Na^+-K^+ pump and metabolic activities of trout erythrocytes during anoxia

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[1]*Departament de Fisiologia, Universitat de Barcelona, E-08028 Barcelona; and* [2]*Departament Ciències Fisiològiques II, Unitat de Biofísica, Universitat de Barcelona, E-08907 L'Hospitalet de Llobregat, Spain*

Pesquero, Jesús, Teresa Roig, Josep Sánchez, and Jordi Bermúdez. Na^+-K^+ pump and metabolic activities of trout erythrocytes during anoxia. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C29–C34, 1999.—Metabolic activity in the red blood cells of brown trout was monitored under conditions of oxygen depletion and chemically induced anoxia. Although metabolic activity was reduced during anoxia to one-third of the normoxic value, these cells maintained their ATP contents stable and were viable for hours in the absence of oxygen. In addition, Na^+-K^+ pump activity was not down-regulated when metabolic activity was reduced during anoxia. The compatibility of this finding with energy equilibrium and ion homeostasis was investigated.

microcalorimetry; potassium flux

IN MAMMAL ERYTHROCYTES, glycolysis is the main source of ATP. However, salmonid erythrocytes possess mitochondria and can produce ATP by oxidative phosphorylation. Trout red blood cells (RBCs) are highly dependent on aerobic metabolism, and when respiration ceases, the increased anaerobic glycolysis does not compensate for the loss of the ATP produced by respiration (8). However, experiments on deoxygenated trout RBCs indicate that these cells tolerate anoxia for several hours, which suggests that trout RBCs adjust their ATP demand to the impaired ATP production (8, 9, 28). In many vertebrate cells Na^+-K^+ pump activity is the main energy-consuming process and is down-regulated under energy-limited conditions. This reduction in pump activity must be accompanied by changes in membrane permeability to maintain ion homeostasis (5, 6, 13).

The aim of the present study was to identify the effect produced by anoxia on Na^+-K^+ pump activity and to discern how trout erythrocytes maintain energy equilibrium. Changes in metabolic activity that were linked to normoxia-to-anoxia transitions were quantified by measuring the heat production of cell suspensions by microcalorimetry. Records of power heat production [power-versus-time (P-*t*) curves] were correlated with specific indexes of the different metabolic pathways. These include the rates of lactate production, oxygen consumption, and cell ATP content (6, 7, 12, 25). The effect of the arrest of oxidative phosphorylation on Na^+-K^+ pump activity was determined by a comparison of the levels of ouabain-sensitive K^+ uptake during normoxia and anoxia, with $^{86}Rb^+$ as a tracer (4, 16, 24).

MATERIALS AND METHODS

Animals. Brown trout (*Salmo trutta*; 250–400 g) were obtained from fish farms (Direcció General de Medi Natural, Generalitat de Catalunya) in the Pyrenees, where they were maintained in open-water circuits, directly connected to a river. Animals were fed ad libitum.

Isolation of RBCs. For each experiment, blood was obtained by caudal puncture from three animals and diluted with heparinized medium (RPMI 1640; Sigma, St. Louis, MO). RBCs were separated from lymphocytes and other mononuclear cells by centrifugation in a gradient of Ficoll (Histopaque-1077; Sigma). The cells were rinsed four times in a modified Cortland buffer (pH 7.4; in mM: 141 NaCl, 3.5 KCl, 1 $MgSO_4$, 3 NaH_2PO_4, 1 $CaCl_2$, and 10 HEPES and 0.3% bovine albumin) (15). The osmolality was adjusted to 305 mosmol/kgH_2O. For measurements, RBCs were resuspended at a final hematocrit of 10% in fresh buffer containing 1 mM glucose (21).

