J. exp. Biol. **143**, 149–164 (1989)
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METABOLIC–MEMBRANE COUPLING IN RED BLOOD CELLS OF TROUT: THE EFFECTS OF ANOXIA AND ADRENERGIC STIMULATION

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Accepted 5 January 1989

Summary

Under oxygenated conditions, *in vitro*, the highly aerobic red cells of the rainbow trout (*Salmo gairdneri*) exhibit tight coupling between energy (i.e. nucleotide triphosphate, NTP)-consuming and NTP-producing metabolic activity, as shown by strict maintenance of red cell NTP: haemoglobin ratios. This coupling is maintained following adrenergic stimulation of oxygenated red cells when the increased NTP demands of ion transporting systems are met by enhanced energy production *via* aerobic metabolism. In unstimulated anoxic red cells, membrane–metabolic coupling is preserved *via* the arrest of NTP-consuming processes. Adrenergic stimulation of anoxic red cells, however, leads to a functional uncoupling of membrane metabolism with the result that NTP levels decline rapidly. At this time, cellular [NTP] is negatively correlated with $[Na^+]_i$ and $[Cl^-]_i$ and positively correlated with $[K^+]_i$. This, in addition to the fact that the pH of the intracellular compartment is also highly dependent on cellular NTP levels, provides evidence for the integration of energy and membrane metabolisms.

Introduction

Mammalian red cells obtain energy primarily through anaerobic glycolysis. The mature nucleated red cells of many lower vertebrates, however, possess mitochondria and therefore the ability to produce cellular energy *via* oxidative phosphorylation (Hunter & Hunter, 1957; Schweiger, 1962). For example, red cells collected from resting, chronically catheterized rainbow trout (*Salmo gairdneri*) are estimated to derive 99 % of their metabolic energy needs from aerobic metabolism (Ferguson *et al.* 1989).

Adrenergic stimulation of rainbow trout red cells *in vivo* or *in vitro* induces an increase in cell volume and a decrease in the proton gradient across the red cell membrane (Nikinmaa, 1982). The response is initiated *via* stimulation of the membrane Na^+/H^+ exchanger (Nikinmaa *et al.* 1987). The degree to which erythrocyte pH is regulated in the face of an extracellular acidosis is proportional

Key words: erythrocyte, pH, β-adrenergic, nucleotide triphosphates, metabolism, trout, anoxia.

to the adrenergically mediated increase in red cell oxygen consumption ($\dot{M}_{O_2}$) in both Atlantic salmon (Ferguson & Boutilier, 1988) and rainbow trout (Ferguson *et al.* 1989). Such increases in $\dot{M}_{O_2}$ are associated with a rapid (within minutes) decline in erythrocyte nucleotide triphosphate (NTP) levels and subsequent maintenance at a new (lower) steady-state level. This leads us to believe that pH regulation in these cells is accompanied by increased NTP turnover (Ferguson & Boutilier, 1988; Ferguson *et al.* 1989). For example, when energy-consuming (red cell membrane) processes were uncoupled from energy-producing processes (by deoxygenation and concomitant incapacitation of aerobic NTP production) pHi regulation remained operative, but NTP levels fell owing to the absence of aerobic NTP production.

The present study was undertaken to evaluate further the role of energy metabolism in the rainbow trout red cell and, in particular, to examine the interrelationships between NTP metabolism and the membrane processes associated with adrenergic stimulation.

Materials and methods

Animals

Freshwater rainbow trout (*Salmo gairdneri*) ranging in size from 200 to 400 g were obtained from a commercial supplier and housed in the Aquatron Facility at Dalhousie University. The fish were maintained in large fibreglass aquaria (1 m × 1 m × 1·5 m) supplied with flow-through dechlorinated tap water (10 ± 2 °C). Prior to experimentation, the animals were fed *ad libitum* on a commercially prepared diet of food pellets.

Surgical preparation

Each fish was removed from the holding aquarium and anaesthetized with 1:2:10000 parts of 3-aminobenzoic acid ethyl ester (Sigma MS-222): $NaHCO_3$: water (pH ≈ 7·5). During surgery the fish was placed on an operating table where the gills were continuously irrigated with buffered MS-222 in fresh water (1/10 strength of the initial concentration of anaesthetic). Following chronic catheterization of the dorsal aorta (Smith & Bell, 1964), the fish was revived and placed in a blackened Perspex box where it recovered for at least 36 h.

Analytical measurements

pH determinations were made using a Radiometer G279/G2 glass capillary electrode and K497 calomel electrode coupled to a Radiometer PHM84 pH meter. The electrodes were thermostatted at 10 °C. Extracellular pH (pHe) was determined on a 40 μl sample of anaerobically collected whole blood. Intracellular pH (pHi) measurements were made on a haemolysate according to the freeze–thaw method of Zeidler & Kim (1977). Samples for metabolite determinations

were prepared by adding a known volume of whole blood to twice its volume of ice-cold 12% (w/v) trichloroacetic acid (TCA). The TCA/blood slurry was immediately vortexed for 10 s and then left to stand on ice for 5 min to enhance protein precipitation. The TCA slurry was then centrifuged (4 min in a Fisher Microfuge) and the supernatant stored in liquid N_2.

