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ADENOSINE RECEPTOR BLOCKADE AND HYPOXIA-TOLERANCE IN RAINBOW TROUT AND PACIFIC HAGFISH

II. EFFECTS ON PLASMA CATECHOLAMINES AND ERYTHROCYTES

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Summary

The purpose of this study was to examine the role of adenosine receptors (ARs) in (1) the regulation of catecholamine secretion and (2) the modulation of blood oxygen capacitance by catecholamines. To this end, we assessed the response of rainbow trout and Pacific hagfish treated with either an AR blocker, theophylline, or saline under hypoxic and normoxic conditions. Compared with the control hypoxic rainbow trout, AR blockade resulted in a smaller increase in haematocrit and haemoglobin (Hb) concentration of the blood, smaller red blood cell transmembrane pH differences and mean cellular [Hb] (MCHC), as well as a 16-fold higher plasma adrenaline concentration after only 10 min of acute hypoxic exposure. In hypoxic hagfish, AR blockade had no effect on the [Hb] of the blood, and there was no regulation of red blood cell pH or changes in MCHC. However, whereas plasma [adrenaline] did not change following exposure to a $P_{W O_2}$ of 1.33 kPa in the hypoxic sham group, the concentration increased 3.8-fold within 10 min in the theophylline-injected group. These results suggest that adenosine modulates the circulating level of catecholamines in both hypoxic rainbow trout and hypoxic Pacific hagfish.

Key words: adenosine, hypoxia, methylxanthines, catecholamines, erythrocytes, haemoglobin, spleen, *Oncorhynchus mykiss*, rainbow trout, *Eptatretus stouti*, Pacific hagfish.

Introduction

In the absence of a capability to depress metabolism, increasing oxygen transport capacity is the most efficient way for a fish to compensate for an acute reduction in O_2 availability. This can be accomplished by increasing ventilation, improving gill O_2-diffusing capacity and increasing blood O_2 capacitance (Jensen *et al.* 1993; Fritsche and Nilsson, 1993). The response of fish to acute hypoxia is also characterized by the release of catecholamines (Boutilier *et al.* 1988; Ristori and Laurent, 1989). This stress response further enhances blood oxygen transport by mediating an elevation of haemoglobin (Hb) concentration and Hb O_2-affinity (Tetens and Christensen, 1987; Randall and Perry, 1992). The O_2-carrying capacity can be increased *via* catecholamine-stimulated contraction of, and release of red blood cells (rbcs) from, the spleen (Holmgren and Nilsson, 1975; Yamamoto *et al.* 1985; Perry and Kinkead, 1989). This response can increase [Hb] by up to 30–35 % but is quite variable, being substantial in some species and almost insignificant in others (Weber and Jensen, 1988). Fish can also increase their O_2-carrying capacity in severe hypoxia by regulation of their rbc pH (Tetens and Christensen, 1987; Nikinmaa, 1992). An increase in the concentration of circulating catecholamines with acute hypoxia, stimulates the rbc Na^+/H^+ exchanger *via* β-adrenergic receptors and results in a net outflow of H^+, which raises rbc pH (Nikinmaa, 1992). Alkalization of the rbcs increases haemoglobin O_2-affinity because the haemoglobin undergoes a Root shift (Tufts and Randall, 1989). Coupled with the H^+ outflow is an inflow of Na^+, followed by Cl^-, which together cause the cell to swell by osmosis (Borgese *et al.* 1987). Adrenergic activation of the Na^+/H^+ exchanger also leads to a rapid decline in levels of ATP and other phosphates (Ferguson and Boutilier, 1989), which may further enhance oxygen binding by reducing their negative allosteric effects (Nikinmaa, 1992).

