

## Inhibition of the sodium pump does not cause a stoichiometric decrease of ATP-production in energy limited fish hepatocytes

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**Abstract.** In isolated goldfish hepatocytes under aerobic conditions the energy requirement for the sodium pump (calculated from  $\text{Rb}^+$  flux) is closely matched by the ouabain-sensitive fraction of oxygen consumption, whereas during in vitro anoxia (cyanide inhibition of the electron transport chain) the measured ATP demand of the sodium pump clearly exceeds ouabain-sensitive ATP production by anaerobic glycolysis. We conclude that when the energy status of cells is low, part or all of the ATP spared by the inhibition of a particular function may be used for fuelling other ATP-consuming functions.

**Key words.** Goldfish hepatocytes;  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ ; protein synthesis; membrane-metabolic coupling.

Generally, the energy status of cells is in dynamic equilibrium, the rates of ATP supply matching the rates of ATP consumption. Short-term mismatches in supply and demand of ATP may be buffered by the adenylate pool. When cells are perturbed by endogenic or exogenic stimuli, transitions in energy status may be brief, the cells returning rapidly to their previous steady state, or a new steady state may be reached, the size of the adenylate pool adjusting to the new steady state<sup>1,2</sup>.

When a cell is considered to be in energetic equilibrium it is implicitly assumed that a reduction in ATP demand will be followed—after a short transition period—by a corresponding reduction in ATP supply, and vice versa. This assumption is the basis of the use of specific inhibitors for estimating the energy costs of metabolic functions, such as the cost of protein synthesis (inhibition by cycloheximide), or the cost of ion pumping by the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  (inhibition by ouabain). Thus energy budgets of cells have been constructed by successively inhibiting protein synthesis, nucleic acid synthesis, and ion transport, and measuring the effect of each step on the total ATP production as deduced from oxygen consumption<sup>3,4</sup>, heat production<sup>5</sup>, lactate formation<sup>6,7</sup>, or inhibition of  $^{14}\text{CO}_2$  production from  $[1\text{-}^{14}\text{C}]\text{-glutamate}$ <sup>8</sup>. Such calculations rest on three major assumptions: 1) specificity of the inhibitors; 2) validity of the stoichiometric factors used between energy-supplying reactions and ATP production; and 3) equilibrium of ATP supply and ATP demand throughout the experiment due to the buffering effect of the adenylate pool. Although problems attach to each of them (see, e.g., refs 9, 10) assumptions 1) and 2) are based on sufficiently firm empirical evidence for checking to be considered unnecessary. For most authors this also applies to assumption 3), but in this case the empirical basis is in fact quite thin.

Logically there is no reason why the ATP spared by inhibiting an energy-consuming cellular function should not be used for other functions. Some circumstantial evidence is available that such transfers of ATP between different functions might occur in metazoan tissues<sup>11–14</sup>. Compelling evidence suggests that in microorganisms<sup>15</sup> as well as in whole animals<sup>16</sup>, excessive energy demand by a particular physiological function (usually growth) is compensated by a reduction in the energy demand of maintenance functions. Consequently, the rate of total ATP production (measured as oxygen consumption or end product formation) may remain more or less constant despite a variable load from the function studied<sup>17,18</sup>.

The question whether, and if so to what extent, trade-offs might exist between different ATP-consuming functions in cells, is of paramount importance for the correct interpretation of experiments in which specific inhibitors have been used for estimating the energy demand of individual cellular functions. In order to answer this question, total ATP production as well as the activity of the function of interest, with and without a specific inhibitor, have to be measured. We reasoned that trade-offs in energy consumption are most likely to occur when the energy status of cells is low, as, for example, during anoxia in otherwise aerobic tissues. We chose the hepatocytes of goldfish, *Carassius auratus* L., for our investigation since this species is capable of surviving long periods of environmental anoxia<sup>19,20</sup>.