Haematocrit was determined in triplicate by spinning blood in microcapillary tubes for 3 min in an Autocrit centrifuge (Clay Adams). Haemoglobin (Hb) was determined in triplicate as described in Sigma Bulletin no. 525.

Cellular ion and water contents were determined by spinning whole blood for 4 min in a previously dried and tared 500 μl Eppendorf tube. The plasma layer was removed following centrifugation and placed in another previously dried and tared tube. The red cell and plasma tubes were then dried to constant weight (72 h at 80°C). A 2·5% plasma trapping correction was routinely made (Houston, 1985).

Dried plasma and red cell pellets were digested in nitric acid (5·5 mol l^{-1}) prior to ion analyses. Na^+ and K^+ contents were determined by flame photometry (Corning 410). The colorimetric method of Zall *et al.* (1956) was used for Cl^- determination.

A Gilford Response narrow-beam UV–VIS spectrophotometer was used for the determination of Hb, Cl^-, NTP and lactate contents. Coupled NAD/NADH enzymatic assays were utilized in the analysis of NTP and lactate (Sigma enzymes and reagents).

Experimental design

Blood was withdrawn from the dorsal aorta of resting cannulated fish, pooled on ice and then transferred (in 5 ml samples) to heparinized round-bottomed (50 ml) flasks. The flasks were placed in an incubation/tonometry apparatus thermostatted at 10°C. Each flask received a humidified (10°C) gas supply of known composition (Wösthoff). The two gas mixtures used in this study were 0·2% CO_2/N_2 and 0·2% CO_2/air. The flasks were shaken so that a thin film of blood formed along the sides of the glass. Each blood sample was incubated with either 200 μl of the Na^+/H^+ exchange blocker, amiloride, in Cortland saline (Sigma A7410: final concentration in blood = 5×10^{-4} mol l^{-1}) or 200 μl of Cortland saline alone. At the end of this 60 min incubation period a control blood sample was removed for analyses. Ten minutes later 100 μl of a 'sham' or 'isoproterenol' (β-adrenergic agonist) solution was added to the blood in each vessel. The latter solution consisted of isoproterenol in Cortland saline (Sigma I6504: final concentration in blood = 1×10^{-5} mol l^{-1}), whereas the sham consisted of Cortland saline alone. This drug treatment regime gave rise to three groups of red cells in both the oxygenated and the deoxygenated conditions: (i) sham- and (ii) isoproterenol-treated red cells preincubated in the amiloride vehicle as well as (iii) isoproterenol-treated red cells preincubated in amiloride. Blood samples were subsequently taken 5, 25 and 60 min after addition of the sham or isoproterenol solution.

Statistical analyses

Data were analysed statistically using Student's *t*-test (paired or two-sample tests as appropriate). Differences were considered significant at $P < 0{\cdot}05$.

Results

Erythrocyte and plasma pH

Isoproterenol treatment induced a marked extracellular acidification which was of equal magnitude in both oxygenated and deoxygenated whole blood preparations (Fig. 1A). The pH of the oxygenated and deoxygenated erythrocytes showed a small, but progressive, alkalinization following addition of isoproterenol and both were significantly higher at 60 than at 5 min.

The transmembrane pH gradient (ΔpH) of oxygenated cells was reduced upon isoproterenol administration, though never to the extent observed in the deoxygenated cells. Most notably, the transmembrane pH gradient of deoxygenated red cells (Fig. 1A) was reversed upon isoproterenol addition. A progressive increase occurred in the ΔpH of deoxygenated cells over the 60 min period following

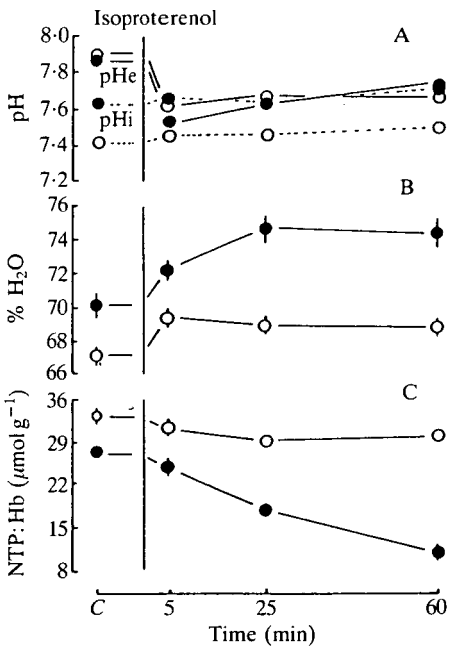

Fig. 1. Response to isoproterenol addition of red cells in aerobic (○) and anaerobic (●) environments. A vertical line shows the point of addition of isoproterenol. Control (*C*) samples were taken 60 min following commencement of aerobic or anaerobic incubation at 10°C. See text for further details. Each point represents the mean ± 1 S.E.M. of six determinations. Note: dashed lines are drawn between pHi means and solid lines are drawn between pHe means in A.