The release of catecholamines in response to acute O_2 shortages is adaptive, since it enables organisms to meet the immediate increased energy demands, but it can also be maladaptive (Barton and Iwama, 1991), because it leads to

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exhaustion and may impair biological processes from the cellular to the organismal level (Adams, 1990). Given the potency of the hormones involved and the multiplicity of their targets, control over the magnitude and the timing of this response to stress is key to its effectiveness. Since adenosine is a potent modulator of neural activity in the central nervous system (CNS) and the peripheral nervous system (Stone and Bartrup, 1991), a hypothetical role for this metabolite in the modulation of the primary stress response may be expected. Adenosine has been shown to have anti-adrenergic actions in various tissues (Dobson *et al.* 1987; Schimmel *et al.* 1987; Mullane and Williams, 1990). In mammals, adenosine has been shown to modulate the secretion of catecholamines from the adrenal medulla (Chern *et al.* 1987, 1992; Tseng *et al.* 1994).

This study investigates whether adenosine plays a modulatory role in the release of catecholamines in fish and the possible interactions between adenosine and the adrenergic control of two mechanisms generally used by fish to increase blood O_2 capacitance under severe hypoxia: (1) rbc pH regulation and (2) splenic release of rbcs. To this end, the responses of rainbow trout and Pacific hagfish to adenosine receptor blockers under normoxic and hypoxic conditions were monitored, and their responses were compared with those obtained from sham groups given saline.

Throughout the study, comparisons are made between rainbow trout and Pacific hagfish, two species with marked differences in haematological characteristics (Table 1). Compared with rainbow trout, hagfish have a large blood volume with a low Hct, [Hb], oxygen content and buffering capacity. Hagfish haemoglobin is monomeric, has a very high oxygen affinity, a small Bohr effect and no Root effect. Other important differences between these two species are the extremely low metabolic rate (Munz and Morris, 1965; Farrell, 1991), the absence of a spleen (Satchell, 1991) and the absence of a band III protein on the rbc membrane of the Pacific hagfish (Ellory *et al.* 1987; Brill *et al.* 1992).

Table 1. *Blood parameters of the Pacific hagfish and the rainbow trout*

Blood parameters	Pacific hagfish *Eptatretus stouti*	Rainbow trout *Oncorhynchus mykiss*
Hct (%)	14.7[1]	30.3[1]
[Hb] (g dl^{-1})	3.1[1]	8.7[1]
MCHC (g dl^{-1})	21.1[1]	28.7[1]
Blood volume (%)	18*[,2]	5–6[2]
Buffering capacity (μmol pH unit^{-1} g^{-1})	4.0*[,2]	9.7[3]
P_{50} (kPa)	0.239 at 18 °C[4]	2.53 at 15 °C[5]
Bohr factor	–0.20[6]	–0.54[7]
Root effect	No[2]	Yes[8]
Oxygen content (vols%)	2.3*[,9]	14.6[1]

References: 1, This study; 2, Satchell (1991); 3, Mattsoff and Nikinmaa (1988); 4, Manwell (1958); 5, Milligan and Wood (1987); 6, Li *et al.* (1972); 7, Eddy (1971); 8, Boutilier *et al.* (1986); 9, Wells *et al.* (1986).

*Value for *Eptatretus cirrhatus*.

[Hb], haemoglobin concentration; MCHC, mean cellular haemoglobin content; Hct, haematocrit.

Materials and methods

Three different experimental series involving the exposure of fish to hypoxic conditions were conducted. Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were exposed either to a $P_{W_{O_2}}$ of 4.00 kPa for 6 h (series I) or to 3.33 kPa for 1 h (series II). Pacific hagfish [*Eptatretus stouti* (Lockington)] were exposed to a $P_{W_{O_2}}$ of either 1.33 or 3.33 kPa for 1 h (series III). Experimental protocol and sampling regimes are the same as those described in Bernier *et al.* (1996) using two different adenosine receptor (AR) blockers: theophylline (1,3-dimethylxanthine) and enprofylline (3-propylxanthine; Sigma, St Louis, MI, USA).

