J. Membrane Biol. 195, 33–42 (2003)
DOI: 10.1007/s00232-003-2042-8

The Journal of
Membrane
Biology





# Pivotal Role of Reduced Glutathione in Oxygen-induced Regulation of the $Na^+/K^+$ Pump in Mouse Erythrocyte Membranes

**A.Y. Bogdanova, O.O. Ogunshola, C. Bauer, M. Gassmann**
Institute of Veterinary Physiology and Institute of Physiology, University of Zürich, Zürich, Switzerland

Received: 18 October 2002/Revised: 2 June 2003

**Abstract.** This study addresses the mechanisms of oxygen-induced regulation of ion transport pathways in mouse erythrocyte, specifically focusing on the role of cellular redox state and ATP levels. Mouse erythrocytes possess $Na^+/K^+$ pump, $K^+$-$Cl^-$ and $Na^+$-$K^+$-$2Cl^-$ cotransporters that have been shown to be potential targets of oxygen. The activity of neither cotransporter changed in response to hypoxia-reoxygenation. In contrast, the $Na^+/K^+$ pump responded to hypoxic treatment with reversible inhibition. Hypoxia-induced inhibition was abolished in $Na^+$-loaded cells, revealing no effect of $O_2$ on the maximal operation rate of the pump. Notably, the inhibitory effect of hypoxia was not followed by changes in cellular ATP levels. Hypoxic exposure did, however, lead to a rapid increase in cellular glutathione (GSH) levels. Decreasing GSH to normoxic levels under hypoxic conditions abolished hypoxia-induced inhibition of the pump. Furthermore, GSH added to the incubation medium was able to mimic hypoxia-induced inhibition. Taken together these data suggest a pivotal role of intracellular GSH in oxygen-induced modulation of the $Na^+/K^+$ pump activity.

**Key words:** $Na^+/K^+$ pump — Mouse erythrocyte — Hypoxia — Glutathione

## Introduction

A number of ion transporters are regulated by oxygen in different tissues. Oxygen-sensitive ion channels are involved in oxygen sensing (Lopez-Barneo, Pardal & Ortega-Saenz, 2001). Hypoxia-induced modulation of a number of ion transport pathways plays an important role in acclimation of organisms to hypoxic conditions. Deactivation of the $Na^+/K^+$ pump coupled to a decrease in plasma membrane passive permeability to sodium and potassium ions (so-called "channel arrest") was shown to be a powerful adaptive strategy in hepatocytes and neurons of hypoxia-tolerant species (Hochachka & Lutz, 2001). Cell volume regulation under conditions of hypoxia-reoxygenation is involved in acute adjustments of hemoglobin oxygen-affinity in erythrocytes of different species (Nikinmaa, 2001). These oxygen-induced volume changes are mediated by joint action of a number of oxygen-sensitive transporters, including sodium-proton exchanger (NHE), potassium-chloride cotransporter (KCC) and sodium-potassium-chloride cotransporter (NKCC) (for review, see Gibson, Cossins & Ellory, 2000).

The inability of cells to sustain ion gradients under hypoxic/ischemic conditions results in rapid and irreversible damage of hypoxia-sensitive tissues (Silver & Erecinska, 1998). However, the exact mechanisms of oxygen-sensitivity of ion transporters are unclear. We have recently suggested that crosstalk between KCC and NHE in response to hypoxia-reoxygenation in trout red blood cells is mediated by reactive oxygen species (ROS), of which hydroxyl radical is the most important (Bogdanova & Nikinmaa, 2001; Nikinmaa, 2002; Nikinmaa, Bogdanova & Lecklin, 2003). However possible targets of reactive oxygen species (ROS) as well as mechanisms of ROS production, remain to be elucidated. Data generated recently suggest involvement of mitochondria in oxygen sensing (Chandel & Schumacker, 2000). Whether manipulating the redox status of a cell (non-protein thiol levels) can mimic hypoxia-reoxygenation remains a matter of debate. Moreover, one cannot exclude the possibility of participation of de novo synthesized proteins in triggering oxygen-induced regulation of ion transporters (e.g., Reeve et al., 2001; Colebrooke et al., 2002; Simkhovich et al., 2002). Although a lot of work

*Correspondence to*: A.Y. Bogdanova, Institute of Veterinary Physiology, Winterthurerstrasse 260, CH-8057, Zürich, Switzerland; email: annab@access.unizh.ch

on oxygen sensitivity of ion transporters has been done in nucleated fish erythrocytes, the system is by far too complicated to dissect the possible involvement of hypoxia-induced gene expression and mitochondria-derived ROS production in regulation of ion transport by oxygen. Therefore, in our current study, we used mammalian erythrocytes, which lack mitochondria and de novo protein synthesis, to eliminate the influence of these factors on oxygen-induced regulation of ion transporters. We concentrated specifically on mouse red blood cells since they possess NKCC and KCC as well as $Na^+/K^+$ pump (Benos, 1980; Benos & Tosteson, 1980), which were shown to be oxygen-sensitive in red blood cells of other species as well as in other cell types. Moreover, erythrocytes of genetically modified mouse strains give a unique possibility to characterize signal transduction pathways involved in oxygen-induced regulation. Very little is known about the oxygen sensitivity of ion transporters in mouse erythrocyte membrane. We monitored unidirectional $K^+$($^{86}$Rb) influx components mediated by NKCC-, KCC- and $Na^+/K^+$ pump, using selective inhibitors of these transport systems under hypoxic and normoxic conditions. Surprisingly, the $Na^+/K^+$ pump was the only transporter in mouse erythrocyte membranes that was inhibited by hypoxic treatment. Activity of both NKCC and KCC remained unaffected by hypoxia-reoxygenation. To date there are no reports on oxygen sensitivity of the pump in erythrocytes, although numerous studies indicate hypoxia-induced inhibition of this transporter in other cell types, including cardiac myocytes (Ziegelhoffer et al., 2000), neurons (Akaike, Jin & Koyama, 1997; Bruer et al., 1997; Tanaka et al., 1997), hepatocytes (Carini et al., 2000), lung epithelial tissue (Mairbaurl et al., 1997, 2002), and chromaffin cells (Inoue, Fujishiro & Imanaga, 1999, 2000). In our studies, hypoxia-induced inhibition of the pump in mouse red cells was rapid and proportional to the degree of deoxygenation. Decrease in the pump activity could not be attributed to ATP depletion since ATP levels remained constant during the experiment. Since the maximal rate of the enzyme function was not altered by hypoxia, decrease in oxygenation most probably affected the affinity of the transporter to substrate. Changes in cellular levels of reduced glutathione (GSH) in response to decreased $pO_2$ were of significant importance in the development of hypoxia-induced inhibition of the $Na^+/K^+$ pump.

## Materials and Methods

### Animals and Cell Preparation

Male C57BL6 mice, 12 to 20 weeks old, weighing 25–30 g were either purchased from RCC (Itlingen, Switzerland), Charles River Laboratories (Wilmington, MA) or raised in sterile breeding facilities at the Institute of Physiology, University of Zürich. The animals were kept on a commercial diet in accordance with institutional guidelines approved by the Veterinary Department of Canton Zürich. According to the same guidelines, animals were euthanized by subjecting them to $CO_2$ and blood taken by cardiac puncture (0.8–1.2 ml) was collected into heparinized syringes immediately after euthanasia. After washing twice in the standard incubation saline, a buffy coat was aspirated. The erythrocyte suspensions of several (2–3) animals were pooled together, washed twice more in the standard incubation saline and stored at 4°C for 30 min. Cells were subsequently resuspended at a hematocrit (Hct) of 35–45% and immediately used for further experiments.