Microcalorimetric measurements. The rate of heat production by trout RBCs was measured at 15°C in a thermal activity monitor (ThermoMetrics AB), which consists of four identical heat conduction calorimeters with a working volume of 3.5 ml (11, 27). A turbine stirrer (100 rpm) ensured efficient mixing of the cell suspension. For normoxic measurements, the calorimetric vessels were partially filled with 2.7 ml of the cell suspension. The remaining air volume (0.8 ml) and the stirring ensured aerobic conditions (1, 22). For anaerobic measurements, the calorimetric vessels were completely filled with 3.5 ml of cell suspension, so that the only oxygen available was that dissolved in the medium. Under these conditions, while the cells were consuming the dissolved oxygen, measurements were aerobic. After complete oxygen exhaustion (Fig. 1*A*), the metabolism became anaerobic. To shorten the duration of the initial aerobiosis, cell suspensions were previously flushed with N_2 to reduce the amount of dissolved oxygen. Injections were performed with a gas-tight Hamilton syringe driven by a stepper motor. The injections of chemical compounds into the microcalorimetric vessel produced exothermic peaks because of mechanical disturbance and the heat of dilution. The P-*t* curves were considered to correspond to the heat produced by cells in the presence of the chemical added once the injection peak was dissipated (Fig. 1 and Fig.5). Data were sampled every second, and the mean values over 180 s were stored. The resulting P-*t* curves indicate the heat produced by the metabolic activity of cell suspensions under the experimental conditions.

Oxygen measurements. Microcalorimeter vessels are designed to incorporate an oxygen electrode, which allows the simultaneous control of the oxygen consumption and heat production of the cell suspension. Accurate measurements of the rate of oxygen consumption were provided by a Clarke-type oxygen electrode thermostated at 15°C (Rank Brothers) and filled with 2.7 ml of the cell suspension. Because the

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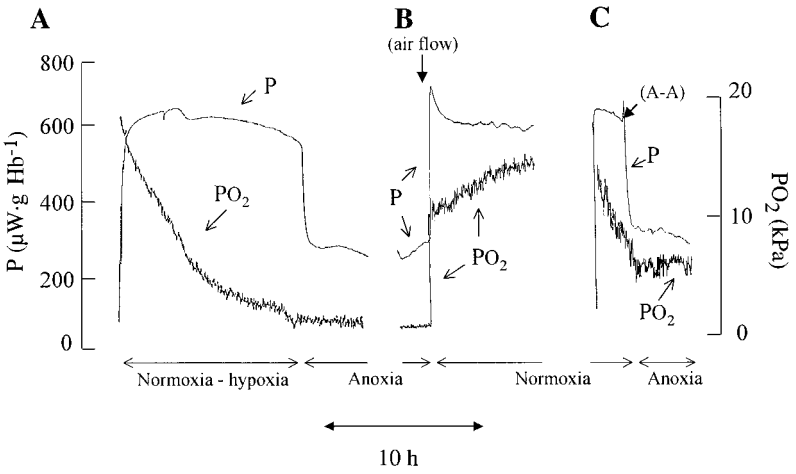

Fig. 1. *A*: effect of varying P_{O_2} values on heat production (P). P_{O_2} values were obtained with a polarographic sensor incorporated inside calorimetric vessel. *B*: recovery of heat production rate after reoxygenation of the anoxic red blood cells (RBCs) by flushing air into measurement vessel. *C*: effect of 20 μM antimycin A (A-A) on aerobic heat production. The exothermic peak is due to mechanical disturbance of injection and heat of dilution of chemicals added. Curves are representative of 5–12 different experiments with similar results. Hb, hemoglobin.

measurements involved erythrocytes, the slope of the polarographic curve reflected the rate of oxygen uptake of the cells only at high P_{O_2} values, at which a change in oxygen tension does not influence the oxygen saturation of hemoglobin. Therefore, changes in the P_{O_2} value may be assumed to represent the rate of oxygen consumption only when hemoglobin is fully saturated with oxygen (80 to 100% air saturation, i.e., 16–20 kPa) (22).

Enthalpy calculations. Comparisons between changes in heat production and in metabolic fluxes were carried out by using the caloric equivalent of carbohydrates: −507.3 kJ/mol O_2 and −80 kJ/mol lactate (10). Data were corrected for buffer protonation (23).

