol (50 mM), which is commonly used to block catalase in mammalian erythrocytes (Ou and Wolff, 1993), did not affect the potassium fluxes either in the presence or absence of 1 mM H₂O₂. These results suggest that the effect of H₂O₂ is on a redox-sensitive group or a system readily accessible from the extracellular compartment.

Effects of NMG on Oxygen-sensitive K^+ Influx

In addition to hydrogen peroxide, other reactive oxygen species that are formed during the reduction of molecular oxygen are superoxide ions and hydroxyl radicals. Although the influence of these on potassium fluxes could not be measured directly, the effect of hydroxyl radicals could be estimated using MPG. MPG acts as a scavenger of free oxygen radicals showing preference for hydroxyl radicals (Bolli et al., 1989; Sekili et al., 1993). Specifically, the EPR spectroscopy studies by Bolli et al. (1989) indicated that MPG removed hydroxyl radicals with little or no effect on superoxide radical or hydrogen peroxide levels. When MPG was added in the incubation during the initial hypoxic period (1% O_2), 30 min before the oxygen level was increased to 5%, the oxygen-induced stimulation of K^+ influx was completely inhibited at millimolar concentrations (Fig. 4). Half-maximal inhibition, as obtained from the nonlinear least squares fit of the equation in Fig. 4, of the oxygen-stimulated potassium flux was reached at 0.304 ± 0.092 mM concentration of MPG. At 5% oxygen, only the chloride-dependent component of potassium influx was inhibited by MPG, as indicated by the observation that the potassium flux in the chloride-free medium was not affected by MPG: the K^+ influx into MPG-treated (1 mM) cells in the chloride-free medium was 0.46 ± 0.047 mmol $l^{-1}h^{-1}$, which is not significantly different from that into control cells (0.51 ± 0.044 mmol $l^{-1}h^{-1}$). The efficiency of MPG as an inhibitor of potassium-chloride cotransport was oxygen-dependent, as shown by the fact that the MPG concentration required for half-maximal inhibition at 21% O_2 was much higher (4.05 ± 0.23 mM) than that at 5% O_2 (0.304 ± 0.092 mM). The 10-fold increase in MPG required for 50% inhibition of the transporter when the oxygen tension is increased fourfold suggests that the rate of hydroxyl radical production between 5 and 21% oxygen increases more rapidly than expected only on the basis of change in oxygen tension.

The inhibition of K^+ flux by MPG was reversible. This was ascertained by treating the cells with 2 mM MPG for 20 min, and then washing them free of the drug before the hypoxia-reoxygenation cycle. There was no significant difference in the K^+ flux between the MPG-pretreated, washed cells and the control cells at 5% oxygen (Fig. 5). Furthermore, the inhibition obtained by adding 1 mM MPG to the MPG-pretreated, washed cells was similar to that obtained by adding 1 mM MPG to control cells. These findings indicate that the inhibition of K^+ flux by MPG is not caused by covalent binding of MPG to the transporter or its regulatory molecules.

In many systems, the thiol alkylating agent NEM activates chloride-dependent potassium transport (Lauf et al., 1992). We ascertained that this is the case also in

our experimental setup by treating air-equilibrated cells (21% oxygen) with 5 mM NEM. As a result, the potassium influx increased fivefold (Fig. 6 A). The activation of transport by NEM was virtually irreversible: the chloride-dependent potassium flux was only slightly reduced by washing the cells free of NEM. The irreversible activation of chloride-dependent potassium fluxes by NEM was not inhibited by MPG (Fig. 6 B). To further evaluate if MPG acts at the same site(s) as NEM, we carried out experiments in which the cells were treated with both MPG and NEM. Studies on the interactions are complicated by the fact that MPG is a thiol compound, and will thus react with NEM. For this reason, we used an excess of NEM (5 mM) in comparison to MPG (1 mM). At this concentration, MPG reduces the potassium fluxes at 21% oxygen significantly. However, NEM caused an activation of K^+ fluxes both when it was added before MPG into the equilibration and when it was added after MPG. This result suggests that MPG is not influencing K^+ transport at the same site as NEM. The observation that the activation by NEM was somewhat smaller in the presence than in the absence of MPG is possibly due to the interactions of NEM with the thiol groups on MPG causing a reduction in the functional concentration of NEM. The suggestion that NEM and MPG act at different sites is further strengthened by the observation that the activation of K^+ flux by NEM was similar both at 21% and 1% oxygen (Fig. 6 A). In contrast, MPG inhibited K^+ flux at 5 and 21% oxygen, but not at 1% oxygen.