### Materials and methods

Goldfish with a mean weight of 90 g were obtained from a commercial supplier and were kept in 100 liter aquaria at 15 °C. The animals were fed trout pellets once a day ad libitum and were fasted 20 hours prior to the experiment. Hepatocytes were isolated from goldfish

acclimated to 15 °C as described<sup>21</sup>. Freshly isolated cells were suspended in a medium containing (mM): NaCl (135), KCl (3.8), KH<sub>2</sub>PO<sub>4</sub> (1.2), CaCl<sub>2</sub> (1.3), MgSO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (10), HEPES (10), 2% bovine serum albumin, pH 7.6. A number of criteria such as trypan blue exclusion, and the stability of oxygen consumption rates and of ATP levels for at least 2 h, indicated that the preparation yielded metabolically competent cells<sup>22</sup>. Prior to each experiment the cells were preincubated in a shaking waterbath at the experimental temperature for 30 min. Oxygen consumption (VO<sub>2</sub>) was measured by means of a Cyclobios Oxygraph in a thermostatted cell with constant stirring at 500 rpm<sup>21</sup>. Ouabain (inhibitor of (Na<sup>+</sup>, K<sup>+</sup>)-ATPase) or cycloheximide (inhibitor of protein synthesis) were added to respiring cells to a final concentration of 1 mM and 15 mM, respectively. Inhibitor-sensitive respiration was calculated from the difference of VO<sub>2</sub> before and after the addition of the inhibitor.

The rate of lactate production during in vitro anoxia (after inhibition with 2 mM sodium cyanide) was measured in cells incubated in a shaking waterbath at 15 °C. Triplicate samples were taken from the cell suspension at the intervals indicated in the figure. The samples were precipitated and extracted in ice-cold 10% metaphosphoric acid and the lactate content was assayed according to standard enzymatic techniques<sup>23</sup>.

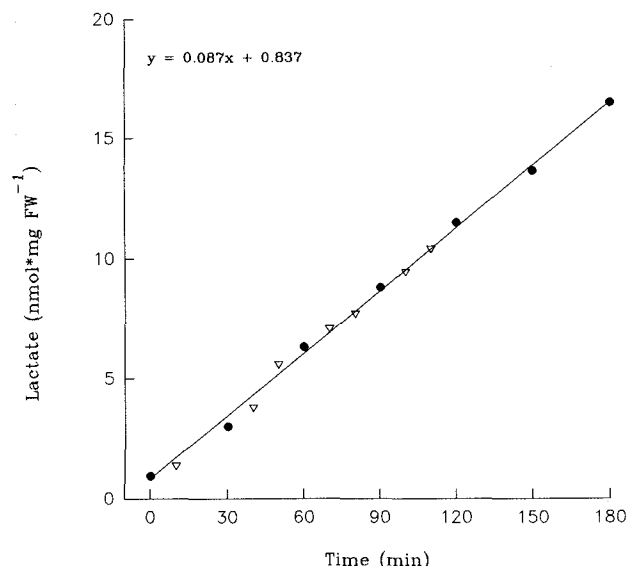
Ouabain-sensitive rubidium uptake was used to measure (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity. Experiments were per-

formed in the Oxygraph chamber under conditions identical to those described for VO<sub>2</sub> measurements. Rates of Rb<sup>+</sup> uptake were determined by incubating cells in assay medium where K<sup>+</sup> was replaced by Rb<sup>+</sup>. After 1, 5, and 9 min of incubation aliquots of the cell suspension were withdrawn and washed 3 times with ice-cold isotonic MgCl<sub>2</sub>-medium. For this purpose the hepatocytes were centrifuged in a micro-centrifuge (3 s at 10,000 × g), the supernatant was removed by aspiration, and the cells were resuspended in the washing medium. The final cell pellet was lysed, the protein precipitated and the supernatant assayed for Rb<sup>+</sup> by flame photometry<sup>24</sup>. Another aliquot of the cell suspension was transferred to a preweighed Eppendorf reaction tube and treated in the same way as for Rb<sup>+</sup> uptake. After 3 washings the vial was wiped dry and weighed to the nearest 0.01 mg.

