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ADRENERGIC STIMULATION OF SEA BREAM (*SPARUS AURATA*) RED BLOOD CELLS IN NORMOXIA AND ANOXIA: EFFECTS ON METABOLISM AND ON THE OXYGEN AFFINITY OF HAEMOGLOBIN

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Summary

The metabolic response of sea bream (*Sparus aurata*) red blood cells to adrenergic stimulation was determined in normoxia and anoxia. In the presence of oxygen, red blood cells swelled and then recovered their resting volume. Continuous monitoring of oxygen uptake displayed the kinetics of the increase in the oxygen affinity of haemoglobin. Cell volume recovery correlated with an activation of ATP consumption, and the energy equilibrium was restored by increasing the rates of respiration and glycolysis. When the respiratory chain was blocked, adrenergic stimulation increased the rates of ATP consumption and glycolysis of red blood cells. Moreover, adrenergic stimulation of deoxygenated erythrocytes also increased cell volume but did not enhance glycolysis or ATP consumption, and the cells remained swollen. Our results suggest that there is an oxygen-linked signal transducer that activates ATP-consuming processes, provided that the adrenergic stimulation occurs in the presence of oxygen.

Key words: erythrocytes, adrenaline, fructose 1,6-bisphosphate, microcalorimetry, sea bream, *Sparus aurata*, normoxia, anoxia.

Introduction

It is well known that adrenergic stimulation of fish red blood cells (RBCs) increases the internal pH and thus enhances the affinity of haemoglobin for oxygen under extreme conditions of hypoxia (Cossins and Richardson, 1985; Thomas and Perry, 1992). The results of *in vitro* and *in vivo* studies have established that the mechanism promoting intracellular alkalization is a catecholamine-mediated activation of the Na^+/H^+ exchanger *via* stimulation of the β-adrenoreceptors (Motais and Garcia-Romeu, 1987; Nikinmaa and Tufts, 1989; Nikinmaa, 1992; Guizouarn *et al.* 1993). The increase in intracellular Na^+ concentration that follows adrenergic stimulation induces a water inflow which causes cell swelling. The RBCs may then recover their resting Na^+ concentration, cell volume and electrochemical potentials by means of passive (Borgese *et al.* 1987*a*; Thomas and Perry, 1992; Guizouarn *et al.* 1993) and active (Tufts and Boutilier, 1991) transport, which increases ATP consumption (Salama and Nikinmaa, 1988; Ferguson and Boutilier, 1988, 1989; Ferguson *et al.* 1989; Tufts and Boutilier, 1991). The energy equilibrium of the cell can be maintained provided that the rate of ATP production rate is increased by an equal amount (Bourne and Cossins, 1982; Ferguson and Boutilier, 1988, 1989). Therefore, the activation of energy metabolism plays an important role in the control and recovery of resting conditions when erythrocytes are adrenergically stimulated (Ferguson and Boutilier, 1988, 1989; Ferguson *et al.* 1989). This metabolic activation is well-characterised in trout RBCs (Ferguson and Boutilier, 1988, 1989; Ferguson *et al.* 1989; Tufts and Boutilier, 1991). In contrast, the adrenergic effects on RBC metabolism in teleosts other than salmonids have been less well investigated (Salama and Nikinmaa, 1988).

The aim of this work was to study RBC metabolism under conditions of adrenergic activation in a novel species, the gilthead sea bream (*Sparus aurata*). These fish face large fluctuations in water salinity and temperature and, consequently, in oxygen availability when they move from the open sea to brackish waters in coastal lagoons. Not only can they adapt to fluctuating environments (Altimiras *et al.* 1994; Tort *et al.* 1994), but they can carry out their life cycle in either habitat. Moreover, they also show a hypoxia-induced release of catecholamines when oxygen tension falls below 7.31 kPa (catecholamine concentration is maximal at 2.13–2.66 kPa) (J. Altimiras and L. Tort, unpublished results). This tolerance of hypoxia is higher than in rainbow trout, where catecholamine release is triggered by oxygen tensions of 8.0–8.6 kPa (Perry and Reid, 1992) and, coupled with a higher blood osmolarity,

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could mean that these are different challenges to RBC metabolism in this species.

To determine the importance of the presence of oxygen for the adrenergic response, sea bream RBCs were stimulated under normoxic and anoxic conditions. The metabolic changes of adrenergically stimulated sea bream erythrocytes were evaluated by correlating total metabolic activity, assessed by calorimetry, with oxygen consumption and cellular metabolite measurements. In addition, a comparison of the continuous oxygen uptake in the presence and in the absence of different metabolic inhibitors allowed us to study the enhancement of the oxygen affinity of haemoglobin and to evaluate the amount of oxygen bound after adrenergic stimulation.