Potassium flux pathways in rainbow trout erythrocytes can also be activated by an increase in cell volume. In the case of isotonic swelling, only the chloride-dependent potassium transport pathway is activated (Guizouarn and Motais, 1999). An isotonic increase in cell volume can be achieved by treating the cells with β -adrenergic drugs such as isoproterenol which activates sodium/proton exchange (Nikinmaa and Huestis, 1984). The cell volume was perturbed at atmospheric oxygen tension (21% air) using 10^{-6} M isoproterenol in the absence and presence of MPG at pH 7.9. Before adrenergic stimulation, the cell water content was 2.25 ± 0.03 kg dcw^{-1} (dry cell weight; mean \pm SEM; $n = 6$ in all cases) in control cells and 2.34 ± 0.03 kg dcw^{-1} in MPG-treated cells; after the stimulation the respective values were 2.51 ± 0.04 and 2.79 ± 0.03 kg dcw^{-1} . The cell water content of MPG-treated cells after stimulation with isoproterenol was significantly ($P < 0.05$) greater than that of untreated cells after stimulation with isoproterenol. It is likely that this difference in volume is due to a greater sodium/proton exchange activity in MPG-treated than in control cells after catecholamine stimulation (Bogdanova, A., and M. Nikinmaa, unpublished data). Since the potassium-chloride cotransport in rainbow trout erythrocytes is strongly acti-