K^+ influx determinations. The unidirectional flux of K^+ was determined by using $^{86}Rb^+$ as a tracer (4, 5, 19). RBCs were placed in Cortland modified buffer supplemented with 1 mM glucose either during normoxia or in the absence of oxygen. Each cell suspension was again subdivided into two groups according to the presence or absence of 0.1 mM ouabain. A similar procedure was followed to determine differences between levels of $^{86}Rb^+$ influx during normoxia and anoxia chemically induced by 20 μM antimycin A. $^{86}Rb^+$ was added to each incubation mixture to a final activity of 1 μCi/ml. Samples were taken in triplicate every 15 min, placed in fresh buffer solution containing 0.1 mM ouabain to reduce new $^{86}Rb^+$ uptake by active transport, and centrifuged. Pellets were then deproteinized, and the activities corresponding to the $^{86}Rb^+$ taken up were measured in a liquid scintillation counter.

Fluxes of K^+ were calculated from the uptake of $^{86}Rb^+$, under the assumption that both ions are analogous in the measurement of active and passive fluxes (16). $^{86}Rb^+$ was provided by Amersham as rubidium chloride in aqueous solution at 0.22 mg Rb^+/ml with an initial activity of 1 mCi/ml. When the sample was used 9 days later, the activity had gone down to 0.61 mCi/ml, equal to 2.76 mCi/mg Rb^+. Then, the concentration of Rb^+ in the medium of the cell suspension necessary to produce an activity of 1 μCi/ml was 4.26×10^{-6} M. This value was 825 times lower than the concentration of K^+. According to our assumption of an analogy between the two ions, 825 mol of K^+ crossed the membrane for every mole of Rb^+. The liquid scintillation counter gave a calibration value of 1.39×10^{-15} mol Rb^+/cpm. Therefore 825 times this value, i.e., 1.14×10^{-12} mol K^+/cpm, allowed us to convert counts per minute into moles of K^+ taken up.

Cell ion content. Cells were suspended in 10 ml $MgSO_4$ solution (1 mM) and carefully mixed for 2 h. Then, 160 μl of 70% (vol/vol) perchloric acid were added to the suspension. After centrifugation at 3,000 rpm for 45 min, the clear supernatant was saved for ion analysis. Measurements were made with a flame photometer (Polyscan 61E; Thermo Jarrell Ash).

Analytical procedures. RBC hemoglobin, lactate production, and intracellular ATP levels were determined as described by Beutler (3). The RBC hemoglobin concentration was 0.22 g/ml of packed cells.

Statistical analysis. Results are expressed as means ± SE of the values obtained from the numbers of experiments indicated in Table 1. Comparisons between groups were performed by ANOVA. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

To assess the dependence of the metabolic activity of trout RBCs on the oxygen available for respiration, microcalorimetric vessels were completely filled with

Table 1. *Heat production, O_2 consumption, lactate and ATP production, and intracellular Na^+ and K^+ concentrations for trout RBCs during normoxia and anoxia with or without ouabain*

	Normoxia	Normoxia + Ouabain	Anoxia	Anoxia + Ouabain
Heat production, μW/g Hb	653 ± 16 (16)	563 ± 14* (8)	216 ± 30 (6)	214 ± 7 (4)
O_2 consumption, μmol·g Hb^{-1}·h^{-1}	2.20 ± 0.15 (7)	1.77 ± 0.05* (5)	0	0
Lactate production, μmol·g Hb^{-1}·h^{-1}	3.2 ± 0.2 (8)	2.6 ± 0.1* (8)	6.8 ± 0.3 (5)	7.0 ± 0.4 (5)
ATP production, μmol·g Hb^{-1}·h^{-1} †	16.4 ± 0.2	13.2 ± 0.1	6.8 ± 0.3	7.0 ± 0.4
Na^+ concn, mmol/g Hb	0.29 ± 0.02 (9)	0.30 ± 0.02 (9)	0.31 ± 0.03 (9)	0.30 ± 0.03 (9)
K^+ concn, mmol/g Hb	0.83 ± 0.06 (9)	0.73 ± 0.06 (9)	0.82 ± 0.07 (9)	0.82 ± 0.06 (9)

Values are means ± SE for nos. of experiments in parentheses. Ionic concentrations were measured after 60 min under indicated experimental conditions. Ouabain concn, 10^{-4} M. *$P < 0.001$ compared with control value (statistically significant). †Calculated from O_2 consumption and lactate production (1 mol ATP/mol lactate; 6 mol ATP/mol O_2). RBC, red blood cells; Hb, hemoglobin.