In series I, four different experimental groups of six fish each at 10 °C were used to investigate the effects of AR blockade on hypoxia-tolerance: (1) normoxic sham; (2) normoxic theophylline; (3) hypoxic sham; and (4) hypoxic theophylline. Throughout the course of a trial, blood samples of 700 μl were taken at 0, 10, 30, 120 and 360 min from the start of hypoxic exposure. Each blood sample was replaced by an equivalent volume of Cortland saline. Theophylline was dissolved in saline to a concentration of 8 mg ml^{-1} and given as a bolus injection at a dose of 4 mg kg^{-1}, followed by 0.2 ml of saline.

Series II involved five different experimental groups of eight fish at 7 °C: (1) normoxic sham; (2) normoxic enprofylline; (3) hypoxic sham; (4) hypoxic enprofylline; and (5) hypoxic theophylline. Throughout the course of a trial, blood samples of 900 μl were taken at 0, 10, 30 and 60 min. Each blood sample was replaced by an equivalent volume of saline. Enprofylline and theophylline were dissolved in saline to a concentration of 1.5 mg ml^{-1} and infused at a rate of 0.5 ml min^{-1}. Both AR blockers were injected at a dose of 4 mg kg^{-1}, followed by 0.2 ml of saline.

Series III involved six different experimental groups of six fish: (1) normoxic sham; (2) normoxic theophylline; (3) 3.33 kPa $P_{W_{O_2}}$ hypoxic sham; (4) 3.33 kPa $P_{W_{O_2}}$ hypoxic theophylline; (5) 1.33 kPa $P_{W_{O_2}}$ hypoxic sham; and (6) 1.33 kPa $P_{W_{O_2}}$ hypoxic theophylline. Throughout the course of a trial, blood samples of 500 μl were taken at 0, 10, 30 and 60 min. Each blood sample was replaced by an equivalent volume of hagfish saline (3.0 % NaCl and heparin at 50 i.u. ml^{-1}). Theophylline was dissolved in hagfish saline to a concentration of 6 mg ml^{-1} and given as a bolus injection at a dose of 4 mg kg^{-1}, followed by 0.2 ml of hagfish saline.

In all experimental series, blood samples were collected in 1.5 ml microcentrifuge tubes. From this initial sample, aliquots of whole blood were taken for measurement of haematocrit (Hct) and haemoglobin concentration ([Hb]). The remaining blood was centrifuged at 11 000 revs min^{-1} for 2 min, and plasma was removed for measurement of blood pH (pHe). In series II and III, plasma aliquots were also removed from each

blood sample, frozen in liquid nitrogen, and stored at −80 °C for later determination of plasma adrenaline (A) and noradrenaline (NA) concentrations. The remaining packed red blood cells (rbcs) from all three experimental series were frozen at −80 °C for later measurement of the intracellular pH (pHi) of rbcs.

Analytical techniques

Hct was determined by centrifuging the blood in heparinized capillary tubes for 5 min at 11 500 revs min^{-1} in a Damon IEC MB microhaematocrit centrifuge. Hb concentration was measured using a Sigma total haemoglobin (525-A) assay kit and the relative absorbency measured at 540 nm in a Shimadzu UV-160 visible recording spectrophotometer. Mean cellular [Hb] (MCHC) was calculated as ([Hb]/Hct)×100. Whole-blood pH (pHe) and intracellular red cell pH (pHi) were measured using a thermostatted Radiometer G297/G2 glass capillary electrode with a PHM71 acid–base analyzer. The fast freeze–thaw technique of Zeidler and Kim (1977) was used to determine rbc pHi. Calibration of the pH electrode was performed using Radiometer precision buffer solution standards S1519 and S1500.

Plasma A and NA levels were determined on alumina-extracted plasma samples using high-pressure liquid chromatography (HPLC) based on Woodward (1982). The HPLC incorporates a Waters 460 electrochemical detector using a glassy carbon electrode, a reverse-phase Waters plasma catecholamine column, a Waters model 510 HPLC pump with pulse dampeners and a Waters U6K universal liquid chromatograph injector (Waters Chromatography Division of Millipore Ltd). Concentrations were calculated by an integrator (Waters 746 data module) connected on-line to the electrochemical detector.