Materials and methods

Animals

Gilthead sea bream (*Sparus aurata*, L.), with a body mass of 300–350 g, were obtained from Aquadelt S.A., a fish farm in the delta of the Ebre river (East Spain). Fish were transported in oxygenated tanks under light anaesthesia and placed in 400 l fibreglass tanks. They were allowed to recover from transport and acclimatised in a closed seawater [12 °C, 38 p.p.t. (3.8 ‰) salinity] circulation system with physical and biological filters for 1 month before the beginning of experiments. When abnormal behaviour, such as a decrease in feeding or any other sign of non-adaptation, occurred, the fish were allowed to recover for longer. Oxygen tension and ammonia and nitrite levels in the water were determined twice a week. A constant photoperiod (12 h:12 h light:dark) was set in the room using appropriate timers. Fish were fed the equivalent of 1 % of their body mass daily with extruded pellets (Purina S.A.).

Isolation of red blood cells

Blood was obtained from the caudal vessels, after rapidly capturing the fish, using heparinized syringes with 0.6 mm×25 mm (23 gauge) needles. Using this method, blood was obtained in less than 20 s, which is fast enough to avoid most of the effects of the stress response that could interfere with our study. The blood was immediately diluted with heparinized RPMI 1640 medium (Sigma Co., USA). Red blood cells were separated from lymphocytes and other mononuclear cells by centrifuging with histopaque-1077 (Sigma Co., USA), following the procedure suggested by the supplier. The cells were rinsed four times in a modified Cortland buffer (Houston *et al.* 1985) (NaCl, 141 mmol l^{-1}; KCl, 3.5 mmol l^{-1}; $MgSO_4$, 1 mmol l^{-1}; NaH_2PO_4, 3 mmol l^{-1}; $CaCl_2$, 1 mmol l^{-1}; $NaHCO_3$, 30 mmol l^{-1}; Hepes, 10 mmol l^{-1}; albumin 0.3 % w/v). pH was adjusted to 7.6 and osmolality to 0.4 osmol kg^{-1}. For measurements, RBCs were resuspended at a final haematocrit of 10 % in fresh buffer containing 1 mmol l^{-1} glucose. Cells were used the day after preparation.

Analytical procedures

Haematocrit was determined by quadruplicate centrifuging of blood samples for 5 min at 12 000 ***g***. Lactate production and haemoglobin, ATP and fructose 1,6-bisphosphate (Fru-1,6-P_2) concentrations were determined according to Beutler (1984).

Microcalorimetric measurements

The rate of heat production of sea bream RBCs was measured at 15 °C in a thermal activity monitor (ThermoMetrics AB, Sweden), which consists of four identical heat conduction calorimeters with a working volume of 3.5 ml (Suurkusk and Wadsö, 1982; Görman *et al.* 1984). A turbine stirrer (100 revs min^{-1}) ensured efficient mixing of the cell suspension. When assembled, the unit was lowered to the measurement position of the calorimeter for four periods of 10 min each. Further equilibration of the calorimeter took 1 h before a steady heat reading was obtained.

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 (Bäckman and Wadsö, 1991; Pesquero *et al.* 1994). For anaerobic measurements, the calorimetric vessels were completely filled with 3.5 ml of cell suspension, so that all the available O_2 was that dissolved in the medium. In these conditions, while the cells were consuming the dissolved oxygen, measurements were aerobic. After complete O_2 exhaustion, the metabolism became anaerobic (see Fig. 5B). To shorten the duration of the initial aerobiosis, cell suspensions were previously flushed with N_2 to reduce the amount of dissolved O_2 present. Injections were performed with a gas-tight Hamilton syringe driven by a stepper motor. Data were sampled every second, and the mean values over 180 s were stored. The resulting power–time curves indicate the heat produced by the metabolic activity of cell suspensions under the experimental conditions.

Oxygen measurements

The rate of oxygen consumption was measured in a Clark-type oxygen electrode thermostatted at 15 °C (Rank Brothers, England) and filled with 2.7 ml of cell suspension. The reading from the Clark electrode provides a measurement of the oxygen tension; that is, the partial pressure of oxygen dissolved in the solution (P_{O_2}). Because the measurements involve erythrocytes, the slope of the polarographic curve reflects the rate of oxygen uptake of the cells only at high P_{O_2} values, where a change in oxygen tension does not influence the oxygen saturation of haemoglobin. At low P_{O_2}, the decrease in oxygen tension is not a reliable indicator of the rate of oxygen consumption since the measurement includes both the oxygen consumption of the cells and the oxygen taken up or released by haemoglobin. This proved to be the case in catecholamine-stimulated cells and, therefore, P_{O_2} measurements may only be assumed to represent the rate of O_2 consumption when haemoglobin is fully saturated with oxygen (80–100 % air saturation, i.e. 16–20 kPa) (Pesquero *et al.* 1994).

The total amount of oxygen taken up by adrenaline-stimulated cells was calculated as the difference between the unperturbed value of P_{O_2} (estimated from the dashed line in

Fig. 1A–C) and the value displayed by the recording 30 min after the addition of adrenaline (stationary conditions).

Enthalpy calculations

Heat production is a global index that integrates the heat produced by all the underlying events of cell metabolism. To evaluate which part of the measured heat production was accounted for by respiration and which by glycolysis, their caloric equivalents ($-507.3\,kJ\,mol^{-1}\,O_2$ for respiration and $-80.5\,kJ\,mol^{-1}$ lactate for glycolysis, respectively) were used (Gnaiger and Kemp, 1990; Bäckman and Wadsö, 1991).