### Chemicals and Solutions

All chemicals were of analytical grade. Inorganic salts were purchased from Fluka Chemie (Buchs, CH) and Sigma (St. Louis, MO). Bumetanide, ouabain, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's Reagent), reduced glutathione (GSH), nystatin, imidazole, trichloracetic acid (TCA), R-(+)-([2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydrol-oxo-1H-inden-5-yl]oxy)acetic acid (DIOA), 1,3-dichloro-4,6-dinitrobenzene (CDNB), sucrose and glucose were from Sigma, St. Louis, MO. $^{86}$Rb was purchased from Perkin Elmer. Calibrated gas mixtures were prepared by PanGas (Winterthur, CH). Stock solutions of ouabain (100 mM), bumetanide (10 mM) and DIOA (5 mM) were made in DMSO. Syringes were heparinized with heparin-Na, 25,000 I.E./5 ml (Braun Medical, Emmenbrücke, CH). All experiments were done at room temperature (20–22°C). Standard incubation saline contained (mM): 125 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 0.15 $MgCl_2$, 10 Tris-MOPS, 10 glucose, 10 sucrose (pH 7.40 at room temperature when equilibrated with a gas mixture containing 5% $CO_2$). When $K^+$ loss from cells into potassium-free medium was studied, KCl in the standard incubation medium was replaced by an isosmotic amount of NaCl. During nystatin treatment, cells were incubated in a $Na^+$-loading medium of the following composition (mM): 50 NaCl, 90 KCl, 15 sucrose, 2.5 HEPES-NaOH, pH 7.40 at 4°C. Washing solution contained (mM): 100 $Mg(NO_3)_2$ and 10 imidazole (pH 7.40 when ice-cold).

### Flux Measurements

To monitor unidirectional $K^+$ influx in murine erythrocytes, cell suspensions with hematocrit of 4–6% were added to standard incubation medium preequilibrated for 15 min with humidified hypoxic or normoxic gas mixtures. The condition termed "normoxic" applies to medium equilibrated with 21% $O_2$, 5% $CO_2$, 74% $N_2$ and "hypoxic"condition denotes medium equilibrated with 0.5% $O_2$, 5% $CO_2$, 94.5% $N_2$, unless stated otherwise. Before adding radioactive tracer ($^{86}$Rb), at the beginning of flux measurements cells were preincubated for 15 min in the presence of ion-transport inhibitors, unless stated otherwise. After 5 and 12 min of incubation with $^{86}$Rb, 0.8 ml aliquots were taken into Eppendorf tubes containing 0.7 ml ice-cold washing saline. Experimental conditions used provided linearity of tracer uptake during flux measurements. After centrifugation (1 min at $4000 \times g$), an aliquot of the supernatant was taken to evaluate the radioactivity of the incubation medium ($A_m$). The cells were washed twice more with ice-cold washing medium before lysis with 0.2 ml $H_2O$ and protein precipitation with 0.5 ml 5% trichloroacetic acid. An aliquot of supernatant was finally taken to estimate the tracer uptake by the cells ($A_c$). To calculate the unidirectional $K^+$ influx rate the following equation was used:

$$J = \frac{\frac{A_c}{A_m}[X]_e}{t}$$

where $A_c$ and $A_m$ are radioactivity of 1 ml of packed cells and 1 ml medium, respectively; $[X]_e$ is $K^+$ concentration in the incubation medium, and $t$ is the equilibration time with the tracer.

To measure $K^+$ loss from the cells, erythrocytes were incubated in $K^+$-free incubation medium for 10, 20, 30, 40 and 60 min at hypoxic or normoxic conditions. Aliquots (0.4 ml) were centrifuged for 2 min at 5000 × *g* and samples of supernatants were used for $K^+$ determination. $K^+$ concentration in the medium was measured by flame photometry (Flame photometer IL 943, Instrumentation Laboratories). The efflux rate coefficient was calculated from the slope of the curve of $\delta[K^+]_{medium}/t$, fitted using a linear regression analysis module (SigmaPlot 2000).

### Cell Ion Content, Nystatin-induced Cell $Na^+$ Loading and ATP Content Measurements

After 10, 20, 30, 40 and 60 min of hypoxic exposure (0.5% $O_2$) and after 10, 30, 60 min of incubation at normoxic conditions, aliquots (0.2 ml) of red blood cell suspension were taken and the cells washed 4 times with $Na^+/K^+$-free $Mg(NO_3)_2$-imidazole washing saline. The cell pellet was dried, weighed, and burned wet in concentrated $HNO_3$ (~65%, TraceSelectUltra, Fluka). Intracellular $Na^+$ and $K^+$ concentrations were determined by flame photometry (flame photometer IL 943).

To modify intracellular $Na^+/K^+$ content, cells were loaded with $Na^+$ in the presence of nystatin, as described elsewhere (Kaji, 1986). Briefly, cells at Hct of 5–8% were incubated for 15 min in $Na^+$-loading medium in the presence of 50 μg/ml of nystatin. Nystatin was then washed away with ice-cold nystatin-free $Na^+$-loading saline and the cells were resuspended in nystatin-free medium and incubated on ice for 15 min. Next, the cells were washed four times at room temperature in $Na^+$-loading medium containing 0.1% desalted albumin, pH 7.40 at 22°C. Finally, the cells were washed three times in standard incubation medium at room temperature. After resuspension in standard incubation medium at Hct of 40–35%, the cells were used for $K^+$ ($^{86}Rb$) influx measurements, as described above.

Aliquots were also taken for ATP measurements after 10, 20, 40 and 60 min of incubation at hypoxic and after 20, 40 and 60 min at normoxic conditions. Red blood cell pellets were washed once with ice-cold washing saline and proteins precipitated with 5% TCA. Supernatants were neutralized with 100 mM Tris and ATP levels assayed. For cell ATP measurements we used the Sigma adenosine 5′-triphosphate bioluminescent assay kit. Luminescence was measured with a luminometer (Lumomat LB 9507, EG&G Berthold).

### Oxyhemoglobin Levels

Erythrocyte suspensions in standard incubation saline were incubated at room temperature in Eschweiler tonometers gassed with humidified mixtures of the following composition: X% $O_2$ (0.5, 3, 4, 5, or 21), 5% $CO_2$, and (100-X)% $N_2$. Suspensions were allowed to equilibrate for 10 min prior to oxyhemoglobin measurements with a CO-oxymeter 282 (Instrumentation Laboratories). Total hemoglobin levels in the suspensions were 1.6–1.7 g/dl, which corresponded to the hematocrit used for the flux experiments.

### Nonprotein Thiol Measurements

Ellman's reagent was used to measure nonprotein thiols in red blood cells, as described previously (Tietze, 1969). In brief, red blood cell suspensions were incubated at hypoxic or normoxic conditions with or without CDNB and samples were taken after 5, 10, 15, 30, 45 and 60 min of incubation. After centrifugation for 3 min at 5000 × *g*, supernatants were aspirated and cell pellets lysed in a precipitation solution, containing 1.67 g of glacial metaphosphoric acid, 0.2 g $Na_2EDTA$ and 30 g NaCl in 100 ml $ddH_2O$. To reach complete hemoglobin denaturation, lysates were left for 10 min at room temperature. After centrifugation (15 min 5000 × *g*), supernatants were assayed for nonprotein thiols (most of which in red blood cells are in the form of reduced glutathione). GSH was evaluated spectrophotometrically using a Lambda 25 UV/VIS spectrometer (Perkin Elmer). Absorption of complexes of GSH with DTNB was measured at 412 nm and a calibration curve made after each experiment with commercially available GSH standard (Sigma).