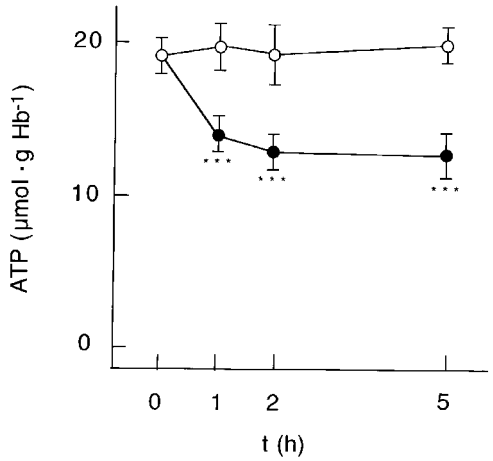

Fig. 2. Time course of intracellular ATP levels under normoxic and anoxic conditions ($n = 5$). Antimycin A was added at $t = 0$. ○, Normoxic controls; ●, anoxia. ***$P < 0.001$ (statistically significant difference).

the cell suspensions. During measurements, cells consumed the oxygen dissolved in the medium. Then P_{O_2} was continuously reduced and finally vanished. In Fig. 1A, the resulting P-*t* curve for a typical experiment is shown, together with the record of P_{O_2} obtained simultaneously from the same vessel. During normoxia RBCs maintained a mean heat dissipation of 653 ± 16 µW/g hemoglobin (Hb) before oxygen exhaustion. ATP production during normoxia, estimated from lactate production and oxygen consumption, was 16.4 ± 1.1 µmol·g $Hb^{-1}\cdot h^{-1}$, 13.2 ± 0.9 µmol·g $Hb^{-1}\cdot h^{-1}$ of which were provided by aerobic metabolism and 3.2 ± 0.2 µmol·g $Hb^{-1}\cdot h^{-1}$ by glycolysis (Table 1).

Figure 1*A* shows that the aerobic plateau in P-*t* curves (normoxia-to-hypoxia period) was sharply interrupted when P_{O_2} reached values below 0.8 kPa (anoxia period), and P-*t* curves fell to a new state in which heat production was one-third of the normoxic value (Table 1). The characteristic profile of the heat production curves at the beginning of anoxia corresponds to the transition to anaerobic metabolism. Lactate production during anaerobiosis was more than twice that during normoxia and accounted for an ATP production of 6.8 µmol ATP·g $Hb^{-1}\cdot h^{-1}$, which was less than half the ATP produced during normoxia (Table 1). Figure 1*B* shows that, after 3 h of anoxia, trout RBCs in air-flushed suspensions recovered their metabolic activity. A similar response to oxygen depletion was obtained when the respiratory chain was inhibited by antimycin A (20 µM final concentration) (Fig. 1*C* and Table 1). The effect of prolonged anoxia on ATP content is shown in Fig. 2, which shows that ATP pools fell by almost 40% and thereafter remained stable for hours.

To determine the effect of the arrest of respiration on the activity of the Na^+-K^+ pump, we compared the ouabain-sensitive fluxes of K^+ during normoxia and anoxia. Results shown in Figs. 3 and 4 indicate that in deoxygenated cells, as well as during chemically induced anoxia, Na^+-K^+ pump activity was similar to that during normoxia. Figures 3*A* and 4*A* show the time courses of total and passive K^+ uptake by control and nonrespiring cells during the first hour of anoxia. Figures 3*B* and 4*B* show the mean values of the corresponding K^+ fluxes during the same period. Mean ouabain-sensitive K^+ fluxes in control and nonrespiring cells were 6.8 ± 0.5 and 6.2 ± 0.6 µmol $K^+\cdot$g $Hb^{-1}\cdot h^{-1}$, respectively; this difference is not statistically significant.

To ascertain whether anoxia and blockage of the Na^+-K^+ pump affected the ion homeostasis of cells, the cationic contents under all the experimental conditions were measured. Results for Na^+ and K^+ contents are shown in Table 1.