Statistical analyses

Results are expressed as mean ± S.E.M. Calorimetric and oxygen curves are representative of similar results obtained in different experiments. Comparisons between groups were performed using analysis of variance (ANOVA) and the Scheffe *post hoc* test. Differences were considered to be significant at $P<0.05$.

Results

Changes in the rate of oxygen uptake

The time courses shown in Fig. 1 indicate the effect of several substances on the rate of oxygen uptake of RBC suspensions. The addition of adrenaline to oxygenated RBCs (Fig. 1A) produced a biphasic response. First, there was a sharp increase in the rate of oxygen uptake to a value as high as $600\,nmol\,O_2\,g^{-1}\,Hb\,min^{-1}$ (where Hb is haemoglobin). The total oxygen uptake during this initial phase (15 min) was $2.2\pm0.1\,\mu mol\,O_2\,g^{-1}$ Hb (Table 1). This large increase in the rate of oxygen uptake did not match the enhancement of metabolic activity reflected in the microcalorimetric records (Table 1; see Fig. 5A).

The second phase of Fig. 1A shows that 15 min after the adrenaline addition the rate of oxygen uptake was stable and had increased by $7.0\pm1.4\,nmol\,O_2\,g^{-1}\,Hb\,min^{-1}$ compared with that of the control cells (Table 1). The enthalpy equivalent of this value, together with that corresponding to the increase in lactate production, fits with the enhancement of heat production observed in normoxic RBCs.

The double-phase effect of Fig. 1A may be reproduced by increasing cyclic AMP synthesis by adding forskolin together with isobutylmethylxanthene (IBMX), which inhibits cyclic AMP degradation to 5′-AMP (Rozenburg, 1986). Fig. 1B shows the rate of oxygen uptake of a RBC suspension following the addition of $25\,\mu mol\,l^{-1}$ forskolin plus $50\,nmol\,l^{-1}$ IBMX.

To determine whether this oxygen uptake was metabolically utilised, adrenaline was added to RBCs preincubated with $20\,\mu mol\,l^{-1}$ antimycin-A to block the respiratory chain. Fig. 1C indicates that, in the presence of antimycin-A, erythrocytes ceased to take up oxygen, and the further addition of adrenaline reproduced only the initial phase of Fig. 1A. Total oxygen uptake by non-respiring cells during the adrenergic stimulus was $1.7\pm0.2\,\mu mol\,O_2\,g^{-1}$ Hb, which is in good agreement with that of the first phase obtained for respiring cells ($2.2\pm0.1\,\mu mol\,O_2\,g^{-1}$ Hb) (Table 1) if the oxygen consumed by respiration during the same period ($0.3\pm0.1\,\mu mol\,O_2\,g^{-1}$ Hb) is taken into account. The addition of adrenaline to RBCs incubated with NaCN did not alter their rate of oxygen uptake (Fig. 1D).

To determine the role played by the Na^+/H^+ exchanger in the oxygen uptake by RBCs during adrenergic stimulation, adrenaline was added to RBC suspensions in which Na^+/H^+ antiport had been inhibited by $1\,mmol\,l^{-1}$ amiloride. Fig. 1E shows a slight inhibition of the rate of oxygen uptake following amiloride addition and no significant change when adrenaline was then added to the cell suspension.

Table 1. *Mean values of heat and lactate production, rate of oxygen consumption and total oxygen uptake in the first 30 min following adrenergic stimulation under different conditions*

	Normoxia		Anoxia		Antimycin-A	
	Control	ADR	Control	ADR	Control	ADR
Heat production ($\mu W\,g^{-1}$ Hb)	581±11 (N=8)	699±10* (N=8)	370±9† (N=4)	367±5† (N=4)	391±11† (N=4)	448±7*,† (N=4)
Rate of lactate production ($\mu mol\,g^{-1}\,Hb\,h^{-1}$)	2.9±0.4 (N=8)	5.2±1.0* (N=8)	12.9±0.7† (N=6)	13.0±0.6† (N=6)	11.9±0.6† (N=6)	13.7±0.8*,† (N=6)
Rate of oxygen consumption ($nmol\,g^{-1}\,Hb\,min^{-1}$)	55.2±1.5 (N=8)	62.2±4.3* (N=8)	–	–	0 (N=5)	0 (N=5)
Total oxygen taken up ($\mu mol\,g^{-1}$ Hb)	–	2.2±0.1 (N=7)	–	–	–	1.7±0.2 (N=3)

Adrenergic stimulus was performed by addition of $0.1\,mmol\,l^{-1}$ adrenaline (ADR) in all conditions.

Inhibition of the respiratory chain was achieved by addition of $20\,\mu mol\,l^{-1}$ antimycin-A.

* indicates a statistically significant difference from the respective control value ($P<0.05$); † indicates a statistically significant difference from the normoxic control value ($P<0.05$).

Values are mean ± S.E.M. for the number of experiments given in parentheses.