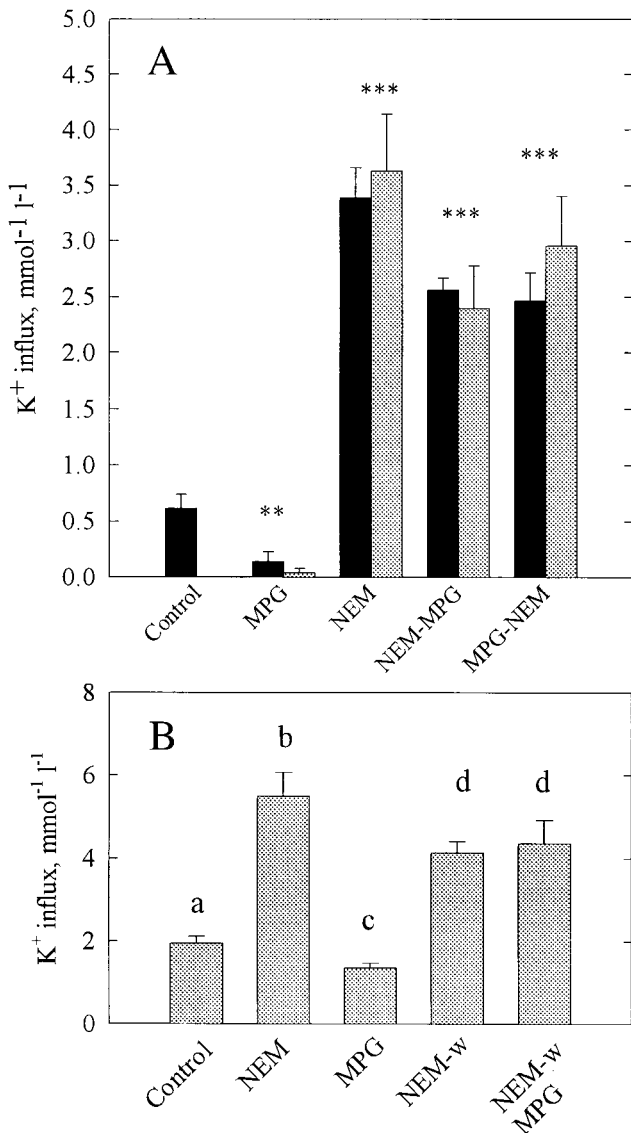


FIGURE 6. (A) The effect of MPG and *N*-ethylmaleimide (NEM) on chloride-dependent potassium fluxes at 21% (black bars) and 1% (gray bars) oxygen. (MPG) Cells equilibrated in the presence of 1 mM MPG; (NEM) cells equilibrated in the presence of 5 mM NEM; (NEM-MPG) cells equilibrated first with 5 mM NEM and after 20 min equilibration 1 mM MPG was added; (MPG-NEM) cells equilibrated first with 1 mM MPG and after 20 min equilibration 5 mM NEM was added. (Means and SEM are given; $n = 4$.) MPG inhibited K^+ influx significantly at 21% oxygen (**, $P < 0.01$), whereas NEM activated transport significantly both at 21% and 1% oxygen (***, $P < 0.001$). Activation by NEM was also observed in the presence of MPG (***, $P < 0.001$). There was no statistically significant difference between fluxes in equilibrations with MPG added first and with NEM added first. ANOVA followed by the LSD test was used for comparisons. (B) The irreversible effect of NEM on chloride-dependent potassium flux. To evaluate the irreversibility and its inhibition, the cells were equilibrated for 20 min with 5 mM NEM, thereafter they were washed twice with 10-ml incubation medium (15 min), and resuspended in the medium in

vated by cell swelling (Guizouarn and Motais, 1999), the observation that the potassium flux measured after stimulation with the catecholamine was greater in MPG-treated cells than in control cells (2.11 ± 0.54 and 0.34 ± 0.15 mmol l⁻¹h⁻¹, respectively) may be explained by the volume dependence of the activity of the transport, although conclusive verification of this suggestion requires more detailed studies. However, from the data presented, it is clear that there are at least two ways of activating chloride-dependent potassium transport that are not inhibited by MPG, i.e., treatment with NEM and cell swelling.

Effect of Copper Ions on K^+ Influx

Although it appears that MPG has a preference for hydroxyl radicals in its scavenging action (Bolli et al., 1989; Sekili et al., 1993), as a thiol group-containing compound, it can react with all reactive oxygen species (Patterson, 1993). To differentiate between the effects of superoxide ions and hydroxyl radicals, we catalyzed the Fenton reaction by exposing the cells to copper ions. In the course of the Fenton reaction (Fig. 7) superoxide ions are converted to hydroxyl radicals in the presence of Fe^{2+}/Fe^{3+} or Cu^+/Cu^{2+} ions. Thus, if hydroxyl radicals are involved in the activation of oxygen-sensitive potassium transport, potassium fluxes should be markedly speeded up when the cells are treated with copper salts. In contrast, if superoxide ions were the species activating the transporter, the addition of copper ions would decrease the oxygen-induced flux changes by shifting the superoxide/hydroxyl radical equilibrium towards hydroxyl radical formation.

Addition of 25 μ M $CuSO_4$ to red cell suspensions resulted in a marked increase in the K^+ influx into trout erythrocytes at all oxygen levels studied (Fig. 8). This ouabain-resistant copper-induced K^+ influx was fully chloride-dependent, since copper ions did not activate transport in chloride-free medium. If the copper-induced activation of potassium fluxes were due to the hydroxyl radicals formed in the Fenton reaction, then it should be inhibited by MPG. Indeed, pretreatment of the cells with 5 mM MPG fully inhibited the copper-induced increase in potassium flux: at 25 μ M $CuSO_4$ the flux at 5% O_2 in the presence of MPG was 0.731 ± 0.061 mmol