Statistical analyses

All data are presented as mean ±1 S.E.M. The statistical significance of observed effects of treatment exposure within a group were tested by one-way repeated-measures analysis of variance (ANOVA). To compare pre-treatment means with means at subsequent sampling times, Dunnett's test was used. Where appropriate, the statistical significance of observed differences between the means from all treatments at a particular sampling time were tested using one-way ANOVA. Since the mean plasma adrenaline and noradrenaline concentrations were positively correlated with the variance, the nonparametric Kruskal–Wallis one-way ANOVA on ranks test was used to determine differences between the means from all treatments at a particular sampling time. To isolate which group(s) differed from the others, a Student–Newman–Keuls test was used. The significance level for all statistical test was $P<0.05$.

Results

Effects of AR blockers on erythrocytes of hypoxic rainbow trout and Pacific hagfish

Results of other aspects of these experiments have been reported in a previous paper (Bernier *et al.* 1996). In the normoxic rainbow trout of series I, there was a gradual decrease in the Hct values throughout the 360 min trials, and no change in the MCHC values of the two normoxic treatments (Table 2). In the hypoxic sham treatment, the Hct values were significantly higher than the control value from 30 min onwards, and the 360 min value was 14.9 % higher than the control value. An initial increase in Hct values was also observed in the hypoxic theophylline treatment. The MCHC values in the hypoxic sham and hypoxic theophylline groups decreased gradually throughout the 360 min trial. Removal of five 700 μl blood samples from rainbow trout (mean mass 461±24 g) resulted in a significant decrease in the [Hb] of all the experimental groups (Fig. 1A). Relative to time 0 values, the 360 min [Hb] values had decreased significantly by 30.2,