Hb, haemoglobin.

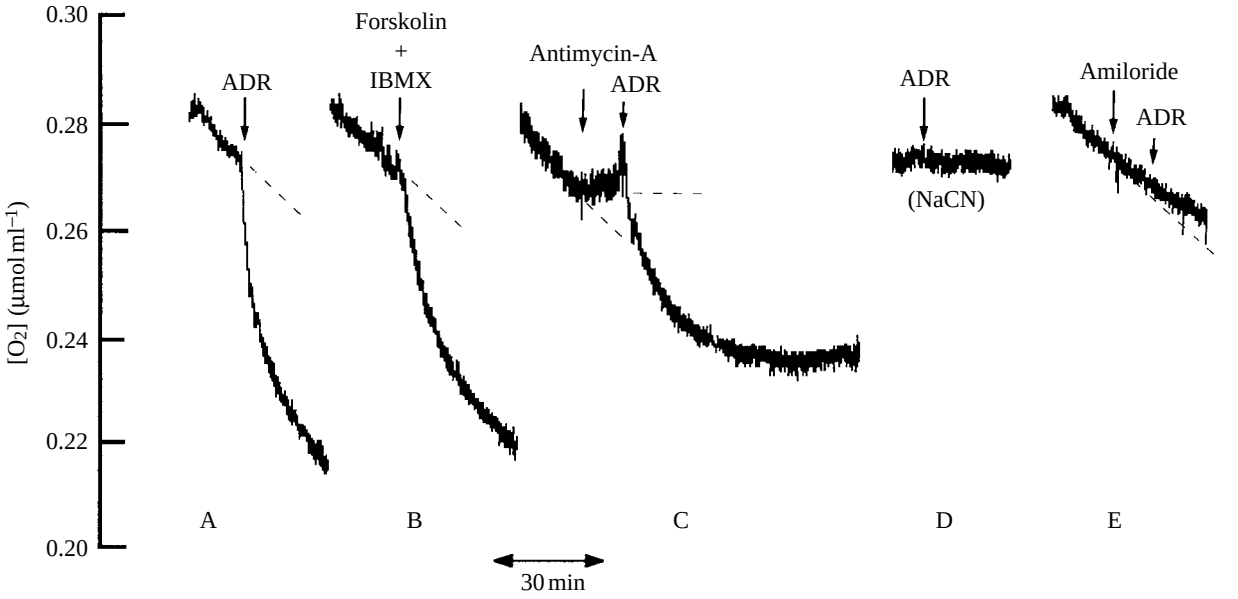

Fig. 1. Modifications in the slopes of the time courses of dissolved oxygen concentration ($[O_2]$) indicate changes in the rate of oxygen uptake by red blood cells (RBCs) due to the addition of 0.1 mmol l^{-1} adrenaline (ADR) (A) or 25 μmol l^{-1} forskolin plus 50 nmol l^{-1} IBMX (B) and when 0.1 mmol l^{-1} adrenaline (ADR) was added in the presence of 20 μmol l^{-1} antimycin-A (C), 0.1 mmol l^{-1} NaCN (D) or 1 mmol l^{-1} amiloride (E). Arrows indicate the time of addition of chemicals. Plots are representative of 4–8 experiments. The dashed lines indicate the unperturbed oxygen concentration.

Metabolic changes

The physiological response of sea bream RBCs to adrenergic stimulus was initially characterised by changes in the cell volume. Fig. 2 shows the increase of sea bream RBC volume following adrenergic stimulation (0.1 mmol l^{-1} adrenaline) under normoxic and anoxic conditions and in the presence of 20 μmol l^{-1} antimycin-A to inhibit the respiratory chain. As observed by Borgese *et al.* (1986), the oxygen concentration of the medium affects the cell volume increase produced by the adrenergic stimulus: the increase in cell volume was greater in the absence of oxygen than in the other conditions. In addition, normoxic cells recovered their control volume within 3 h of adrenaline addition, whereas in the absence of oxygen the increased cell volume was maintained and in chemically achieved anoxia showed only slight recovery.

Most of the previous studies of the mechanisms of adrenergic stimulation of fish erythrocytes have used isoproterenol, a purer β-agonist than adrenaline (Borgese *et al.* 1986, 1987*a*; Ferguson and Boutilier, 1989; Ferguson *et al.* 1989; Nikinmaa *et al.* 1990; Guizouarn *et al.* 1993). Since we focused our study on metabolic changes produced by adrenergic stimulation, we used the physiological agonist (Nikinmaa, 1982). Although the dose of adrenaline used to stimulate RBCs *in vitro* was several times higher than physiological concentrations, we observed that the aerobic response of RBCs was qualitatively similar in the range 10^{-4}–10^{-7} mol l^{-1} adrenaline (data not shown).