## Results

### Effect of Hypoxia on Different Ion Transport Pathways in Mouse Erythrocytes

The effect of hypoxic exposure on different components of $K^+$ influx in mouse erythrocytes was studied. The absolute value of total $K^+$ influx, as well as its active component, showed marked inter-individual variation (5 mmol $l^{-1}$ $h^{-1}$ vs 2–2.5 mmol $l^{-1}$ $h^{-1}$ for total influx). However, hypoxic responses of the active $K^+$ influx were identical and independent of the absolute values of fluxes. Influx measurements in the presence of selective inhibitors of the $Na^+/K^+$ pump (1 mM ouabain), NKCC (50 μM bumetanide) and KCC (25 μM DIOA) revealed that all ion transporters contribute to the total $K^+$ influx under normoxic conditions (Fig. 1). Reducing oxygen levels from 21% to 0.5% resulted in a decrease of total $K^+$ influx due to inhibition of the $Na^+/K^+$ pump (Fig. 1). Both KCC and NKCC in mouse erythrocytes were not significantly affected by hypoxia. Moreover, neither cotransporter responded to reoxygenation (*data not shown*). Therefore, further attention was concentrated on hypoxia-induced inhibition of the $Na^+/K^+$ pump.

### Characteristics of Hypoxia-induced Inhibition of the $Na^+/K^+$ Pump

Hypoxia-induced inhibition of the active $K^+$ influx was statistically significant already after 3 min of incubation of the cells at hypoxic conditions (Fig. 2). Half-inhibition was achieved after 3.9 ± 0.9 min and maximal inhibition of the $Na^+/K^+$ pump was reached already after 15 min of incubation at 0.5% $O_2$ (Fig. 2). In a separate set of experiments we were able to show partial reversibility of hypoxia-induced inhibition of the pump by reoxygenation. Active $K^+$ transport decreased from 1.08 ± 0.23 to 0.42 ± 0.14 mmol $l^{-1}$ $h^{-1}$ after 20 min of hypoxic exposure but resumed to 0.68 ± 0.06 mmol $l^{-1}$ $h^{-1}$ after 20 min reoxygenation at 21% $O_2$.

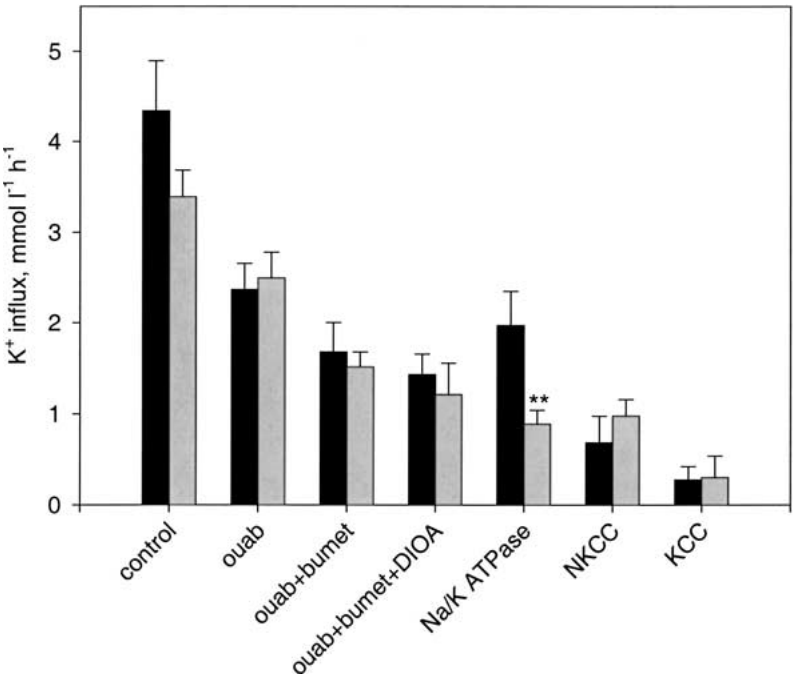


**Fig. 1.** Effect of hypoxia on different components of unidirectional $K^+$ influx. Mouse erythrocytes were incubated in media equilibrated to normoxic (21% $O_2$, *black bars*) or hypoxic (0.5% $O_2$, *gray bars*) conditions in the presence or absence of inhibitors of $Na^+/K^+$ pump (ouabain, 1 mM); $Na^+$-$K^+$-$2Cl^-$ cotransporter (bumetanide, 50 μM); $K^+$-$Cl^-$ cotransporter (DIOA, 25 μM). Presented values are means of 6 independent experiments ± SEM. **denotes $p < 0.01$.

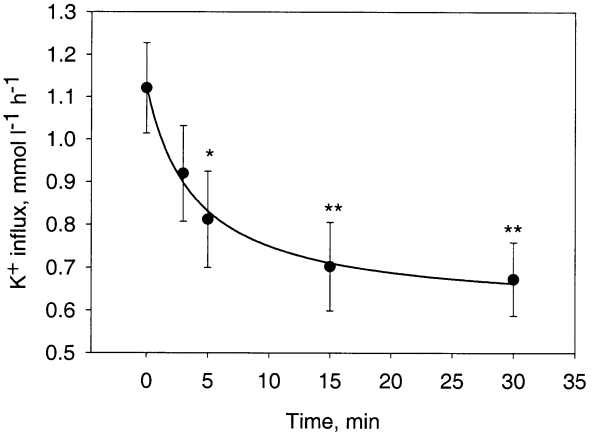


**Fig. 2.** Time dependence of hypoxia-induced inhibition of active $K^+$ influx. Cells were incubated for various time periods in media equilibrated with 0.5% $O_2$. Data are means of 4 independent experiments ± SEM. *denotes $p < 0.05$, and ** stands for $p < 0.01$.

We found that ouabain-sensitive (active) $K^+$ influx was a function of the $pO_2$ (Fig. 3). Ouabain-resistant $K^+$ influx remained oxygen-independent, whereas active $K^+$ influx showed half-maximal inhibition at 5% $O_2$, with full inhibition reached at 3% $O_2$ (Fig. 3*A*). Notably, in venous blood, $pO_2$ values correspond to 30–40 mmHg, i.e., well within the range of oxygen concentrations where $Na^+/K^+$ pump inhibition was observed.

Whether oxygen-induced regulation of ion transporters in erythrocytes of different species is directly linked to conformational changes of hemoglobin when changing from oxy- to deoxy-conformation is a matter of debate. In mouse red cells active transport was a sigmoid function of oxygen level (Fig. 3*B*). When compared with the oxy-hemoglobin formation profile in the range of oxygen levels of 0.5–21% $O_2$ no correlation could be observed between hypoxia-induced inhibition of the pump and bulk hemoglobin oxygen saturation. At $pO_2$ corresponding to 50% inhibition of the active $K^+$ influx, oxyhemoglobin levels reached 92% saturation (Fig. 3*B*).