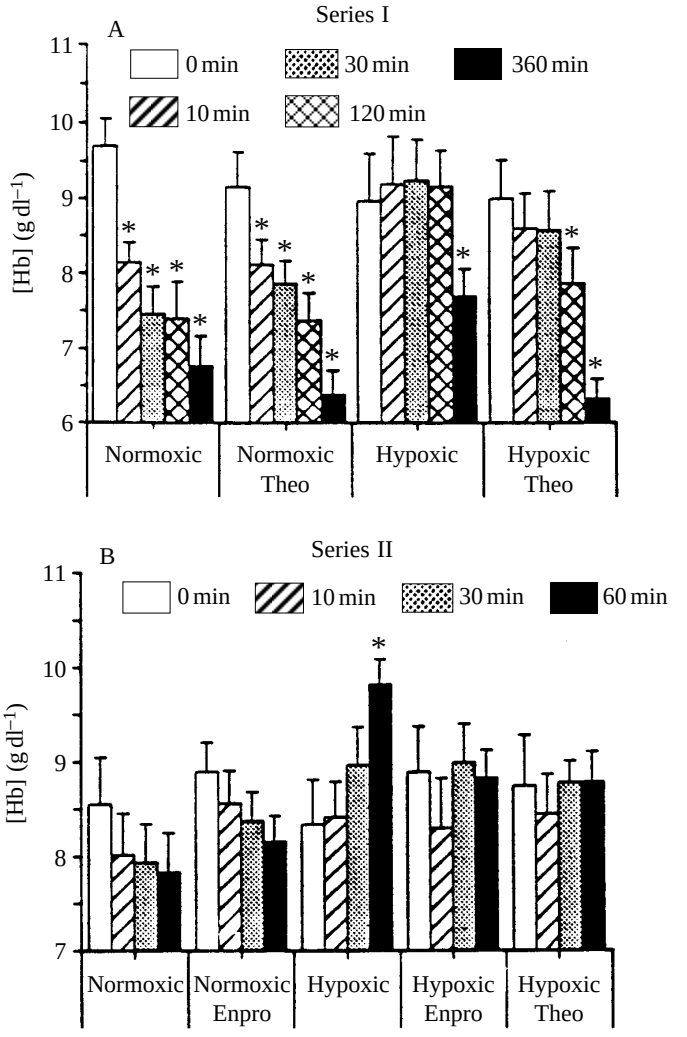

Fig. 1. Haemoglobin (Hb) concentration of rainbow trout in relation to exposure duration to normoxia (A) and hypoxia (B) (P_{WO_2}=4.00 kPa in series I, P_{WO_2}=3.33 kPa in series II). Animals were injected with saline, enprofylline (Enpro) or theophylline (Theo). Time 0 values are controls. * indicates a significant difference from the control value in a given treatment ($P<0.05$). Values are means ±1 S.E.M.; values of N are given in the Materials and methods section.

Table 2. *Haematocrit, mean cellular haemoglobin content, red blood cell pH (pHi), and the difference between whole-blood pH (pHe) and pHi of rainbow trout, in relation to exposure duration to normoxia and hypoxia (P_{wO_2}=4.00 kPa)*

Experimental condition	Time (min)	Haematocrit (%)	MCHC (g dl^{-1})	pHi	pHe−pHi
Normoxic sham	0	30.0±0.7	32.3±0.6	7.46±0.04	0.56±0.07
	10	25.8±1.1*	31.6±0.8	7.42±0.03	0.68±0.03
	30	23.4±1.1*	31.9±0.4	7.38±0.02	0.65±0.03
	120	22.9±1.3*	32.2±0.9	7.40±0.02	0.67±0.05
	360	20.6±1.1*	32.8±0.5	7.38±0.02	0.74±0.03
Normoxic theophylline	0	29.3±0.7	31.1±1.0	7.42±0.02	0.60±0.04
	10	26.3±0.8	31.0±1.1	7.42±0.02	0.60±0.04
	30	25.8±0.9	30.6±1.0	7.37±0.02	0.60±0.04
	120	23.6±0.8*	31.3±1.2	7.41±0.03	0.63±0.06
	360	19.8±1.0*	32.3±0.8	7.42±0.03	0.61±0.06
Hypoxic sham	0	26.3±1.3	34.0±1.4	7.44±0.02	0.66±0.04
	10	28.6±1.2	32.2±1.7	7.69±0.02*	0.32±0.06*
	30	32.1±1.0*	28.8±1.2*	7.71±0.03*	0.31±0.07*
	120	34.1±2.1*	27.2±1.5*	7.69±0.05*	0.29±0.06*
	360	30.9±2.8*	25.7±1.6*	7.52±0.06	0.29±0.06*
Hypoxic theophylline	0	27.6±1.7	32.9±1.8	7.39±0.01	0.63±0.06
	10	30.3±2.0	28.5±1.2*	7.68±0.02*	0.07±0.05*,†
	30	33.9±2.4*	25.5±1.2*	7.62±0.03*,†	0.11±0.04*,†
	120	32.0±1.6*	24.6±1.1*	7.60±0.02*	0.10±0.06*,†
	360	26.2±0.5	24.3±1.3*	7.49±0.04	0.17±0.03*

Animals were infused with either saline or theophylline.
Values are means ± 1 S.E.M., values of *N* are given in Materials and methods.
*Significantly different from control time 0 values (P<0.05).
†Significantly different from hypoxic sham value (P<0.05).
MCHC, mean cellular haemoglobin content; pHi, red blood cell pH; pHe, whole-blood pH.

30.3 and 29.6 % in the normoxic sham, normoxic theophylline and hypoxic theophylline groups, respectively. In the hypoxic sham group, [Hb] was maintained through the first four sampling times and decreased by 14.2 % at 360 min. There was no difference between the mean body mass of all four groups.