To determine whether the differences in the normoxic and anoxic responses to adrenaline were reflected in the energy metabolism of the RBCs, the content of a key glycolytic effector (Fru-1,6-P_2) (Kirtley and McKay, 1977) was determined (Fig. 3). No change in the concentration of this bisphosphorylated compound was measured after the addition of adrenaline in the absence of oxygen. However, in oxygenated cells, a significant transient increase in the

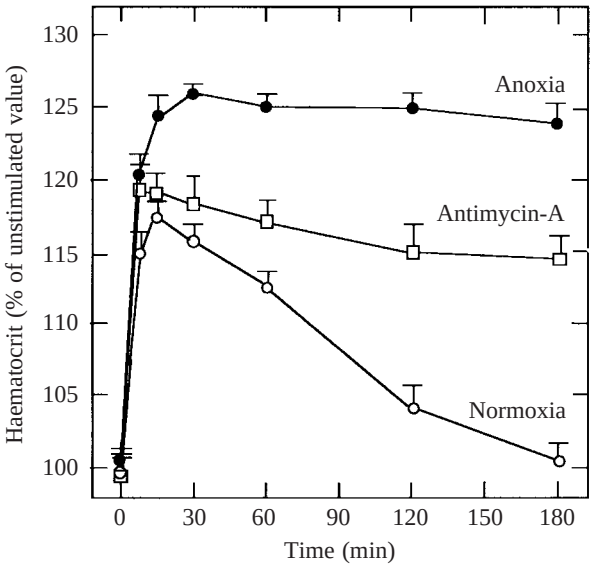

Fig. 2. Haematocrit expressed as a percentage of the respective control values after the addition of 0.1 mmol l^{-1} adrenaline to RBCs in normoxia (○) and in anoxia (●) and to normoxic cells incubated with 20 μmol l^{-1} antimycin-A (□). All chemicals were added at time 0. Values are mean + S.E.M. for 5–6 experiments.

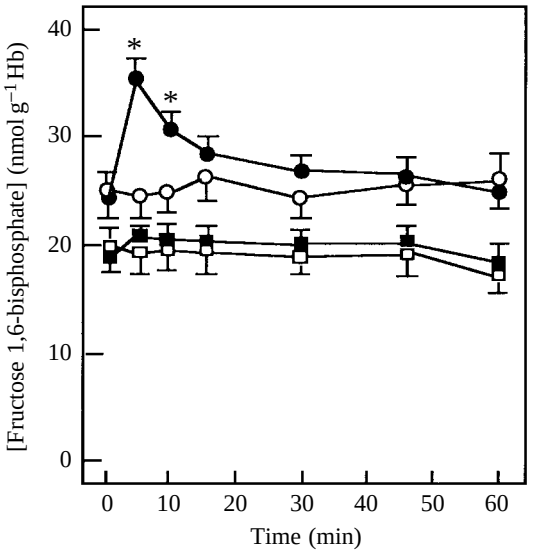

Fig. 3. Fructose 1,6-bisphosphate content in control normoxic RBCs (○), in control anoxic RBCs (□), in the presence of 0.1 mmol l^{-1} adrenaline in normoxia (●) and in anoxia (■). Adrenaline was added at time 0 and fructose 1,6-bisphosphate concentrations were measured at different incubation times. Values are mean ± S.E.M. for 3–4 experiments. *Significantly different from the respective control value (P<0.01). Hb, haemoglobin.

concentration of intracellular Fru-1,6-P_2 occurred after adrenaline addition (Fig. 3).

These results indicate that carbohydrate metabolism was activated when adrenaline was added to oxygenated RBC suspensions. The changes induced in the cell energy charge were determined by measuring variations in the ATP pool during the adrenergic response. Fig. 4A shows that adrenergic stimulation under aerobic conditions induced a significant 20 % decrease in cell ATP content; [ATP] returned to control levels 60 min after adrenaline addition. Fig. 4B indicates a lower ATP content in anoxic than in normoxic RBCs (cf. Fig. 4A) and that the addition of adrenaline had no effect on this value. Moreover, Fig. 4C shows that adrenergic stimulation of RBCs in the presence of antimycin-A increased the rate at which their ATP content declined compared with that of normoxic cells.

Changes in all metabolic activity induced by adrenergic stimulation of sea bream RBCs were monitored using a microcalorimeter. Fig. 5A–C shows the metabolic responses of sea bream RBCs corresponding to the experiments shown in Figs 2 and 4. The mean values of heat production are displayed in Table 1. Adrenergic stimulation of normoxic cells increased heat production by 118±2 μW g^{-1} Hb (Fig. 5A; Table 1). However, no significant change was observed when adrenaline was added under anaerobic conditions (Fig. 5B; Table 1). When antimycin-A was added to the RBC suspensions, heat production was reduced to the anaerobic level. Addition of adrenaline in these conditions increased heat production significantly by 57±4 μW g^{-1} Hb (Fig. 5C; Table 1).