The contribution of Na^+-K^+ pump activity to the metabolic activity of trout RBCs was ascertained by determining the inhibition in power heat production and in metabolic fluxes produced when the ionic pump was arrested. The addition of ouabain to cell suspensions under aerobic conditions produced a reduction of 90 ± 14 µW/g Hb in heat production (Fig. 5*A* and Table 1). This value is within the range of the 75 ± 26 µW/g Hb of heat production calculated from the reduction of lactate production (0.6 ± 0.1 µmol lactate·g $Hb^{-1}\cdot h^{-1}$) and oxygen consumption (0.43 ± 0.15 µmol $O_2\cdot$g $Hb^{-1}\cdot h^{-1}$) (Table 1). This metabolic reduction ac-

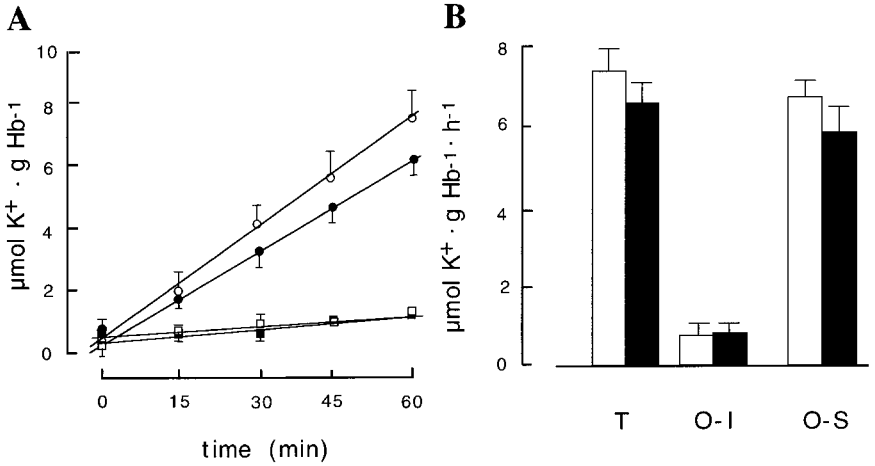

Fig. 3. Effect of absence of oxygen on total, ouabain-sensitive, and ouabain-insensitive K^+ uptake by trout RBCs. *A*: time courses of K^+ uptake. Total K^+ uptake during normoxia (○) and anoxia (●) and ouabain-insensitive K^+ uptake during normoxia (□) and anoxia (■) are shown. Values are means ± SE of at least 4 independent experiments. Symbols overlap on occasion. *B*: mean total (T), ouabain-sensitive (O-S), and ouabain-insensitive (O-I) fluxes of K^+ ± SE measured in experiments shown in *A*. Open bars, measured fluxes in normoxic trout erythrocytes; solid bars, corresponding fluxes measured during anoxia.