Table 1. *Haematological data for oxygenated and deoxygenated red cells preincubated for 60 min in amiloride or its corresponding sham (vehicle)*

		Oxygenated (0·2 % CO_2/air)		Deoxygenated (0·2 % CO_2/N_2)	
	Time	Sham	Amiloride	Sham	Amiloride
pHe	*C*	7·771 ± 0·051	7·821 ± 0·017	7·833 ± 0·055	7·861 ± 0·020
	5	7·856 ± 0·043	7·805 ± 0·030	7·824 ± 0·034	7·824 ± 0·023
	25	7·848 ± 0·027	7·764 ± 0·033[a]	7·827 ± 0·053	7·834 ± 0·029
	60	7·817 ± 0·037[c]	7·721 ± 0·024[a]	7·895 ± 0·029[c]	7·794 ± 0·028
pHi	*C*	7·414 ± 0·039	7·404 ± 0·032	7·559 ± 0·011[b]	7·577 ± 0·058[b]
	5	7·399 ± 0·029	7·433 ± 0·041	7·621 ± 0·011[b]	7·602 ± 0·055[b]
	25	7·394 ± 0·015	7·430 ± 0·030	7·609 ± 0·036[b]	7·585 ± 0·047[b]
	60	7·397 ± 0·038	7·488 ± 0·028[a]	7·665 ± 0·039[b]	7·618 ± 0·034[b]
% H_2O ($g\,100\,g^{-1}$ red blood cells)	*C*	67·43 ± 0·37	67·69 ± 1·35	69·66 ± 0·75[b]	68·95 ± 1·22
	5	67·30 ± 0·58	66·74 ± 0·91	70·60 ± 0·95[b]	69·63 ± 1·37
	25	66·90 ± 0·47	67·02 ± 1·16	70·57 ± 0·62[b]	69·44 ± 1·35
	60	67·25 ± 0·51	68·38 ± 1·09	69·79 ± 0·67[b]	69·36 ± 1·19
[NTP]:[Hb] ($\mu mol\,g^{-1}$ Hb)	*C*	32·90 ± 1·12	32·80 ± 1·10	27·04 ± 1·35[b]	29·48 ± 0·95[b]
	5	32·54 ± 0·65	33·37 ± 0·58	28·94 ± 1·59[b]	27·75 ± 1·29[b]
	25	32·88 ± 0·53	30·94 ± 1·43	26·38 ± 1·75[b]	23·66 ± 1·28[a,b]
	60	34·18 ± 0·41[c]	30·82 ± 1·04	22·30 ± 1·44[a,b]	23·15 ± 0·98[a,b]

Control (*C*) samples were collected at the end of a 60 min preincubation period: i.e. 10 min prior to treatment of 'Amiloride' cells with isoproterenol and treatment of 'Sham' cells with the isoproterenol vehicle. Samples were then collected at 5, 25 and 60 min following these treatments. See text for further details.

These data were also collected for isoproterenol-treated cells preincubated in a sham solution (see Fig. 1).

Each value is the mean ± 1 S.E.M. of six determinations.

Values are marked if different from sham (*a*); if different from oxygenated (*b*) and if different from amiloride (*c*).

β-adrenergic stimulation, such that the gradient at 60 min was not significantly different from zero.

The pHe and pHi values for red cells treated with sham and amiloride solutions are presented in Table 1. Whereas pHi was consistently higher in deoxygenated than in oxygenated red cells, there were no significant differences in pHe between these groups of cells. A small, but significant, increase in pHi was observed at 60 min for oxygenated red cells treated with amiloride (Table 1).

Erythrocyte water content

Preincubation under conditions of deoxygenation led to an increased red cell water content (expressed as grams of water per 100 g of red cells) in the sham-treated red cells but not in the amiloride-treated red cells (Fig. 1B; Table 1). No significant changes in red cell water content were observed as a result of sham or

isoproterenol–amiloride treatment of oxygenated and deoxygenated red cells (Table 1). Water contents of oxygenated and deoxygenated red cells before and after the addition of isoproterenol are shown in Fig. 1B. Isoproterenol induced a significant increase in the cell water content of oxygenated red cells which was complete at 5 min. In deoxygenated cells, the uptake of water continued beyond the 5 min sampling period, such that the magnitude of the response was much greater than in oxygenated cells; at 60 min the increases in water content were $4{\cdot}18 \pm 0{\cdot}95$ and $1{\cdot}61 \pm 0{\cdot}83$ g H_2O 100 g^{-1} red cells for deoxygenated and oxygenated red cells, respectively.

Erythrocyte NTP content

Red cell nucleotide triphosphate content (expressed as μmol NTP g^{-1} Hb) remained constant following sham and amiloride treatment in oxygenated conditions, whereas there was a steady decline observed for deoxygenated cells treated with sham and amiloride solutions (Table 1). Treatment of deoxygenated red cells with isoproterenol caused a much greater decline in NTP: Hb than did the sham treatment (Fig. 1C). In fact, NTP:Hb levels in isoproterenol-treated deoxygenated cells were lower than in all other treatments after 60 min (i.e. $10{\cdot}94 \pm 1{\cdot}62$ μmol NTP g^{-1} Hb). Although isoproterenol also stimulated a decline in NTP:Hb in oxygenated cells, NTP levels did not decrease as much as in deoxygenated cells.