To determine how adrenergic stimulation affects different

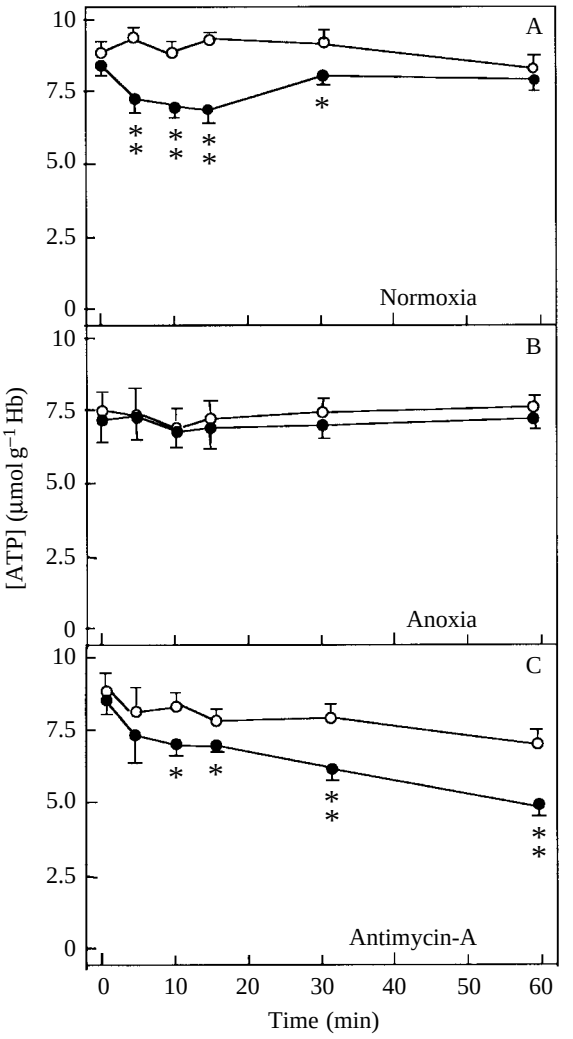

Fig. 4. ATP content in control RBCs (○) and in adrenergically (0.1 mmol l^{-1} adrenaline) stimulated cells (●) under normoxia (A), under anoxia (B) and in the presence of 20 μmol l^{-1} antimycin-A (C). All chemicals were added at time 0, and ATP concentrations were measured at the indicated incubation times. Values are mean ± S.E.M. for 3–4 different experiments. Asterisks indicate significant differences from the respective control levels; *P<0.01, **P<0.001. Hb, haemoglobin.

metabolic pathways, in addition to the respiratory activity assessed by oxygen consumption (Fig. 1), we determined the rate of lactate production as an index of anaerobic glycolysis (Table 1). The rate of lactate production in normoxia was significantly increased by 2.3±0.3 μmol g^{-1} Hb h^{-1} after adrenaline addition. In anoxic cells, as well as in cells where respiration was blocked by antimycin-A, the rate of lactate production was at least four times the value obtained under normoxia. The addition of adrenaline did not change the rate of lactate production of deoxygenated RBC suspensions but significantly increased the rate of lactate production of antimycin-treated non-respiring cells by 1.8±0.6 μmol g^{-1} Hb h^{-1} (Table 1). The enthalpy change associated with this increase in lactate production was