## Effect of Hypoxia on Cellular $Na^+/K^+$ Content

The $Na^+/K^+$ pump is involved in maintenance of transmembrane $Na^+/K^+$ gradients. The possible impact of hypoxia-induced inhibition of the pump on cellular $Na^+/K^+$ content was evaluated in the next set of experiments. First, $K^+$ loss from mouse erythrocytes into potassium-free medium was measured with no significant difference observed in $K^+$ loss from the cells incubated under hypoxic (0.5% $O_2$) and normoxic conditions for one hour (0.093 ± 0.007 $h^{-1}$ in normoxia *vs.* 0.104 ± 0.006 $h^{-1}$ under hypoxic conditions). These observations suggest that hypoxia resulted in a gradual net $K^+$ loss from the cells. However, measurements of intracellular $Na^+$ and $K^+$ content revealed no significant loss of $K^+$ from the cells during 45 min of equilibration at hypoxic conditions or during reoxygenation (Fig. 4). When we compared $K^+$ influx and efflux rates, it was evident that $K^+$ loss occurs during hypoxic conditions at a very low rate that could not be reliably measured under our experimental conditions. Approximately 5 mmol $l^{-1}$ $h^{-1}$ of $K^+$ entered the cells and about

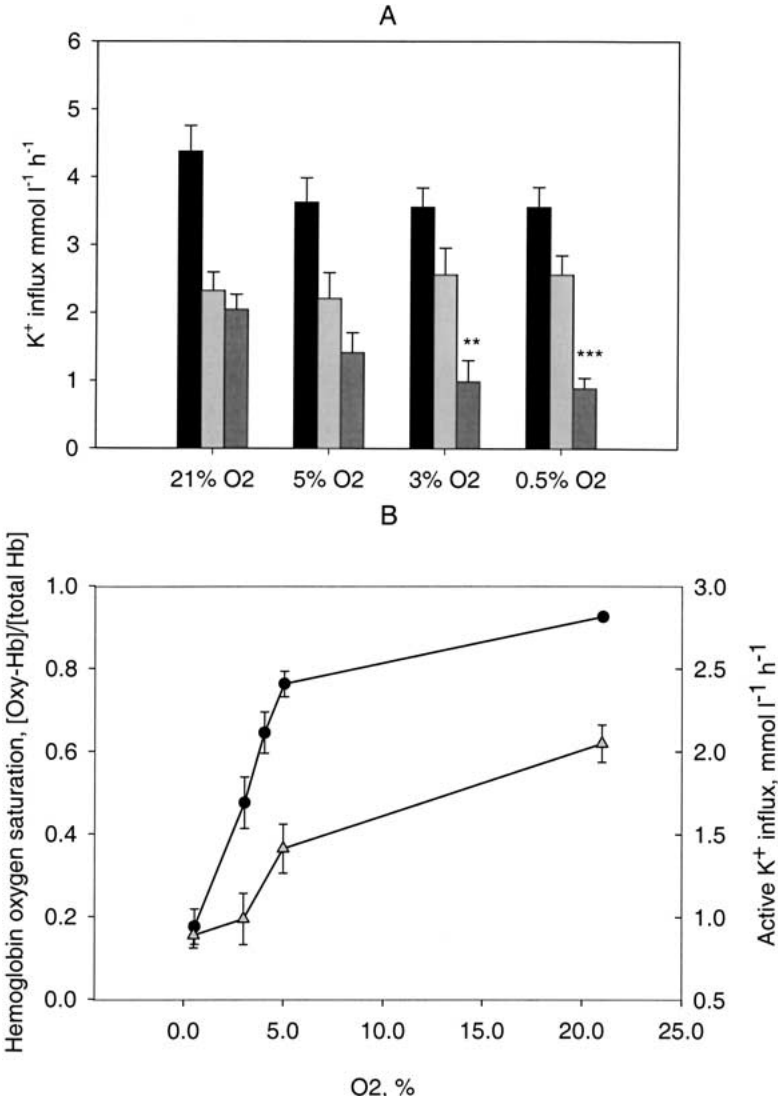


**Fig. 3.** Activity of the $Na^+/K^+$ pump in mouse red cells as a function of oxygen levels. (*A*) Effect of decreased oxygen levels on total $K^+$ uptake (*black bars*), ouabain-insensitive (*light-gray bars*) and active, ouabain-sensitive (*dark-gray bars*) components of $K^+$ influx. Flux was evaluated at various $pO_2$ after preequilibration with 1 mM ouabain for 15 min. Values are means of 5 independent experiments. (*B*) The $Na^+/K^+$ pump activity (*triangles*, right ordinate) and oxyhemoglobin levels (*circles*, left ordinate) in mouse erythrocytes at different oxygen concentrations. Values are means of 5 independent experiments. ** denotes $p < 0.01$, *** denotes $p < 0.001$ compared to normoxic control.

10–12 mmol $l^{-1}$ $h^{-1}$ was lost under hypoxic conditions, making the net $K^+$ loss about 5 mmol $l^{-1}$ $h^{-1}$. These changes account for the net $K^+$ loss of about 4% of total cell $K^+$ content in 1 h and therefore are hard to monitor.

## Effect of Hypoxia on the Pump under Saturated Conditions

To test if hypoxia-induced inhibition of the pump holds true under conditions of transporter saturation, the cells were loaded with $Na^+$ in the presence of nystatin (*see* Methods). Final sodium concentration in the cells after loading was 53 ± 3 mmol $l_{cells}^{-1}$compared to 12.8 ± 0.8 mmol $l_{cells}^{-1}$ in non-treated cells. No significant changes in cell (Na + K)-content occurred after loading, revealing no changes in cell volume (84.5 ± 8.5 mmol $l_{cells}^{-1}$ in control cells vs 95.7 ± 14.5 mmol $l_{cells}^{-1}$ in the cells after $Na^+$ loading). Ouabain-resistant $K^+$ influx was increased in nystatin-treated cells without hemolysis and the flux levels remained stable within the time of incubation. The active component of $K^+$ influx increased from 1.02 ± 0.17 mmol $l^{-1}h^{-1}$ to 3.40 ± 0.22 mmol $l^{-1}h^{-1}$ in the cells loaded with $Na^+$ under normoxic conditions (Fig. 5). Hypoxic exposure of the $Na^+$-loaded cells did inhibit the pump (3.40 ± 0.22 under normoxic conditions *vs.* 3.49 ± 0.32 mmol $l^{-1}$ $h^{-1}$ for the cells equilibrated at 0.5% $O_2$).

## ATP Levels in RBC at Hypoxic Conditions

Oxidative phosphorylation does not occur in enucleated erythrocytes. However, the activity of some glycolytic enzymes was shown to be dependent on the oxygen level in the environment (Sukhomlinov, Antoniak & Ikpu, 1990). Therefore, one possible mechanism of hypoxia-induced decrease in activity of the $Na^+/K^+$ pump could be a significant decrease in cellular ATP levels. Intracellular ATP concentrations

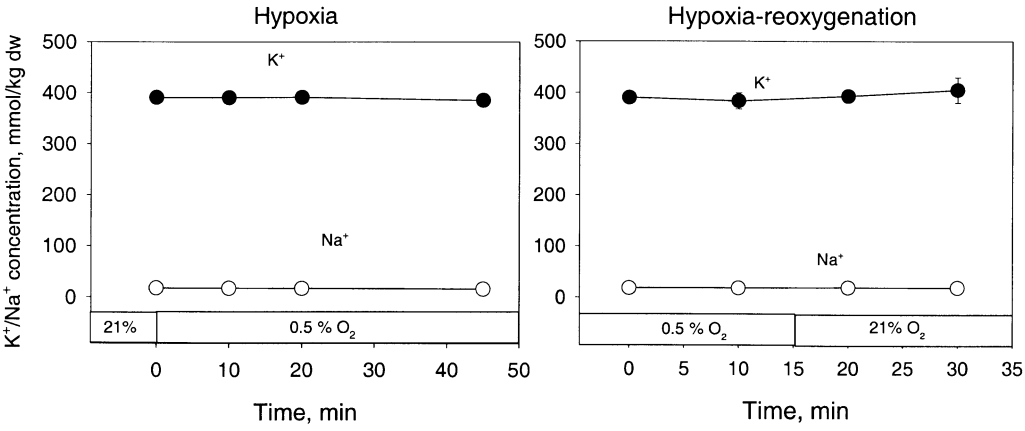


**Fig. 4.** Effect of hypoxia-reoxygenation on $Na^+$ (*empty circles*) and $K^+$ (*filled circles*) contents of mouse erythrocytes. Cells were equilibrated either at 21% $O_2$ (normoxic conditions, reoxygenation) or at 0.5% $O_2$ (hypoxic conditions). Bars indicate appropriate treatments in the course of experimental run. Data are averages of 4 independent experiments ± SEM.