Erythrocyte ion concentrations

Intracellular Na^+ concentrations ($[Na^+]_i$ in mmol l^{-1} cell water) remained constant in all cells except those receiving isoproterenol in the absence of amiloride (Table 2; Fig. 2A). Deoxygenation itself had no influence on control $[Na^+]_i$; however, it was accompanied by a much greater increase in $[Na^+]_i$ following β-adrenergic stimulation than observed for oxygenated red cells (Fig. 2A). At 60 min, the sodium concentration of isoproterenol-treated deoxygenated red cells was about 40 % greater than in the sham-treated cells, whereas similarly treated oxygenated red cells showed only a 20 % increase.

Intracellular K^+ concentrations ($[K^+]_i$) remained constant in all red cells except those treated with isoproterenol in the deoxygenated condition. In these cells, a substantial decrease in $[K^+]_i$ was observed (Fig. 2B).

Intracellular Cl^- concentrations ($[Cl^-]_i$) were higher in deoxygenated than in oxygenated erythrocytes (Fig. 2C; Table 2). Upon stimulation with isoproterenol, the $[Cl^-]$ of the oxygenated red cells rose quickly to a significantly higher value than that observed for the control. In deoxygenated red cells isoproterenol treatment also led to a rise in $[Cl^-]_i$, but the increase was not as sharp and only became significant after 60 min of adrenergic stimulation. The net movements of chloride into oxygenated and deoxygenated red cells stimulated by isoproterenol treatment are presented in Fig. 3. Adrenergic stimulation resulted in a net influx of chloride which, in less than 5 min, had established a new steady-state level. I

Table 2. *Intracellular ion concentrations (in mmol l^{-1} red cell water) for oxygenated and deoxygenated red cells preincubated for 60 min in amiloride or its corresponding sham (vehicle)*

		Oxygenated (0·2 % CO_2/air)		Deoxygenated (0·2 % CO_2/N_2)	
	Time	Sham	Amiloride	Sham	Amiloride
$[Na^+]_i$	*C*	32·05 ± 3·89	33·75 ± 3·46	34·05 ± 2·68	33·62 ± 4·91
(mmol l^{-1})	5	33·23 ± 3·38	33·66 ± 3·68	32·43 ± 2·18	33·48 ± 2·91
	25	30·24 ± 2·04	30·30 ± 2·33	35·76 ± 3·30	36·17 ± 5·11
	60	31·84 ± 1·66	36·95 ± 3·59	32·85 ± 2·32	34·75 ± 2·60
$[K^+]_i$	*C*	144·7 ± 4·5	139·2 ± 5·0	139·5 ± 9·3	133·2 ± 6·0
(mmol l^{-1})	5	138·6 ± 6·0	138·6 ± 7·8	135·4 ± 6·4	129·8 ± 6·8
	25	140·1 ± 5·7	140·8 ± 6·2	136·1 ± 3·7	137·5 ± 4·4
	60	146·8 ± 7·2	144·1 ± 10·0	141·7 ± 4·1	133·9 ± 6·9
$[Cl^-]_i$	*C*	36·23 ± 7·56	37·64 ± 6·51	55·24 ± 3·00^b	49·73 ± 3·29^b
(mmol l^{-1})	5	32·84 ± 1·04	34·61 ± 4·08	52·54 ± 2·85^b	52·70 ± 1·59^b
	25	31·24 ± 3·97	38·02 ± 3·65	57·44 ± 2·89^b	53·00 ± 3·64^b
	60	36·84 ± 4·08^c	47·74 ± 2·78	54·50 ± 3·21^b	57·85 ± 2·10^{a,b}

See Table 1 and text for further details.

These data were also collected for isoproterenol-treated cells preincubated in a sham solution (see Fig. 2).

Each value is the mean ± 1 S.E.M. of six determinations.

Values are marked if different from sham (*a*); if different from oxygenated (*b*) and if different from amiloride (*c*).

comparison, chloride movement into the anoxic cells was retarded (Fig. 3), as shown by the lack of a rapid establishment of a new steady-state level.

Because impermeable intracellular polyanionic phosphate compounds are important determinants of the Donnan equilibrium, it is useful to consider the influence of NTP on transmembrane ion and water distributions. Cellular water, ion concentrations and pH are expressed in relation to cellular NTP in Fig. 4.

Erythrocyte lactate production

Lactate production rates ($\dot{M}_{lactate}$) for the different red cell treatments are presented in Table 3. The accumulation of lactate was linear over the period of sample collection for all treatments (data not shown). In general, deoxygenated red cells had higher $\dot{M}_{lactate}$ than their oxygenated counterparts. However, there was no significant difference between the oxygenated and deoxygenated cells treated with amiloride (in addition, the $\dot{M}_{lactate}$ of deoxygenated amiloride cells was significantly lower than that of the deoxygenated sham cells). Compared to their sham counterparts, addition of isoproterenol did not significantly change $\dot{M}_{lactate}$ in either the oxygenated or the deoxygenated preparations.