the presence or absence of 1 mM MPG thereafter K^+ influx was determined. ([NEM-w] washed, NEM-treated cells; [NEM-w-MPG] washed, NEM-treated cells in the presence of MPG). In the same patch of cells, K^+ influx was determined for control cells (Control), for cells treated for 20 min with 5 mM NEM (NEM) and for cell treated for 20 min with 1 mM MPG (MPG). Means and SEM are given ($n = 4$); different letters above the bars indicate that the means differ at the 0.05 probability level. ANOVA followed by the LSD test was used for comparisons.

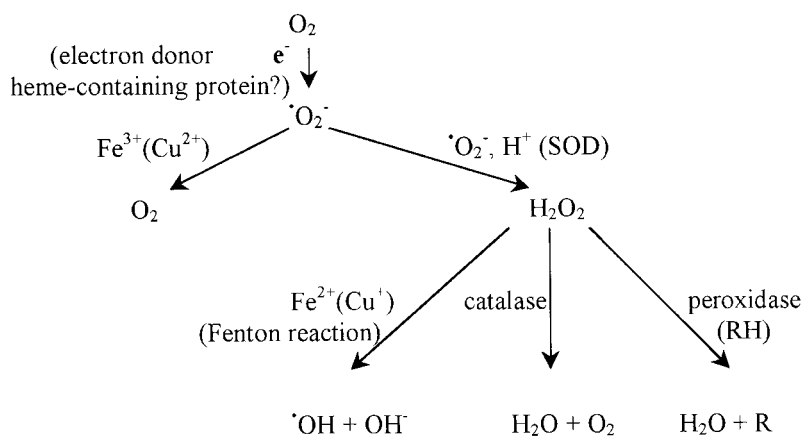


FIGURE 7. Schematic representation of the sequence of events involved in Fenton reaction. Initially, electron donors can convert oxygen to superoxide anion ($\bullet\text{O}_2^-$) which is rapidly converted to hydrogen peroxide. Hydrogen peroxide can further form hydroxyl radicals ($\bullet\text{OH}$) and hydroxyl ions in the actual Fenton reaction in the presence of ferrous or cuprous ions (which are simultaneously oxidized to ferric or cupric ions). SOD, superoxide dismutase; RH/R, reducing agent in oxidized and reduced form.

$1^{-1}\text{cells h}^{-1}$ in copper-treated cells and 0.709 ± 0.066 $\text{mmol l}^{-1}\text{cells h}^{-1}$ in control cells.

DISCUSSION

The present results show that the activity of the oxygen-sensitive potassium-chloride cotransporter in trout erythrocytes can be regulated by reactive oxygen species. It appears especially that hydroxyl radicals influence the transporter activity for the following reasons. First, removal of hydroxyl radicals with MPG inhibits the activation of the transporter by oxygen; and, second, catalysis of the hydroxyl radical-producing Fenton reaction by copper ions results in a dramatic activation of the transporter even at low O_2 levels.

The conclusion that hydroxyl radicals are removed

by treatment with MPG is based on several lines of evidence. The observations that the inhibition of transport by MPG is reversible and that MPG does not prevent NEM-induced activation of transport indicate that the inhibitory effect of MPG is not due to covalent binding of the molecule either to the transporter or proteins in the regulatory pathway of transporter activation. Furthermore, the observation that MPG does not inhibit the activation of potassium transport by NEM or volume disturbance indicates that the effect is specific for oxygen-dependent activation. Thus, it is probable that the effect of MPG is, indeed, due to removal of reactive oxygen species. With regard to the reactive oxygen species involved, EPR spectroscopy studies by Bolli et al. (1989) indicated that MPG removed hydroxyl radicals with little or no effect on superoxide radical or hydrogen peroxide levels.