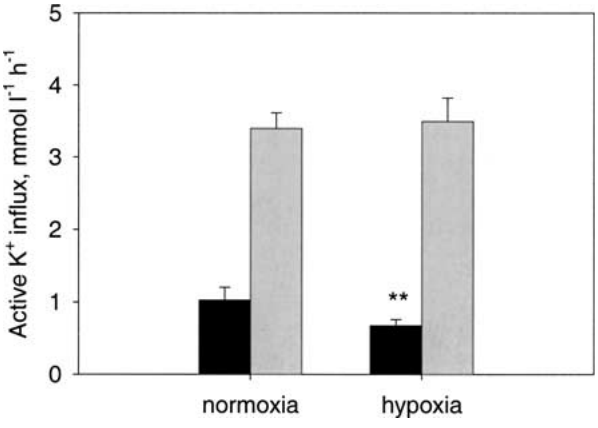


**Fig. 5.** Effect of hypoxia on $V_{max}$ of the $Na^+/K^+$ pump in mouse red blood cells. Cells were preloaded with 50 mM $Na^+$ in the presence of nystatin and incubated with or without 1 mM ouabain at normoxic (21% $O_2$) or hypoxic (0.5% $O_2$) conditions. Black bars denote control cells and gray bars represent $Na^+$-preloaded cells. Values are means of 5 independent experiments ±SEM, **denotes $p < 0.01$ compared to normoxic control.

were assayed in cells incubated at normoxic and hypoxic conditions for one hour. Samples were taken every 10 min but no statistically significant changes in cellular ATP level were observed within the experimental run. Final values after 1 h of exposure to 21% and 0.5% $O_2$ were 1.93 ± 0.29 and 2.02 ± 0.22 $mmol/l_{cells}$ respectively.

## Effect of Hypoxia on GSH Levels in the Cells and GSH-dependence of the Pump Activity

Measurements of nonprotein thiol concentrations revealed that GSH levels in the cells remained stable under normoxic conditions for one hour (Fig. 6*A*). Hypoxic exposure resulted in a rapid ($t_{1/2}$ = 6.5 ± 1.5 min) increase in cellular nonprotein thiol levels, which are mostly represented by glutathione in red cells. This rapid response was not due to an increase in GSH synthesis, since inhibition of gamma-glutamyl-cystein synthetase by buthionine sulfoximine (BSO) did not abolish the effect (Griffith & Meister, 1985). However, in erythrocytes, which are rich in ferrous ions and reducing enzymes, significant amounts of oxygen undergo reduction, forming ROS in the amounts proportional to $pO_2$ (Comporti, 2002). Therefore, reduction in oxygen levels would also decrease the amount of ROS and prevent GSH oxidation.

To study the possible involvement of cellular GSH in modulation of hypoxia-induced inhibition of the $Na^+/K^+$ pump, cells were depleted of GSH by treatment with a non-oxidative conjugating agent, l-chloro-2, 4-dinitrobenzene (CDNB), that forms dinitrophenolic ethers with GSH in the presence of glutathione S-transferase. CDNB at concentrations of 0.2–2 mM has been broadly used in studies of the role of GSH in regulation of KCC in erythrocyte membrane, e.g., Lauf, Adragna & Agar, 1995; Adragna & Lauf, 1997; Fujise et al., 2001. In mouse erythrocytes, cellular GSH concentrations fell by 90% in the presence of 1 mM CDNB under normoxic conditions, with $t_{1/2}$ of 2.3 ± 0.3 min. The effect was also profound under hypoxic conditions (Fig. 6*A*). Marked reduction in GSH was followed by a decrease in active $K^+$ influx at both high and low $pO_2$ so that the levels of $Na^+/K^+$ pump in GSH-depleted cells did not differ from those for CDNB-untreated cells under hypoxic conditions.

Since treatment with 1 mM CDNB mimicked oxidative stress, GSH titration was carried out with lower doses of CDNB in parallel with hypoxic exposure. As can be seen in Fig. 7*B*, GSH levels in cells incubated with 0.1 mM CDNB for 5–30 min under hypoxic conditions did not differ significantly from that in normoxic cells. Further increase in CDNB

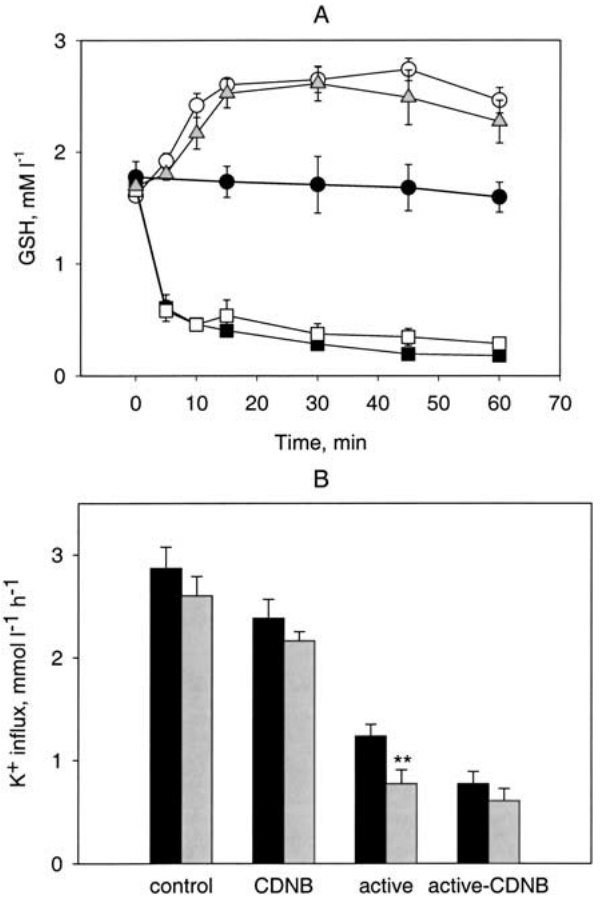


**Fig. 6.** Effect of hypoxia on cellular GSH levels and interaction between hypoxia-induced changes in GSH levels and the $Na^+/K^+$ pump activity. (*A*) Cellular GSH content under normoxic (*filled circles*) or hypoxic (*empty circles*) conditions. The cells were per-incubated with 1 mM BSO (*triangles*) for 20 min before hypoxic insult. Treatment with the GSH-depleting agent CDNB (1 mM) under normoxic (*filled squares*) or hypoxics (*empty squares*) conditions started at $t_0$. Results are means of 5 independent experiments ± SEM. (*B*) Total, ouabain-resistant and ouabain-sensitive components of $K^+$ influx under normoxic (*black bars*) or hypoxic (*gray bars*) conditions with or without 1 mM CDNB. Results are means of 5 independent experiments, **denotes $p < 0.01$ compared to normoxic control.