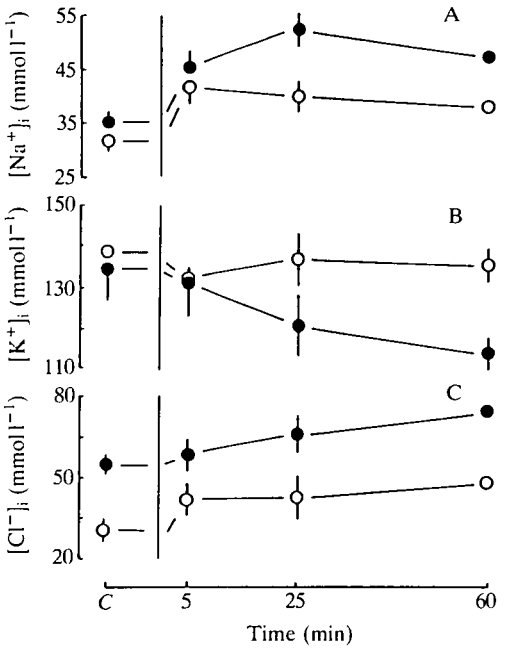

Fig. 2. Intracellular ion concentrations prior to and following isoproterenol addition as designated by vertical lines. Cells were incubated under either aerobic (○) or anaerobic (●) conditions. See Fig. 1 and text for further details.

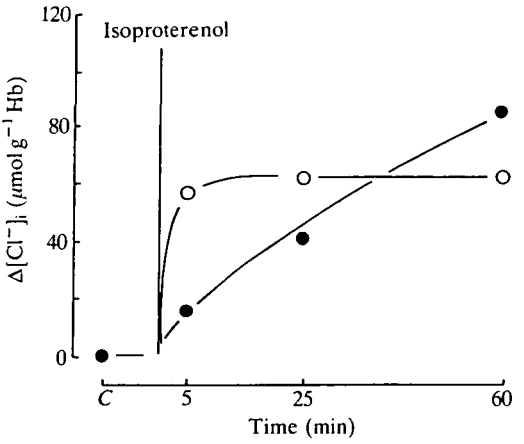

Fig. 3. Changes in net chloride ion content of oxygenated (○) and deoxygenated (●) red cells treated with isoproterenol above that exhibited by their respective sham-treated counterparts. Each point represents the mean of six determinations. See text for further details.

Discussion

Aerobic and anaerobic metabolism of erythrocytes

Aerobic metabolism is estimated to account for over 90% of the NTP production of salmonid erythrocytes under oxygenated conditions *in vitro* (Ferguson & Boutilier, 1988) and *in vivo* (Ferguson *et al.* 1989). The oxygen consumption ($\dot{M}_{O_2}$) of red cells collected from chronically catheterized rainbow trout (at 10°C)

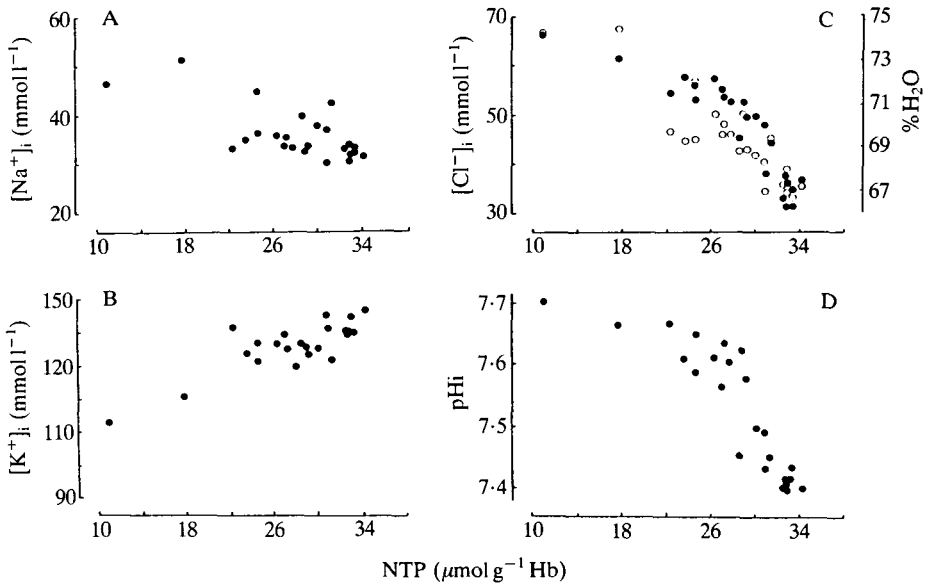

Fig. 4. Relationships between cellular NTP:Hb and cellular (A) $[Na^+]$, (B) $[K^+]$, (C) $[Cl^-]$ (○) and H_2O (●), and (D) pH. Each point represents the mean of six determinations.