The role of hydroxyl radicals in the activation of transport was strengthened by experiments with copper ions. Copper ions can act as catalysts for the Fenton reaction (Fig. 7). In the Fenton reaction, the proportion of superoxide anions and hydrogen peroxide is decreased, whereas the proportion of hydroxyl radicals is increased. Since K^+ flux was markedly increased as a result of this treatment, the result suggests that the hydroxyl radical is the dominant reactive oxygen species involved in the activation of potassium chloride cotransport. If either superoxide or hydrogen peroxide had been the active species, then copper treatment should have decreased the K^+ flux.

It has been suggested previously that the oxygen sensitivity of both sodium/proton exchange (Motais et al., 1987) and potassium-chloride cotransporter (Jensen, 1990; Borgese et al., 1991) in teleost erythrocytes would be controlled by oxy-deoxy transitions of hemoglobin within the cells. However, Berenbrink et al. (2000) observed that the oxygen dependency of transporter activity and of hemoglobin function differed from each other markedly. Thus, they concluded that the oxy-deoxy transition of cytoplasmic bulk hemoglobin is not

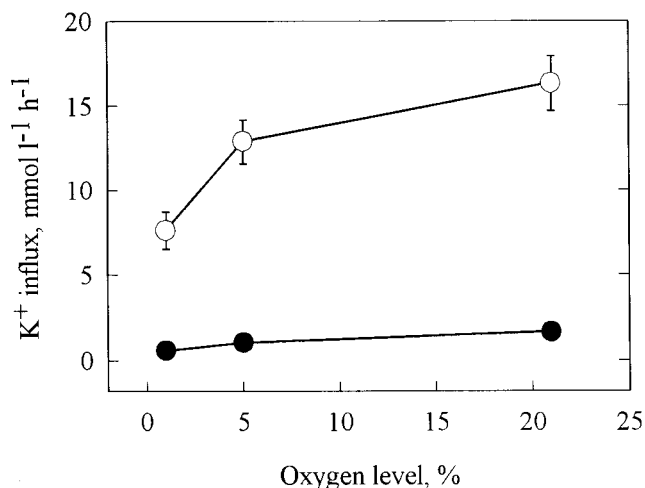


FIGURE 8. Chloride-dependent potassium fluxes in the presence of $25 \mu\text{M CuSO}_4$ as a function of oxygen (open circles). Closed circles indicate the chloride-dependent potassium fluxes in the absence of copper. (Means \pm SEM is given; $n = 4$.) The flux in the presence of copper was significantly ($P < 0.01$) greater at every oxygen level than the flux in the absence of copper. ANOVA followed by the LSD test was used for comparisons.

involved in the oxygen sensing and consequent activation of potassium-chloride cotransporter.

The present studies suggest that pathways leading to increased production of reactive oxygen species, especially hydroxyl radicals, could be involved in oxygen sensing. A role for reactive oxygen species in controlling oxygen-sensitive channel function in excitable cells has been demonstrated previously (Acker, 1994; Acker and Xue, 1995; Duranteau et al., 1998). The role of reactive oxygen species (ROS) production in modulating oxygen-sensitive ion transport requires that the production of oxygen radicals is proportional to the oxygen tension within the cells. Fandrey et al. (1994) have demonstrated that this is the case for a hepatocyte cell line, and we (Bogdanova, A.Y., and M. Nikinmaa, unpublished data) have found this to be the case also for fish hepatocytes. Unfortunately, data are not available for erythrocytes.