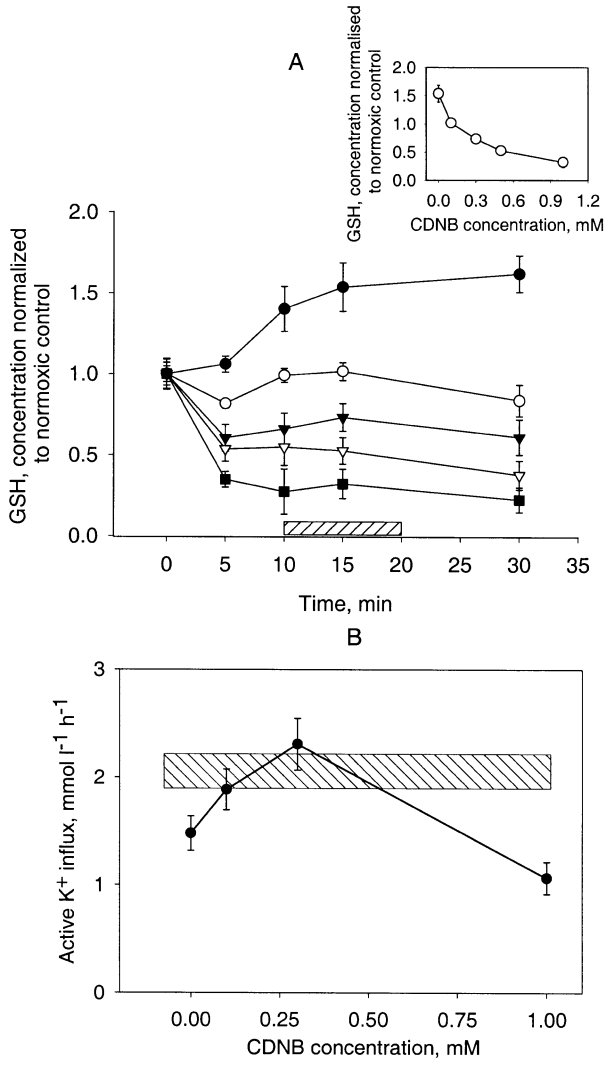


**Fig. 7.** Active $K^+$ influx as a function of intracellular GSH concentration under hypoxic conditions. (*A*) Kinetics of GSH depletion in cells treated with various CDNB concentrations under hypoxic conditions. After 15 min equilibration to normoxic condition, GSH levels were measured at $t_0$ and all the following measurements were normalized to the initial levels. At $t_0$ CDNB was added to the cell suspension with simultaneous decrease in oxygen levels to 0.5% $O_2$. *Filled circles* denote GSH concentration in CDNB-free cells under hypoxic conditions. Alternatively, the cells were exposed to hypoxia in the presence of 0.1 mM (*empty circles*), 0.3 mM (*filled triangles*), 0.5 mM (*empty triangles*), or 1 mM (*filled squares*) CDNB. The hatched bar indicates the time interval used for $K^+$-influx measurements shown in panel *B*. *Inset* presents GSH levels in cells treated with various concentrations of CDNB for 30 min under hypoxic conditions. Data are means of 4–7 independent experiments. (*B*) Active (ouabain-sensitive) $K^+$ influx into the cells incubated in the presence of different CDNB concentrations under hypoxic conditions (0.5% $O_2$). The hatched bar indicates the interval of active $K^+$ transport rates for the cells under normoxic conditions (21% $O_2$) incubated in CDNB-free media. Values are means of 4–7 experiments.

concentrations caused a dose-dependent decrease in GSH levels. Active $K^+$ influx was evaluated in the cells under conditions similar to those used for GSH determination within the period indicated in Fig. 7*A* (hatched bar). Figure 7*B* shows a decrease in active $K^+$ influx that occurred when cellular GSH levels were altered compared to those maintained under normoxic conditions (observed in cells treated with 0.1 and 0.3 mM CDNB under hypoxic conditions). Notably, when applied to normoxic cells, all concentrations of CDNB used suppressed the pump activity (*data not shown*).

Thus our data suggests an important role of GSH in regulation of the $Na^+/K^+$ pump in mouse erythrocytes. To further support this hypothesis, active $K^+$ influx was measured in cells pretreated for 40 min with 5 mM extracellular GSH, which resulted in a 10–15% increase in cellular GSH levels. It has previously

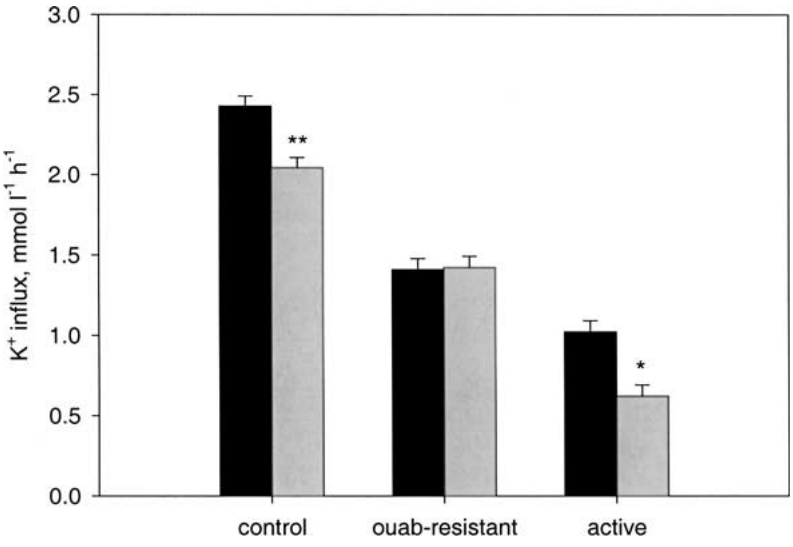


**Fig. 8.** $K^+$ influx in cells incubated under normoxic conditions (21% $O_2$) in the presence (*light-gray bars*) or in the absence (*black bars*) of 5 mM GSH. Cells were incubated for 40 min with GSH prior to the flux measurements. *denotes $p < 0.05$, **denotes $p < 0.01$. Values are means of 4 independent experiments.

been shown that GSH is able to be slowly and passively transported through the cell membrane (Griffith & Meister, 1985). As shown in Fig. 8, GSH treatment resulted in a decrease in total $K^+$ influx, which was solely due to a partial inhibition of the active $K^+$ flux component. Notably, N-acetyl cysteine, which has much higher permeability rates but has to be deacetylated to cysteine prior to GSH synthesis, at a concentration of 5 mM did not have any inhibitory effects on the $Na^+/K^+$ pump (*data not shown*). Therefore, our data suggest that GSH specifically, rather than any reduced thiol, is a regulator of the pump activity.

## Discussion

Our study is the first to show oxygen sensitivity of the $Na^+/K^+$ pump in an erythrocyte membrane. A second important message from our findings is that changes in cellular GSH levels, rather than ATP depletion or de novo protein synthesis, control hypoxia-induced pump inhibition in mouse red blood cell membrane.

### Characterization of the Hypoxia-induced Inhibition of the $Na^+/K^+$ Pump

So far in red blood cells no response of the $Na^+/K^+$ pump to hypoxia-reoxygenation has been reported. Our observation that in mouse red blood cells the $Na^+/K^+$ pump is the only transporter affected by reduction in oxygen level is remarkable per se. Hypoxic responses of the pump in mouse red blood cells were observed in the physiological range of $pO_2$ (half-inhibition at 5% $O_2$, Fig. 3). This hypoxia-induced inhibition was not complete, but to approximately half of the normoxic operation rate of the pump, and could be reversed by reoxygenation. Increased intracellular $Na^+$ levels abolished responses of the pump to hypoxia (Fig. 5). This would lead to reduction in cellular ATP consumption under hypoxic conditions, but at the same time prevent irreversible dissipation of transmembrane $Na^+/K^+$ gradients. The fact that hypoxia-induced inhibition of the pump in mouse erythrocytes is not linked to ATP depletion is another very important message. ATP depletion was suggested to be the cause of hypoxia-induced pump inhibition in cardiac myocytes (Ziegelhoffer et al., 2000) and chromaffin cells (Inoue et al., 1999, 2000). From our findings, lack of ATP is not an imperative prerequisite for hypoxia-induced inhibition of the $Na^+/K^+$ pump in red blood cells and, therefore, existence of alternative regulatory mechanisms in other cell types should be strongly considered.