Table 3. *Lactate production rates of oxygenated and deoxygenated red cells determined over a 55 min period following sham treatment or treatment with isoproterenol (i.e. over the period following collection of the control sample)*

	Oxygenated (0·2% CO_2/air)	Deoxygenated (0·2% CO_2/N_2)
Sham	41·09 ± 12·55	114·18 ± 23·82[b,c]
Isoproterenol	18·36 ± 10·18	75·09 ± 16·24[b]
Amiloride	6·73 ± 29·45	56·00 ± 11·64[a]

Red cells treated with sham solution only are designated Sham; red cells preincubated in sham solution prior to isoproterenol incubation are designated Isoproterenol, and red cells preincubated in amiloride solution prior to isoproterenol incubation are designated Amiloride.

Lactate production rate is expressed as nmol of lactate produced per gram of haemoglobin per minute. See text for further details.

Each value is the mean ± 1 S.E.M. of six determinations.

Values are marked if different from sham (*a*); if different from oxygenated (*b*), and if different from amiloride (*c*).

is typically of the order of 100 nmol g^{-1} Hb min^{-1} (Ferguson *et al.* 1989; Eddy, 1977). Assuming carbohydrate as the preferred substrate (or a metabolic derivative such as pyruvate, Houston *et al.* 1985) and a P/O ratio of 2 for NADH-linked oxidations, each mole of oxygen consumed by the red cell can be translated into about 4·0 equivalents of ATP produced (Hochachka & Somero, 1984). Even if fat and/or protein were the preferred substrates, the ATP yield per mole of O_2 consumed would be very similar. Based on these estimates, oxidative metabolism would account for an ATP production rate of approximately 400 nmol g^{-1} Hb-min^{-1}, whereas that arising from anaerobic metabolism (e.g. $\dot{M}_{lactate}$ under oxygenated conditions, Table 3) would be 41 nmol g^{-1} Hb min^{-1}.

Under oxygenated conditions *in vitro*, NTP-producing and NTP-consuming processes are matched such that cellular NTP can remain constant for up to 3 or 4 h (Ferguson & Boutilier, 1988; R. G. Boutilier, G. Dobson, U. Hoeger & D. J. Randall, unpublished results; Table 1). However, deoxygenation caused red cell NTP levels to decay more or less linearly over the 2 h incubation period (see Table 1). Nevertheless, the rate of NTP decline in deoxygenated cells is much lower than expected. There are two possible explanations for this observation, the first being that the NTP-consuming processes are supported, following deoxygenation, by anaerobic metabolism (i.e. lactate production). Indeed, $\dot{M}_{lactate}$ production is increased by about threefold during anoxia (Table 3). However, had the anoxic cells continued to consume energy at the aerobic rate, while maintaining [NTP] relatively constant, it would mean that $\dot{M}_{lactate}$ would have had to increase by a factor of 10. The fact that it did not leads us to the second, and more plausible, explanation: salmonid red cells possess characteristics of O_2-conforming systems in which NTP turnover rates become suppressed during O_2 lack. Had NTP consumption of the anoxic cells proceeded at the aerobic rate, NTP:Hb should have fallen to zero well within the 70 min incubation period which followed 'control' sample collection. This indicates that certain NTP-consuming processes within the cell could be shut down, or at least slowed, by the onset of O_2 lack.

There are many NTP-consuming processes in any given cell, including those that require NTP directly (e.g. transcriptional and translational events), and those that use transmembrane electrochemical gradients established by NTP-dependent ion pumps (ATPases). Although little is known of the former, the fact that ion gradients are maintained under deoxygenated conditions (Fig. 2 and Table 2) suggests to us that some adaptational responses to hypoxia may be occurring at the level of the cell membrane. Maintenance of ionic gradients usually requires a considerable fraction of the metabolic energy budget of a cell. Mammalian reticulocytes, for example, produce 25% of their NTP for use by the Na^+/K^+-ATPase alone (Rapoport, 1985). Linkages between membrane function and metabolism (energy status) are also known to occur in other vertebrate cell types (Spruce *et al.* 1985; Aw & Jones, 1985), including resealed mammalian red cell membranes (Kennedy *et al.* 1986). Regulation of the ionic permeability of the erythrocyte membrane would be a beneficial adaptation to oxygen limitation. By making the membrane less leaky, the cell would be able to reduce the normally

high energy costs associated with ion transport whilst still preserving the membrane–metabolic coupling. Only when this is achieved do cells display hypoxia tolerance (Hochachka, 1986*a*,*b*, 1988).

In fact, such a strategy may be present in the red cells studied here. For example, Na^+ and K^+ concentrations in the deoxygenated erythrocytes show remarkable preservation (i.e. at 2 h) at levels comparable to those in their oxygenated counterparts (Table 2). This contrasts sharply with hypoxia-sensitive cells, such as those found in the mammalian brain, where ischaemia and concomitant anoxia lead to a rapid exhaustion of the ATP pool (within minutes) and subsequent dissipation of the Na^+ and K^+ gradients (Hansen, 1987).