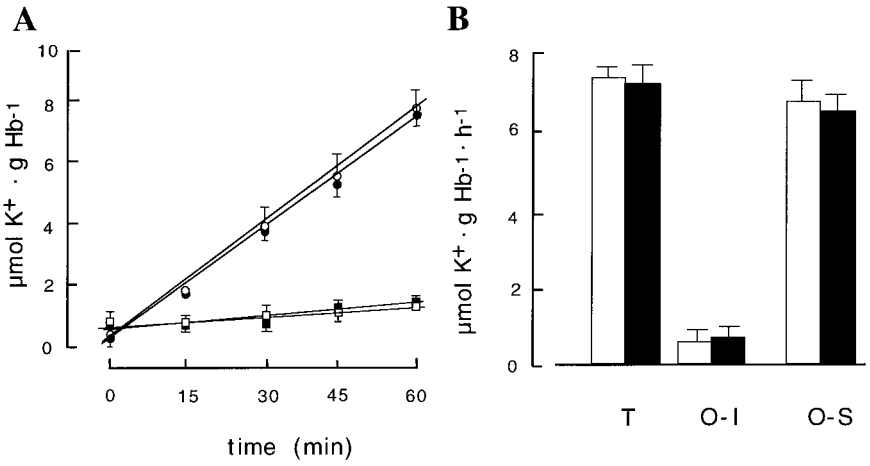

Fig. 4. Effect of anoxia, chemically induced by 20 µM antimycin A, on total, ouabain-sensitive, and ouabain-insensitive K^+ uptake by trout RBCs. *A*: time courses of K^+ uptake. Total K^+ uptake during normoxia (○) and anoxia (●) and ouabain-insensitive K^+ uptake during normoxia (□) and anoxia (■) are shown. Values are means ± SE of at least 4 independent experiments. Symbols overlap at several times. *B*: mean total (T), ouabain-sensitive (O-S), and ouabain-insensitive (O-I) fluxes of K^+ ± SE measured in experiments shown in *A*. Open bars, measured fluxes in normoxic trout erythrocytes; solid bars, corresponding fluxes in nonrespiring cells due to presence of 20 µM antimycin A.

counted for a global drop in ATP production of 3.2 ± 1.0 µmol ATP · g $Hb^{-1} \cdot h^{-1}$.

The arrest of the Na^+-K^+ pump during anoxia did not produce significant changes in the metabolic activity, as measured by heat production (Fig. 5, *B* and *C*). The maintenance of lactate production (Table 1) confirmed that the glycolysis rate did not change when ouabain was added to anoxic trout RBCs. These results suggest that the overall ATP turnover remained unaffected when the Na^+-K^+ pump was arrested in nonrespiring RBCs.

DISCUSSION

P-*t* curves produced by trout RBCs during oxygen depletion (Fig. 1*A*) indicate that the metabolic activity of these cells was almost unaffected by the decrease of the oxygen level of the medium for a wide range of P_{O_2} values. When P_{O_2} reached a value close to 0.8 kPa, the metabolic activity of RBCs suddenly decreased by 60% and reached an anoxic level that became stable after a transitional period. Normoxia values can be recovered by the reoxygenation of cell suspensions after ≥2 h of anoxia (Fig. 1*B*), which suggests that trout RBCs maintained their integrity and homeostasis. Results in Table 1 show that Na^+ and K^+ contents did not significantly change during 1 h of anoxia.

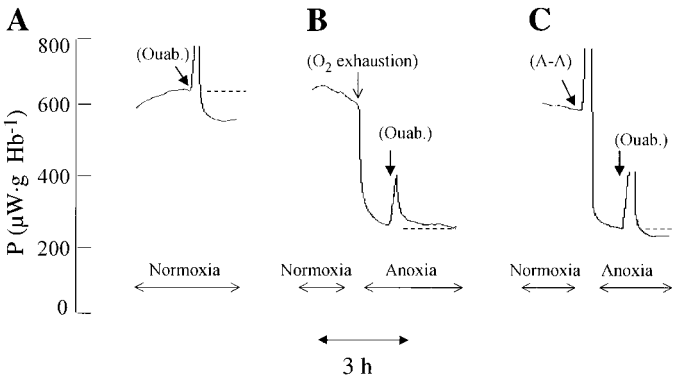

Fig. 5. Effect of 10^{-4} M ouabain (ouab.) on heat production by trout RBCs during normoxia (*A*), in absence of oxygen (*B*), and during anoxia chemically induced by 20 µM antimycin A (A-A; *C*). Exothermic peaks are due to mechanical disturbance of injections plus heat of dilution of chemicals added. Dashed lines indicate the heat production rate before ouabain addition. Curves represent 4–7 different experiments with similar results.

The contribution of glycolysis and oxidative phosphorylation to metabolic activity can be calculated by measuring lactate production and oxygen consumption (Table 1). Our results indicate that oxidative phosphorylation accounted for 80% of the ATP derived from carbohydrates in aerobic trout RBCs. When respiration ceased, glycolysis increased its rate two- to threefold, but the ATP produced was less than half that produced during normoxia. The initial reduction of the ATP pool over the time course of Fig. 2 indicates that, at the beginning of anoxia, energy consumption did not equal energy production. Thereafter, a new steady state was reached, as may also be observed from the P-*t* curves. The maintenance of this steady state required 70% more glucose consumption than that during normoxia (Table 1). In the anoxic steady state, energy equilibrium, which entails energy consumption being downregulated to match energy production, must be assumed. In a previous study, Ferguson and Boutilier (8) suggested that the Na^+-K^+ pump accounted for an important fraction of that energy consumption reduction, because ion-active transport is the main destination of ATP production in salmonid erythrocytes. If so, a concomitant drop in ion leakage through the membrane would be necessary to preserve ion homeostasis, as reported for several types of hypoxia-adapted cells (13, 14, 17).