Notably, mouse red blood cells do not have mitochondria or protein-synthesis machinery. Thus, hypoxia-induced inhibition of the pump can occur without mitochondria-derived ROS generation or production of hypoxia-inducible protein factors affecting the pump activity.

### Possible Mechanisms of Hypoxia-induced Inhibition of the Pump

The question arises, what is a possible mechanism behind hypoxia-induced changes in the pump activity? Signal transduction pathways should not include too many intermediate steps nor result in irreversible damage of the transporter since the pump activity changes abruptly and can be at least partially reversed (*see* Results section and Fig. 2). Although at present we do not possess enough information to suggest a signalling sequence, we would like to speculate on some possible mechanisms.

Our results suggest that cellular GSH levels play an important role in signaling to the pump. Indeed,

we have shown that in mouse red cells activity of the pump was intimately linked to the amount of intracellular GSH. The latter in turn was a function of $pO_2$ with hypoxic exposure resulting in a rapid increase in GSH levels. Quite unexpectedly, both decreases and increases in cellular GSH levels compared to "normoxic" levels resulted in a decrease in pump activity. On the basis of these results we suggest that hypoxia-induced increases in GSH caused hypoxia-induced inhibition of the pump. In all studies known to the authors GSH-dependence of ion transporters was investigated only under conditions of GSH depletion, focusing on susceptibility of different ion transport pathways to oxidative stress. Oxidative stress results in inhibition of the $Na^+/K^+$ pump in different cell types (Boldyrev & Bulygina, 1997; Kurella et al., 1997; Dobrota et al., 1999). In particular, in cardiac myocytes, the $Na^+/K^+$ pump activity (measured as $P_i$ production) was shown to be a function of intracellular GSH (Haddock, Woodward & Hearse, 1995). Similar to mouse red blood cells, in cardiomyocytes, GSH depletion using conjugating agents under normoxic conditions caused a decrease in the $Na^+/K^+$ pump activity to about half of initial levels. Pump function could be rescued by addition of permeable GSH derivative to SH-depleted cells (glutathione isopropyl ester).

Whether GSH can directly interact with SH groups of the transporter or target another intermediate effector (e.g., redox-sensitive kinase) that would then modulate the pump's activity remains to be investigated (Miller et al., 1990; Ferrari et al., 1991; Sies et al., 1998). It is tempting to speculate that PKC, shown to play an important role in coupling between hypoxia-induced responses of the $Na^+/K^+$ pump and cation channel activity in hepatocytes of hypoxia-tolerant species, such as *Chrysemys picta bellii* (western painted turtle), might also be regulated by hypoxia-driven changes in GSH levels (Buck & Hochachka, 1993). Modulation of phosphorylation of the alpha subunit of the pump by PKC is known to be involved in reduction of ATP consumption under hypoxic conditions in rat convoluted tubule (Feraille et al., 1995). Notably, the activity of a number of PKC isoforms was indeed shown to be GSH-dependent (Domenicotti et al., 2000). Our ongoing studies are aimed at evaluation of the possible role of PKC in modulation of activity of the $Na^+/K^+$ pump.

### Passive $K^+$ Transport Pathways in Mouse Erythrocyte Membrane Are Oxygen-insensitive

Interestingly, in mouse red blood cells neither KCC nor NKCC responded to hypoxia-reoxygenation. Responses of ion transporters to the changes in $pO_2$ in erythrocyte membranes were proven to be highly species-specific (for review *see* Gibson et al., 2000). However, it remains unclear why in avian red cells NKCC is activated under hypoxic conditions while remaining unaffected by oxygen in erythrocytes of other species studied, including mouse. In red blood cells of trout and horse, activity of KCC is abolished completely in response to hypoxia; in sheep red cells, a substantial fraction of $Cl^-$-dependent $K^+$ transport is still present under hypoxic conditions, whereas in mouse red cells KCC did not respond to hypoxic treatment (Campbell & Gibson, 1998; Berenbrink et al., 2000). Since many parameters contribute to regulation of these transporters, including pH, DPG and free $Mg^{2+}$ levels as well as multiple phosphorylation-dephosphorylation steps, it is hard to select single variables responsible for development of oxygen-induced responses (Gibson et al., 2000). Notably, GSH depletion results in decrease in activity of both NHE (Cutaia & Parks, 1994) and KCC (Lauf et al., 1995; Adragna & Lauf, 1998; Fujise et al., 2001) in different cell types, suggesting that GSH is able to tune activity of several ion transporters at the same time. To date no data are available on the effects of increased cellular GSH on activity of these transporters.

In conclusion, this study provides evidence for a profound importance of cellular GSH levels in regulation of the pump under conditions of hypoxia-reoxygenation. Further investigations are needed to evaluate the chemistry of the GSH-$Na^+/K^+$ pump interaction. The alpha subunit of the $Na^+/K^+$ pump is represented by the alpha-1 isoform in erythrocyte membranes of most species (Sweadner, 1989), which is also the one ubiquitously expressed in all cell types. However, it remains to be established if a mechanism of hypoxia-induced inhibition of the pump observed for mouse red cells is specific for erythrocytes, or the same regulatory mechanism is present in other cell types, such as cardiac myocytes and neurons.

This study was supported by Swiss National Science Foundation (No 31-56743.99) for M.Gassmann, Olga Mayenfisch scholarship for A.Bogdanova and EMDO-scholarship for O.Ogunshola. We also thank Thomas Hofer for useful comments on the manuscript.

## References

Adragna, N.C., Lauf, P.K. 1997. Oxidative activation of K-Cl cotransport by diamide in erythrocytes from humans with red cell disorders, and from several other mammalian species. *J. Membrane Biol.* **155:**207–217

Adragna, N.C., Lauf, P.K. 1998. Role of nitrite, a nitric oxide derivative, in K-Cl cotransport activation of low-potassium sheep red blood cells. *J. Membrane Biol.* **166:**157–167

Akaike, N., Jin, Y.H., Koyama, S. 1997. Functional interaction between $Na^+$-$K^+$ pump and KATP channels in the CNS neurons under experimental brain ischemia. *Jpn. J. Physiol.* **47:**S50–S51

Benos, D.J. 1980. Intracellular analysis of sodium, potassium, and chloride in mouse erythrocytes. *J. Cell Physiol.* **105:**185–187

Benos, D.J., Tosteson, D.C. 1980. Cation movements across mouse red blood cells. *Biochim. Biophys. Acta* **601:**167–179

Berenbrink, M., Volkel, S., Heisler, N., Nikinmaa, M. 2000. $O_2$-dependent $K^+$ fluxes in trout red blood cells: the nature of $O_2$ sensing revealed by the $O_2$ affinity, cooperativity and pH dependence of transport. *J. Physiol.* **526:**69–80

Bogdanova, A.Y., Nikinmaa, M. 2001. Reactive oxygen species regulate oxygen-sensitive potassium flux in rainbow trout erythrocytes. *J. Gen. Physiol.* **117:**181–190

Boldyrev, A.A., Bulygina, E.R. 1997. Na/K-ATPase and oxidative stress. *Ann. N. Y. Acad. Sci.* **834:**666–668

Bruer, U., Weih, M.K., Isaev, N.K., Meisel, A., Ruscher, K., Bergk, A., Trendelenburg, G., Wiegand, F., Victorov, I.V., Dirnagl, U. 1997. Induction of tolerance in rat cortical neurons: hypoxic preconditioning. *FEBS Lett.* **414:**117–121

Buck, L.T., Hochachka, P.W. 1993. Anoxic suppression of $Na^+$-$K^+$-ATPase and constant membrane potential in hepatocytes: support for channel arrest. *Am. J. Physiol.* **265:**R1020–R1025