### Results and discussion

Under control (= aerobic) conditions the ouabain-sensitive fraction of ATP production closely matched the ATP consumption of the (Na<sup>+</sup>, K<sup>+</sup>)-ATPase, as deduced from Rb<sup>+</sup> flux measurements (table). The slight underestimate of Na<sup>+</sup> pump activity by ouabain-sensitive VO<sub>2</sub> might have been due to the contribution of aerobic glycolysis to the energy supply of the pump which previous experiments have shown to be about 6% of total ATP production<sup>22</sup>. In vitro anoxia, produced by the addition of sodium cyanide (CN) to cell suspensions, strongly stimulated lactate production, the ATP output of which amounted to about 25% of the aerobic control value (table). Rubidium uptake (i.e. ATPase activity), on the other hand, was unaltered by the addition of CN at 15 °C, and decreased to about 80% of the control level at 25 °C (table). Thus lactate production ought to be strongly reduced by the addition of ouabain, since from 35% (25 °C) to 50% (15 °C) of the steady state ATP production of the hepatocytes are consumed by the Na pump under these conditions. However, as shown in the table and figure, inhibition of the (Na<sup>+</sup>, K<sup>+</sup>)-ATPase did not result in a proportional decrease in the rate of anaerobic glycolysis at either temperature. At 15 °C lactate production was totally unaffected by the addition of ouabain, whereas at 25 °C the addition of ouabain reduced the rate of lactate production by  $11.5 \pm 7.8\%$  (mean  $\pm$  SD,  $n = 12$ ), which is only one-third of the expected value based on the measurement of Rb<sup>+</sup>-flux (table).

This finding, in conjunction with the high degree of variability observed in these experiments (c.v. = 73.2% as compared to 23.5% under aerobic conditions), indicates that when the cells are energy-limited, ATP consumption by the Na<sup>+</sup> pump competes with other energy-consuming processes. In fact, when cycloheximide, an inhibitor of protein synthesis, was added to the cells at the same time as ouabain, lactate production



Representative example of several experiments ( $n = 11$ ) in which lactate production of hepatocytes at 15 °C was measured in the presence of 2 mM cyanide with and without ouabain. Cyanide was added at time zero, ouabain was added at 0, 30, 60, and 90 min to cyanide-treated cells. Triplicate samples for lactate were taken every 30 min for cyanide-treated cells (solid circles) and in 10 min intervals after the addition of 1 mM ouabain (open triangles). The equation in the upper left corner describes lactate production, as calculated by linear regression through cyanide-treated cells only.

## ATP production and ATP consumption rates of isolated goldfish hepatocytes under control conditions and in vitro anoxia

	Treatment	Total	OB-sens	ATPase	CH-sens	OB + CH-sens
15 °C	control	0.720	0.073	0.092	-	-
		±0.169 (6)	±0.021 (6)	±0.019 (8)	-	-
	anoxia	0.178	0	0.090	-	-
		±0.048 (12)	(11)	±0.021 (4)	-	-
25 °C	control	1.709	0.196	0.212	0.870	-
		±0.133 (5)	±0.046 (5)	±0.072 (6)	±0.096 (5)	-
	anoxia	0.479	0.056	0.171	0.109	0.184
		±0.059 (12)	±0.041 (12)	±0.064 (6)	±0.071 (11)	±0.051 (8)

ATP production was monitored by measuring oxygen consumption (control) and lactate production (anoxia) in the presence and absence of 1 mM ouabain and of 15 mM cycloheximide. In vitro anoxia was maintained by the inhibition of electron transport with 2 mM CN. Stoichiometries of  $\text{ATP/O}_2 = 6$  and  $\text{ATP/lactate} = 1.5$  were assumed.  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$  activity was converted to ATP consumption rates assuming the transport of 2  $\text{Rb}^+$  per ATP. Results are expressed as means  $\pm$  SD ( $\text{nmol}\cdot\text{mgFW}^{-1}\cdot\text{min}^{-1}$ ), with numbers of experiments given in parentheses. Total, OB-sens, and CH-sens refer to total, ouabain-sensitive, and cycloheximide-sensitive ATP production, respectively. Inhibitor-sensitive fractions were calculated from the difference in ATP production before and after the addition of the inhibitor. ATPase denotes ATP consumption by the  $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ .

was inhibited by  $36.7 \pm 10.3\%$  which closely approximates the calculated ATP requirement of the  $\text{Na}^+$  pump (table). Interestingly, when cycloheximide was added alone, the variability in the suppression of lactate production was just as great (c.v. = 65.1%) as with ouabain alone (c.v. = 73.2%).

Thus it has to be assumed that under conditions of energy limitation the allocation of metabolic energy to different functions is a dynamic and flexible property of the cells, the supply and demand of ATP depending strongly on spatial and temporal priorities which might vary between individuals.

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