Significant decreases in Hct and no changes in MCHC values were also observed in the two normoxic treatments of rainbow trout in series II (Table 3). Compared with their respective control time 0 values, there was a significant increase in Hct in all three hypoxic treatments. In the hypoxic sham group, a decrease in MCHC was only seen at the 30 min sampling period. In contrast, in the two hypoxic groups treated with an AR blocker, the decrease in MCHC was progressive throughout the hypoxic exposure and the values were significantly lower than in the hypoxic sham group at 60 min. Removal of four 900 μl blood samples from rainbow trout (mean mass 1144±32 g) did not significantly decrease the [Hb] in any of the experimental groups (Fig. 1B). Whereas, a 15.2 % increase in [Hb] was recorded after 60 min of hypoxic exposure in the sham group, no change in [Hb] was observed in the hypoxic enprofylline and hypoxic theophylline groups. Once again, there were no significant differences between the mean body masses of all five experimental groups used in this series.

In series III, contrary to results for series I and II with rainbow trout, the Hct and [Hb] of hagfish decreased significantly in the hypoxic sham (P_{wO_2}=1.33 kPa) and hypoxic theophylline (P_{wO_2}=4.00 kPa) treatments (Table 4). No changes were observed in the MCHC values under normoxic or hypoxic conditions. In a separate study, removal of four 500 μl blood samples from hagfish (mean mass 183±7 g) decreased the mean control haematocrit by 10.6 % compared with an overall decrease in haematocrit of 11.5 % in this study.

In series I and series II, rbc pH (pHi) and the rbc transmembrane pH difference (pHe−pHi) remained unchanged throughout the normoxic treatments (Tables 2, 3). Under hypoxic conditions, pHi increased significantly in all the treatments of both series; although rbc pH remained elevated for the 60 min hypoxic exposure in series II, it returned to control values by 360 min in series I. A significant reduction in pHe−pHi was observed in all hypoxic treatments of both series, but the reduction was greater in the groups treated with an adenosine receptor blocker. Unlike the results obtained for rainbow trout, no increase in rbc pH was observed in the hagfish exposed to either 4.00 kPa or 1.33 kPa P_{wO_2} for 60 min. On the contrary, pHi decreased significantly in the 1.33 kPa P_{wO_2} hypoxic theophylline treatment. A small but significant decrease in rbc transmembrane pH difference was also only

Table 3. *Haematocrit, mean cellular haemoglobin content, red blood cell pH, and the difference between whole-blood pH (pHe) and pHi of rainbow trout, in relation to exposure duration to normoxia and hypoxia ($P_{W_{O_2}}$=3.33 kPa)*

Experimental condition	Time (min)	Haematocrit (%)	MCHC (g dl^{-1})	pHi	pHe–pHi
Normoxic sham	0	29.5±1.5	29.0±0.6	7.45±0.02	0.46±0.03
	10	27.3±1.4*	29.4±0.6	7.45±0.03	0.45±0.03
	30	26.9±1.2*	29.5±0.7	7.43±0.03	0.50±0.03
	60	27.4±1.2*	28.5±0.5	7.44±0.03	0.49±0.03
Normoxic enprofylline	0	30.6±1.0	29.1±0.5	7.37±0.02	0.53±0.02
	10	29.7±1.3	28.9±0.4	7.40±0.03	0.50±0.03
	30	28.9±1.2*	29.1±0.5	7.44±0.02	0.49±0.02
	60	27.9±1.0*	29.3±0.4	7.41±0.01	0.54±0.03
Hypoxic sham	0	30.3±1.3	27.6±1.2	7.43±0.03	0.48±0.03
	10	31.8±1.0	26.5±0.8	7.67±0.03*	0.29±0.02*
	30	35.5±0.9*	25.3±1.0*	7.65±0.03*	0.31±0.04*
	60	37.5±0.9*	26.3±0.8	7.63±0.03*	0.32±0.04*
Hypoxic enprofylline	0	30.3±1.3	29.4±0.7	7.46±0.02	0.47±0.04
	10	31.4±1.8	26.4±0.5*	7.70±0.03*	0.03±0.07*,†
	30	36.7±1.4*	24.5±0.5*	7.69±0.04*	0.09±0.08*,†
	60	37.8±1.1*	23.4±0.4*,†	7.67±0.04*	0.21±0.07*
Hypoxic theophylline	0	30.6±1.6	28.6±0.3	7.44±0.02	0.53±0.04
	10	32.0±1.3	26.4±0.5*	7.64±0.03*	0.07±0.07*,†
	30	36.0±1.0*	24.4±0.4*	7.61±0.04*	0.16±0.08*
	60	38.1±1.6*	23.2±0.6*,†	7.62±0.03*	0.17±0.07*