It is unlikely that erythrocytes of salmonids ever experience prolonged bouts of anoxia but, even so, they appear to exhibit some capacity for arresting their metabolism in response to O_2 lack. In this regard it would be of interest to know more about the red cell metabolism of exceptionally good anaerobes such as the goldfish and turtle. For example, the Na^+ and K^+ concentrations of erythrocytes from (apparently hypoxic) turtles submerged at 3 °C for up to 9·5 weeks are similar to those of normoxic animals at 24 °C (Maginniss & Hitzig, 1987). If the strategy in such hypoxic or anoxic animals were to accelerate the anaerobic metabolism of the erythrocytes so as to make up for the energetic shortfall resulting from the loss of aerobic metabolism, the result could put the functioning of the cell at a disadvantage upon subsequent oxygenation. Presumably, the lowered pH and increased erythrocyte swelling resulting from a large Pasteur effect would exacerbate haemoglobin reoxygenation.

Adrenergic pH regulation of oxygenated and deoxygenated erythrocytes

Adrenergic stimulation of salmonid erythrocytes *in vitro* evokes enhanced Na^+/H^+ exchange (it can be blocked by amiloride, Table 1), which leads to an extracellular acidification and an increase in red cell pH (Nikinmaa, 1982, 1983, 1986; Nikinmaa & Huestis, 1984; Baroin *et al.* 1984; Cossins & Richardson, 1985; Borgese *et al.* 1987*a*; Nikinmaa & Tufts, 1989). *In vivo*, the system operates (through stress-induced catecholamine release) to maintain red cell pH constant during extracellular acidoses. Under oxygenated conditions *in vitro* or *in vivo*, the extent to which salmonid red cell pH is adrenergically regulated is positively correlated to red cell $\dot{M}_{O_2}$, such that NTP-consuming processes are matched with NTP-producing processes (Ferguson & Boutilier, 1988; Ferguson *et al.* 1989). Thus, the membrane ion transport processes (notably Na^+/H^+ exchange) stimulated during adrenergic pH regulation are associated with, in oxygenated cells, a highly aerobic process that involves a tight coupling between membrane transport functions and cellular metabolism.

The activity of the adrenergically mediated Na^+/H^+ exchanger varies according to the intracellular pH (Borgese *et al.* 1987*b*; Heming *et al.* 1987; Nikinmaa *et al.* 1987) and oxygen tension (Motais *et al.* 1987; Salama & Nikinmaa, 1988; Ferguson *et al.* 1989; Figs 2, 3). At an extracellular pH of 7·6, the net sodium uptake in trout erythrocytes was calculated to be approximately four times greater in an anaerobic

atmosphere than in an oxygen environment (Motais *et al.* 1987). A much greater uptake of sodium in anoxic red cells was also observed in the present study (Fig. 2). The reason for the greater activation of Na^+/H^+ exchange in deoxygenated cells is not yet clear. Because the adrenergically mediated increase in cyclic AMP level in trout erythrocytes (Mahé *et al.* 1985) is evidently unaffected by deoxygenation (Motais *et al.* 1987), it has been suggested that oxygen modulates Na^+/H^+ exchange indirectly by way of a transducer, such as haemoglobin (Motais *et al.* 1987).

In this regard, there is recent evidence showing that the deoxy t-form of human haemoglobin has a greater electrostatic affinity for the cytosolic segment of band 3 protein than does oxyhaemoglobin, and that the strength of the interaction is negatively correlated with the abundance of the allosteric modifier 2,3-diphosphoglycerate (Chetrite & Cassoly, 1985). Our data show that the stimulation of anoxic red cells by isoproterenol results in a reversal of the transmembrane pH gradient, whereas no such reversal occurs when the cells are oxygenated (Fig. 1A). Interestingly, Nikinmaa *et al.* (1987) report that isoproterenol induced a similar reversal in the transmembrane pH gradient of red cells incubated at atmospheric P_{O_2} in the presence of the anion exchange blocker 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS). If, in the case of the deoxygenated condition, flux through the chloride/bicarbonate exchanger was retarded by its interaction with haemoglobin, it would have contributed (as does DIDS above) to the observed pH reversal. Net chloride accumulation in the adrenergically stimulated red cells is influenced by anoxia (Fig. 3), though the reason(s) for this is presently unclear. It could be due to some form of inhibition of the Cl^-/HCO_3^- exchanger or be caused by O_2-mediated changes in the influx and efflux components of chloride ion movements. Certainly, the observed pH effects presented in Fig. 1 arise in part from the different pHi set points observed prior to stimulation. Deoxygenation and the concomitant Haldane effect lead to a higher pHi set point for the anoxic red cells than for the normoxic red cells (cf. Fig. 1 and Jensen, 1986). This, together with the accelerated Na^+/H^+-mediated extracellular acidification (Figs 1 & 2; Motais *et al.* 1987), could lead to the reversal of the pH gradient in anoxic red cells.