Although the present studies cannot indicate conclusively which step in the production of ROS, and, especially, hydroxyl radical formation, is the oxygen sensitive step, they suggest a possible new role of hemoglobin. Hydroxyl radicals can be formed from other ROS during the oxidation of hemoglobin to methemoglobin in the Fenton reaction (Sadrzadeh et al., 1984; Repka and Hebbel, 1991; Van Dyke and Saltman, 1996). Thus, regardless of the actual oxygen-sensitive step, the hemoglobin–methemoglobin conversion will increase the transporter activity via speeding up hydroxyl radical formation. Naturally, formation of methemoglobin itself may be oxygen-sensitive, although data on the oxygen dependence of hemoglobin–methemoglobin conversion are lacking. Several pieces of evidence are compatible with the role of hemoglobin–methemoglobin conversion in the formation of hydroxyl radicals, and activation of the potassium-chloride cotransporter. First, the methemoglobin levels of fish erythrocytes can be quite high: in rainbow trout 3–17% of the total hemoglobin is methemoglobin (Paa-jaste and Nikinmaa, 1991); and even higher levels (up to 27%) have been reported in other species of teleosts (Graham and Fletcher, 1986). Second, nucleated erythrocytes possess a powerful methemoglobin reduction pathway, which appears to be largely membrane-bound (for review see Nikinmaa, 1990). Third, Muzyamba et al. (2000), working on equine erythrocytes, observed that oxidant treatments that caused an accumulation of methemoglobin and depletion of reduced glutathione caused an increase in potassium chloride cotransport activity even in deep hypoxia. Thus, although it is becoming increasingly unlikely that the oxy–deoxy conformational changes of bulk hemoglobin would transduce the effects of oxygen on membrane transporters in fish erythrocytes, a possibility that oxidation–reduction reactions between hemoglobin and methemoglobin could be involved, is emerging. Alternatively, it is

possible that the actual oxygen sensitivity is conferred by a minor component of hemoglobin (Gibson et al., 2000) or by a heme protein altogether distinct from hemoglobins (Berenbrink et al., 2000).

Hydrogen peroxide was able to activate potassium-chloride cotransport in the trout red cell membrane. Similar activation has been observed for mammalian erythrocytes earlier (Bize et al., 1998); and hydrogen peroxide is capable of activating potassium transport pathways in excitable cells (Acker and Xue, 1995) and in alveolar epithelial cells (Heberlein et al., 2000). However, our present results suggest that H_2O_2 is not the primary transducer molecule of oxygen effects. To be effective, it appears that H_2O_2 needs to have access to heme Fe^{2+} to be converted to hydroxyl radicals (e.g., in the Fenton reaction catalyzed by membrane-bound hemoglobin) in close contact with a membrane moiety associated with the activation of the transporter. Since the half-life of hydroxyl radicals is very short (10^{-9} s), they need to be produced close to the target site, which is either the transporter itself, or a kinase/phosphatase-regulating transporter activity by phosphorylation. Notably, a role for membrane-bound hemoglobin in the activation of ion transport across erythrocyte membranes has been suggested in several studies (Motaïs et al., 1987; Jensen, 1992; Gibson et al., 2000).

Modification of sulfhydryl groups and of intracellular reduced glutathione levels generally influence potassium-chloride cotransport activity (Lauf, 1985; Lauf et al., 1992; Gibson et al., 2000). It is possible that some of these effects are direct effects on the sulfhydryl groups of the transporter required for activation (Lauf, 1985). However, some of the targets affected by sulfhydryl reagents are within the phosphorylation/dephosphorylation sequence involved in the activation of the transporter (Cossins et al., 1994; Flatman et al., 1996). On the basis of the present results, it is probable that the effects of hydroxyl radicals do not involve covalent sulfhydryl modification, since the effect was removed by washing, and since MPG did not prevent NEM-induced activation of transport.

In conclusion, the present results suggest that the oxygen-dependent activation of potassium-chloride cotransport in rainbow trout erythrocytes is caused by hydroxyl radicals that may be produced in a reaction catalyzed by membrane-bound hemoglobin. The present experiments were mainly carried out using 5% oxygen (oxygen tension ~ 5 kPa). This oxygen level is well within the range of physiological oxygen tensions in the blood of rainbow trout with arterial values ranging from 10 to 15 kPa and venous tensions ranging from 3 to 6 kPa (Nikinmaa and Soivio, 1982). Thus, the regulation of transport by oxygen (and hydroxyl radicals) is likely to be a part of normal physiological control of ion permeability.

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