Campbell, E.H., Gibson, J.S. 1998. Oxygen-dependent $K^+$-fluxes in sheep red cells. *J. Physiol.* **506**:679–688

Carini, R., De Cesaris, M.G., Splendore, R., Bagnati, M., Albano, E. 2000. Ischemic preconditioning reduces $Na^+$ accumulation and cell killing in isolated rat hepatocytes exposed to hypoxia. *Hepatology* **31:**166–172

Chandel, N.S., Schumacker, P.T. 2000. Cellular oxygen sensing by mitochondria: old questions, new insight. *J. Appl. Physiol.* **88:** 1880–1889

Colebrooke, R.L., Smith, I.F., Kemp, P.J., Peers, C. 2002. Chronic hypoxia remodels voltage-gated $Ca^{2+}$ entry in a human airway chemoreceptor cell line. *Neurosci. Lett.* **318:**69–72

Comporti, M. 2002. Introduction-serial review: iron and cellular redox status. *Free Radic. Biol. Med.* **32:**565–567

Cutaia, M., Parks, N. 1994. Oxidant stress decreases $Na^+/H^+$ antiport activity in bovine pulmonary artery endothelial cells. *Am. J. Physiol.* **261:**L649–L659

Dobrota, D., Matejovicova, M., Kurella, E.G., Boldyrev, A.A. 1999. Na/K-ATPase under oxidative stress: molecular mechanisms of injury. *Cell. Mol. Neurobiol.* **19:**141–149

Domenicotti, C., Paola, D., Vitali, A., Nitti, M., d'Abramo, C., Cottalasso, D., Maloberti, G., Biasi, F., Poli, G., Chiarpotto, E., Marinari, U.M., Pronzato, M.A. 2000. Glutathione depletion induces apoptosis of rat hepatocytes through activation of protein kinase C novel isoforms and dependent increase in AP-1 nuclear binding. *Free Radic. Biol. Med.* **29:**1280–1290

Feraille, E., Carranza, M.L., Buffin-Meyer, B., Rousselot, M., Doucet, A., Favre, H. 1995. Protein kinase C-dependent stimulation of $Na^+$-$K^+$-ATP epsilon in rat proximal convoluted tubules. *Am. J. Physiol.* **268:**C1277–C1283

Ferrari, R., Ceconi, C., Curello, S., Cargnoni, A., Alfieri, O., Pardini, A., Marzollo, P., Visioli, O. 1991. Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. *Am. J. Med.* **91:**95S–105S

Fujise, H., Higa, K., Kanemaru, T., Fukuda, M., Adragna, N.C., Lauf, P.K. 2001. GSH depletion, K-Cl cotransport, and regulatory volume decrease in high-K/high-GSH dog red blood cells. *Am. J. Physiol.* **281:**C2003–C2009

Gibson, J.S., Cossins, A.R., Ellory, J.C. 2000. Oxygen-sensitive membrane transporters in vertebrate red cells. *J. Exp. Biol.* **203:**1395–1407

Griffith, O.W., Meister, A. 1985. Origin and turnover of mitochondrial glutathione. *Proc. Natl. Acad. Sci. USA* **82:**4668–4672

Haddock, P.S., Woodward, B., Hearse, D.J. 1995. Cardiac $Na^+$/$K^+$-ATPase activity and its relation to myocardial glutathione status: studies in the rat. *J. Mol. Cell Cardiol.* **27:**1185–1194

Hochachka, P.W., Lutz, P.L. 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **130:**435–459

Inoue, M., Fujishiro, N., Imanaga, I. 1999. $Na^+$ pump inhibition and non-selective cation channel activation by cyanide and anoxia in guinea-pig chromaffin cells. *J. Physiol.* **519:**385–396

Inoue, M., Fujishiro, N., Imanaga, I. 2000. Retardation of cation channel deactivation by mitochondrial dysfunction in adrenal medullary cells. *Am. J. Physiol.* **278:**C26–C32

Kaji, D. 1986. Volume-sensitive K transport in human erythrocytes. *J. Gen. Physiol.* **88:**719–738

Kurella, E., Kukley, M., Tyulina, O., Dobrota, D., Matejovicova, M., Mezesova, V., Boldyrev, A. 1997. Kinetic parameters of Na/K-ATPase modified by free radicals in vitro and in vivo. *Ann. N. Y. Acad. Sci.* **834:**661–665

Lauf, P.K., Adragna, N.C., Agar, N.S. 1995. Glutathione removal reveals kinases as common targets for K-Cl cotransport stimulation in sheep erythrocytes. *Am. J. Physiol.* **269:**C234–C241

Lopez-Barneo, J., Pardal, R., Ortega-Saenz, P. 2001. Cellular mechanism of oxygen sensing. *Annu. Rev. Physiol.* **63:**259–287

Mairbaurl, H., Mayer, K., Kim, K.J., Borok, Z., Bartsch, P., Crandall, E.D. 2002. Hypoxia decreases active Na transport across primary rat alveolar epithelial cell monolayers. *Am. J. Physiol.* **282:**L659–L665

Mairbaurl, H., Wodopia, R., Eckes, S., Schulz, S., Bartsch, P. 1997. Impairment of cation transport in A549 cells and rat alveolar epithelial cells by hypoxia. *Am. J. Physiol.* **273:**L797–L806

Miller, R.M., Sies, H., Park, E.M., Thomas, J.A. 1990. Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthine oxidase-initiated S-thiolation. *Arch. Biochem. Biophys.* **276:**355–363

Nikinmaa, M. 2002. Oxygen-dependent cellular functions—why fishes and their aquatic environment are a prime choice of study. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **133:**1

Nikinmaa, M., Bogdanova, A.Y., Lecklin, T. 2003. Oxygen dependency of the adrenergic Na/H exchange in rainbow trout erythrocytes is diminished by a hydroxyl radical scavenger. *Acta. Physiol. Scand.* **178:**1–6

Reeve, H.L., Michelakis, E., Nelson, D.P., Weir, E.K., Archer, S.L. 2001. Alterations in a redox oxygen sensing mechanism in chronic hypoxia. *J. Appl. Physiol.* **90:**2249–2256

Sies, H., Dafre, A.L., Ji, Y., Akerboom, T.P. 1998. Protein S-thiolation and redox regulation of membrane-bound glutathione transferase. *Chem. Biol. Interact.* **111-112:**177–185

Silver, I., Erecinska, M. 1998. Oxygen and ion concentrations in normoxic and hypoxic brain cells. *Adv. Exp. Med. Biol.* **454:**7–16

Simkhovich, B.Z., Abdishoo, S., Poizat, C., Hale, S.L., Kedes, L.H., Kloner, R.A. 2002. Gene activity changes in ischemically preconditioned rabbit heart gene: discovery array study. *Heart Dis.* **4:**63–69

Sukhomlinov, B.F., Antoniak, G.L., Ikpu, B.O. 1990. [The effect of hypoxic hypoxia on the activity of glycolysis enzymes in rat erythrocytes]. *Ukr. Biokhim. Zh.* **62:**90–93

Sweadner, K.J. 1989. Isozymes of the $Na^+/K^+$-ATPase. *Biochim. Biophys. Acta* **988:**185–220

Tanaka, E., Yamamoto, S., Kudo, Y., Mihara, S., Higashi, H. 1997. Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. *J. Neurophysiol.* **78:**891–902

Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27:**502–522

Ziegelhoffer, A., Kjeldsen, K., Bundgaard, H., Breier, A., Vrbjar, N., Dzurba, A. 2000. Na,K-ATPase in the myocardium: molecular principles, functional and clinical aspects. *Gen. Physiol. Biophys.* **19:**9–47