Metabolic- and membrane-coupled functions of adrenergically stimulated erythrocytes

The adrenergic regulation of intracellular pH in salmonid erythrocytes is a tightly balanced energy-consuming process (Ferguson & Boutilier, 1988) and, in oxygenated red cells, increased ATP turnover is evidently linked to membrane ion transporting processes (Ferguson *et al.* 1989; Boutilier & Ferguson, 1989). Metabolically, the situation is quite different in adrenergically stimulated anoxic cells. For example, whilst the adrenergically stimulated ion exchange processes still operate as in the oxygenated erythrocytes, the anoxic cells are apparently incapable of simultaneously increasing ATP turnover through anaerobic metabolism (Table 3; Ferguson *et al.* 1989). The net effect is that ATP consumption is no

matched with ATP production and, therefore, NTP concentrations fall much more rapidly in deoxygenated than in oxygenated red cells after adrenergic stimulation (Fig. 1). This accelerated decay of NTP in the adrenergically stimulated anoxic cells is indicative of an uncoupling of membrane and energy-producing metabolisms. Thus, unlike in the sham-treated anoxic cells, energy production does not approach energy demand in their adrenergically stimulated counterparts (Table 3).

As a consequence of the enhanced Na^+/H^+ exchange and simultaneous metabolic suppression of the deoxygenated cells, it becomes possible to relate the ionic exchange processes to energy expenditure in a direct way (Fig. 4A,B). Thus, it is clear that a rise in $[Na^+]_i$ or a decrease in $[K^+]_i$ leads to increased expenditure of NTP, as shown by the consequent decline in the NTP: Hb ratio. Presumably, the elevated expenditure of energy following adrenergic stimulation is the result of an increased activity of the Na^+/K^+ pump, as has been suggested by the results of other studies (e.g. Bourne & Cossins, 1982). Fig. 5 is a simplified model of an adrenergically stimulated trout erythrocyte, indicating how membrane transport processes and metabolism may be linked in these cells. As shown in the model, the adrenergically mediated uptake of Na^+ presumably stimulates corrective actions

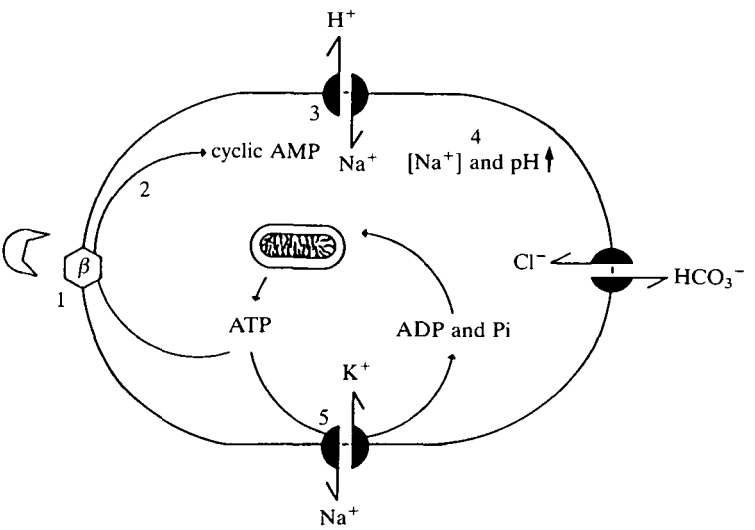

Fig. 5. Hypothetical model of a rainbow trout cell being stimulated adrenergically. We suggest the following sequence of events following adrenergic stimulation: first, β-adrenergic receptor stimulation (1) leads to a rise in intracellular concentrations of cyclic AMP (2), which triggers an activation of the Na^+/H^+ exchanger such that protons are extruded from the cell in exchange for Na^+ (3). The net effect is a cell alkalinization and an increase in intracellular $[Na^+]$ (4). At this stage, the rise in intracellular $[Na^+]$ stimulates Na^+/K^+ exchange and the increased consumption of energy (5). In aerobically incubated cells, aerobic metabolism is tightly coupled to energy-consuming (membrane) processes. Depletion of the NTP pool is observed in red cells in which membrane and energy-producing metabolisms have been uncoupled. See text for further details.

by the Na^+/K^+ pump, thereby leading to increased consumption of NTP (cf. Fig. 4). How this system is regulated by metabolism is currently being explored.

pH regulation and metabolism

Following adrenergic stimulation, the pH of the erythrocyte is determined by the extent of Na^+/H^+ exchange in the short term, and by the consequent levels of intracellular NTP in the long term. For example, under oxygenated conditions, the increased energetic demands of the Na^+/K^+ pump are met through increases in aerobic metabolism so as to maintain the NTP concentrations and, presumably, the fixed negative charge at a relatively constant level (Fig. 1; Ferguson & Boutilier, 1988). The virtue of matching energy supply with demand in the long term is that the fixed negative charge and, therefore, pHi are unaltered in the steady state. Mismatches between energy supply and demand can, for example, be associated with large perturbations in the pH gradient between red cell and plasma (Fig. 1). Certainly, the pH of the erythrocyte appears to be largely dependent on the corresponding levels of intracellular NTP (e.g. Fig. 4D; Wood & Johansen, 1973; Hladky & Rink, 1977), giving further evidence for the coupling of metabolism and pHi regulation in these cells.

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