This study investigated the dependency of the Na^+-K^+ pump on the ATP provided by glycolysis and oxidative phosphorylation. It is widely accepted that ATP produced by glycolysis is the preferred source for energy-consuming processes located at the cell membrane, such as Na^+-K^+ pump and ATP-sensitive K^+ channel activity (2, 20, 26, 29). It has also been postulated that some plasma membrane-associated glycolytic enzymes could be the coupling mechanism between glycolysis and Na^+-K^+-ATPase activity in erythrocytes (2, 26, 29). In trout RBCs the measured activity of the Na^+-K^+ pump (Figs. 3 and 4) involves ATP consumption of 3.4 ± 0.3 µmol ATP · g $Hb^{-1} \cdot h^{-1}$, which represented 20% of the total ATP production during normoxia (Table 1). To calculate how much pump activity depended on respira-

tion, we measured the effect that the induction of anoxia had on active K^+ flux. Results shown in Figs. 3 and 4 indicate that Na^+-K^+ pump activity was basically unaffected by the inhibition of oxidative phosphorylation. Moreover, any ATP supply that the pump might have lacked because of oxidative phosphorylation inhibition can be replaced by the increase in the glycolytic flux. This finding reinforces the hypothesis of linkage between glycolysis and the Na^+-K^+ pump and rules out any mechanism to regulate ion permeability in the plasma membranes of anoxic trout RBCs.

According to Balaban and Bader (2), the load of the Na^+-K^+ pump on each metabolic flux can be estimated by its reduction after pump inhibition. However, Krumschnabel and Wieser (18) argued that this hypothesis failed during limited ATP production, because other cellular functions may compete for this substrate. If so, the noninhibited processes may use the ATP spared by the arrest of the Na^+-K^+ pump. That avoids an equivalent reduction of the metabolic fluxes and produces an underestimation of the ATP utilized by the pump. Conversely, a mismatch between the ATP required by the Na^+-K^+-ATPase and the reduction of metabolic fluxes is indicative of competition with other consuming processes.

Results shown in Table 1 indicate that the addition of ouabain reduced the lactate production and oxygen consumption of respiring cells by almost 20%. The total amount of ATP saved by this metabolic inhibition fits well with the ATP utilized by the Na^+-K^+-ATPase. However, glycolysis inhibition only accounted for 0.6 ± 0.2 μmol ATP·g $Hb^{-1} \cdot h^{-1}$, which represented <20% of the ATP required by the pump. This mismatch was greater in anoxic cells, because the addition of ouabain did not modify the glycolytic rate (Table 1). This underestimation suggests that glycolytic ATP was allocated to Na^+-K^+-ATPase activity as a priority when this competed with the other consuming processes. If so, the demand for glycolytic ATP by the ouabain-insensitive functions could drive glycolysis after pump inhibition. This shift of the ATP consumed from that produced by mitochondria to that provided by glycolysis may explain the observed inhibition in the oxygen uptake that followed the arrest of the Na^+-K^+ pump. Consistent with this interpretation are the results obtained with anoxic RBCs. Results shown in Figs. 3 and 4 indicate that glycolysis supplied the Na^+-K^+ pump at a rate similar to that observed during normoxia although ATP production fell by more than one-half when respiration ceased (Table 1). Therefore, other cellular functions had to reduce their demand to maintain energy equilibrium, as suggested by results shown in Fig. 2. Moreover, results shown in Fig. 5, *B* and *C*, and Table 1 suggest that these suboptimally supplied functions could increase their ATP demand and maintain ATP turnover after the arrest of the pump.

In conclusion, our results indicate that in trout RBCs the Na^+-K^+-ATPase is favored in the allocation of the ATP produced by glycolysis and that pump activity is unaffected by the arrest of oxidative phosphorylation. Moreover, ion homeostasis and energy equilibrium are maintained under prolonged anoxia.

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