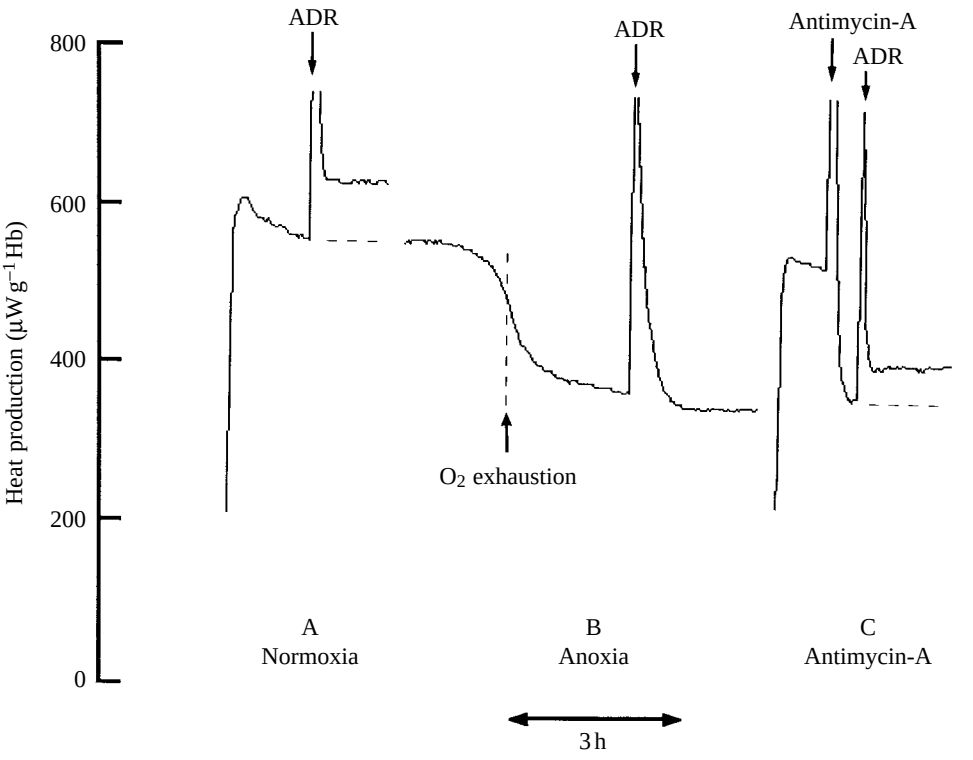

Fig. 5. Plots displaying changes in heat production of RBCs due to the addition of 0.1 mmol l^{-1} adrenaline under normoxia (A) and anoxia (B) and in the presence of 20 μmol l^{-1} antimycin-A (C). The horizontal dashed lines indicate the heat production of the control cell suspensions. In B, anaerobic conditions were achieved after oxygen exhaustion because of cell oxygen consumption. Arrows indicate the addition of chemicals. Exothermic peaks correspond to the heat produced by mechanical disturbances plus the heat of dilution associated with each injection. Plots are representative of 4–8 different experiments. Hb, haemoglobin.

40±14 μW g^{-1} Hb, which corresponds, with acceptable accuracy, to the enhancement of the heat production recorded experimentally (Fig. 5C; Table 1).

Discussion

Adrenergic stimulation increases the oxygen-carrying capacity of erythrocytes

When RBCs were adrenergically stimulated in normoxia (Fig. 1A), the rate of oxygen uptake initially showed a dramatic increase followed by a steady consumption which was 7 nmol O_2 g^{-1} Hb min^{-1} higher than that observed in control cells (Table 1). The metabolic significance of these changes in the rate of oxygen uptake can be explained when Fig. 1A is compared with the corresponding record of heat production (Fig. 5A). The rate of oxygen uptake immediately following the adrenergic stimulus was ten times that observed in non-stimulated cells. If all this oxygen uptake were utilised metabolically, it would account for a higher heat production than that observed. However, only a moderate increase in the heat production was recorded (Fig. 5A), which correlates quite well with the enthalpy equivalents of the increases measured in lactate production and in oxygen consumption (Table 1). Moreover, when metabolic utilisation of oxygen was blocked by antimycin-A (Fig. 1C), the adrenergic stimulus produced an increase in the rate of oxygen uptake similar to the initial phase of Fig. 1A. These results indicate that the initial increase in oxygen uptake, caused by adrenergic stimulation, was not metabolically used by cells. Results reported previously (Nikinmaa, 1983; Cossins and Richardson, 1985; Salama and Nikinmaa, 1988; Reid *et al.* 1993) have indicated that the oxygen affinity of haemoglobin may be enhanced (Bohr and Root effects) when intracellular pH increases (see Thomas and Perry, 1992, for references). The traces shown in Fig. 1A–C display the same kinetics for oxygen uptake as were found for proton extrusion by Cossins and Richardson (1985) when trout erythrocytes were adrenergically stimulated in unbuffered solutions. These findings suggest that the initial phases of the oxygen plots, obtained when sea bream erythrocytes were adrenergically stimulated (Fig. 1A–C), correspond to an increase in haemoglobin oxygen-affinity and the consequent increase in the oxygen saturation of haemoglobin, because of the intracellular pH increase that follows activation of the Na^+/H^+ exchanger (Nikinmaa, 1983; Salama and Nikinmaa, 1988; Thomas and Perry, 1992; Reid *et al.* 1993).

This interpretation was reinforced by the results obtained in the presence of NaCN (Fig. 1D) and amiloride (Fig. 1E). We observed that cells stimulated in the presence of NaCN (not shown) showed similar swelling to that observed in the presence of antimycin-A (Fig. 2). This swelling suggests the activation of the Na^+/H^+ exchanger and, consequently, an intracellular alkalization. However, cyanhaemoglobin cannot bind to oxygen and, therefore, the increasing pH does not modify the oxygen affinity of haemoglobin, so that the initial rate of oxygen uptake displayed in Fig. 1A is not seen in Fig. 1D. Moreover, this initial phase did not occur when adrenaline was added to oxygenated cells in the presence of amiloride (Fig. 1E) because inhibition of the Na^+/H^+ exchanger prevents changes in intracellular pH and, consequently, modifications of the oxygen affinity of oxyhaemoglobin.

Metabolic response of sea bream erythrocytes to adrenergic stimulation

A comparison of the plots shown in Fig. 1A,B indicates that the adrenergic response (Fig. 1A) can be simulated by increasing intracellular [cyclic AMP] with forskolin and IBMX (Fig. 1B). This finding provides corroboration for the proposal that an intracellular accumulation of cyclic AMP triggers the adrenergic response in sea bream RBCs, as it does in salmonid (Mahé *et al.* 1985) and carp (Salama and Nikinmaa, 1990) erythrocytes. Moreover, when the Na^+/H^+ exchanger was inhibited by amiloride, the adrenergic response was completely inhibited (Fig. 1E). This fact suggests (i) that, in sea bream RBCs, the activity of Na^+/H^+ exchanger is increased by cyclic AMP, and (ii) that the increased activity of this antiporter produces swelling, a change in internal pH and intracellular Na^+ accumulation which may evoke the activation of both passive and energy-consuming mechanisms for recovery and to maintain resting levels of ions (Mahé *et al.* 1985; Motais *et al.* 1987). This enhanced energy consumption must be balanced by an increase in metabolic activity to restore the energy equilibrium after adrenergic stimulation. This hypothesis was analysed by examining the response of sea bream RBCs stimulated by adrenaline. In normoxia, there was a complete recovery to the resting volume within 3 h of the addition of adrenaline. The increase in the intracellular Fru-1,6-P_2 concentration observed immediately after addition of adrenaline (Fig. 3) suggests that metabolic fluxes were activated which led to an increase of heat production (Fig. 5A; Table 1). Direct measurements of lactate production and oxygen consumption (Table 1) indicate that the rates of both glycolysis and respiration increased after adrenergic stimulation of oxygenated RBCs. The transient reduction of the ATP content (Fig. 4A) means that the adrenergic stimulus also increased the rates of some ATP-consuming processes, such as the Na^+/K^+ pump, which may be activated by the accumulation of internal Na^+ (Borgese *et al.* 1987*b*; Thomas and Perry, 1992). A balance between ATP consumption and ATP production was reached again within an hour of adrenergic stimulation.

The addition of adrenaline to deoxygenated cells produced a significantly greater cell volume increase than in normoxia. Similar differences have been reported for salmonid RBCs (Motais *et al.* 1987; Reid and Perry, 1991; Reid *et al.* 1993) and were related to the higher activity of the Na^+/H^+ exchanger in the presence of deoxyhaemoglobin than in the presence of oxyhaemoglobin (Motais *et al.* 1987). Moreover, this increase in cell swelling was accompanied by a lack of volume recovery (Fig. 2). This was also observed in trout RBCs and was related to the existence of a Cl^--dependent K^+ efflux, whose activity depended on the presence of oxyhaemoglobin as a trigger (Borgese *et al.* 1991). In addition, no enhancement of metabolic activity was recorded microcalorimetrically (Fig. 5B), and Fru-1,6-P_2 content (Fig. 3) and the rate of lactate production did not increase (Table 1). Consequently, the maintenance of ATP levels (Fig. 4B) indicates that no ATP-consuming process increased its rate. Therefore, in the absence of oxygen, the sequence of processes evoked by adrenergic stimulation seems to be interrupted after activation of the Na^+/H^+ antiporter.

The results obtained using blockers of the respiratory chain demonstrated that the lack of metabolic activation when adrenaline was added to deoxygenated cell suspensions depended intrinsically on the absence of oxygen and not on the inability of the cells to respire. Fig. 5C shows the increase in heat production observed when RBCs were adrenergically stimulated in the presence of antimycin-A. This increase in metabolic activity can only be accounted for by the enhancement of glycolysis (Table 1). Moreover, the decline in the ATP content in these conditions (Fig. 4C) indicates that some ATP-consuming processes were activated although, in spite of metabolic activation, the new situation was not in energy equilibrium. This lack of energy balance in the absence of respiration indicates that anaerobic glycolysis of these cells cannot work quickly enough to match the energy obtained when NaCN instead of antimycin-A was used to block the respiratory chain (data not shown).

The different metabolic responses to adrenergic stimulation in normoxia and in anoxia suggest the existence of a signal transducer, similar to that reported by Motais *et al.* (1987) and Borgese *et al.* (1991), whose conformational state is directly affected by P_{O_2} or by an oxygen-linked phenomenon that acts as a trigger for the active response of sea bream erythrocytes in the presence of oxygen. In a recent study, Land and Hochachka (1995) described an oxygen-sensing mechanism, based on a heme group, controlling anoxic metabolism in turtle hepatocytes. The existence of such a mechanism in sea bream RBCs would avoid the adrenergic activation in anoxia of those ATP-consuming processes that could not be balanced in the absence of oxygen.

These results differ substantially from those obtained with salmonid erythrocytes stimulated with 10^{-7} mol l^{-1} isoproterenol (Ferguson and Boutilier, 1989; Ferguson *et al.* 1989). These authors did not observe glycolytic activation in normoxia and they reported an ATP depletion during the adrenergic response in anoxia. Although some discrepancies may be related to the physiological differences between salmonids and sea bream, the stimulation produced by 10^{-7} mol l^{-1} isoproterenol may be significantly different from that produced by adrenaline at the same concentration. We observed that isoproterenol concentration must be reduced to 10^{-9}–10^{-10} mol l^{-1} to produce a similar haematocrit response in sea bream RBCs to the response to adrenaline at physiological concentrations (data not shown).

In summary, the kinetics of enhancement of the oxygen-binding capacity of haemoglobin, produced by adrenergic stimulation, was clearly displayed in polarographic records of the oxygen uptake and allowed an evaluation of the amount of bound oxygen. The presence of oxygen appeared to be a key factor in determining the metabolic response of sea bream RBCs, since cells showed increased metabolic fluxes when they were adrenergically stimulated during normoxia. In contrast, activation of ATP-consuming processes and metabolism when adrenaline was added to deoxygenated cells

seems to be switched off by a signal transducer associated with the presence of oxygen.

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