Animals were infused with saline, enprofylline or theophylline.
Values are means ± 1 S.E.M., values of *N* are given in Materials and methods.
*Significantly different from control time 0 value (P<0.05).
†Significantly different from hypoxic sham value (P<0.05).
MCHC, mean cellular haemoglobin content; pHi, red blood cell pH; pHe, whole-blood pH.

seen in the 1.33 kPa $P_{W_{O_2}}$ hypoxic theophylline group (Table 4).

Effects of AR blockers on hypoxia-induced catecholamine release in rainbow trout and Pacific hagfish

In rainbow trout (series II), the 60 min hypoxic exposure trials resulted in significant increases in plasma [adrenaline] in all three hypoxic treatments (Fig. 2A). Overall, the increase in [adrenaline] was most pronounced in the hypoxic theophylline group, intermediate in the hypoxic enprofylline group, and smallest in the hypoxic sham group. After 10 min of hypoxic exposure, the [adrenaline] in the theophylline and enprofylline treatments were, respectively, 16-fold and fourfold higher than in the hypoxic sham treatment. The difference between the three groups was much smaller after 30 min of hypoxia, and only the theophylline treatment had a significantly higher [adrenaline] than the hypoxic sham treatment after 60 min. No changes were observed in the resting [adrenaline] values throughout the 60 min sampling regime, either within or between the two normoxic treatments. The mean values were 1.73±0.35 and 2.77±0.34 nmol l^{-1} in the normoxic sham and normoxic enprofylline groups, respectively. In hagfish (series III), although plasma [adrenaline] remained at the control time 0 value in the 1.33 kPa $P_{W_{O_2}}$ hypoxic sham group throughout the trial, [adrenaline] increased 3.8-fold within 10 min in the hypoxic theophylline-treated fish and returned to control levels by 60 min (Fig. 2B). Relative to their control values, [adrenaline] did not change in all the other treatments, and the mean values were 1.99±0.19, 2.59±0.21, 2.47±0.22 and 2.33±0.19 nmol l^{-1} in the normoxic sham, normoxic theophylline, 4.00 kPa $P_{W_{O_2}}$ hypoxic sham and 4.00 kPa $P_{W_{O_2}}$ hypoxic theophylline groups, respectively.

Plasma [noradrenaline] increased in all rainbow trout hypoxic treatments of series II, but there were no significant differences between them at any given sampling time (Fig. 3A). No significant changes were observed for the two normoxic treatments, either within a group throughout the trial or between the two groups. The mean noradrenaline concentrations were 3.35±0.59 and 3.33±0.39 nmol l^{-1} in the normoxic sham and normoxic enprofylline groups, respectively. Plasma [noradrenaline] also increased in the two hagfish groups exposed to a $P_{W_{O_2}}$ of 1.33 kPa, but whereas the increase was significant after 10 min in the theophylline-treated fish, it took 60 min for [noradrenaline] to increase above the control value in the sham group (Fig. 3B). No changes were observed in the plasma [noradrenaline] of all the other treatments, and the mean values were 3.53±0.44, 3.24±0.25, 4.16±0.61 and 3.42±0.33 nmol l^{-1} in the normoxic sham,

Table 4. *Blood pH, red blood cell pH and transmembrane pH difference, haematocrit (Hct), haemoglobin concentration and mean cellular haemoglobin content of Pacific hagfish, in relation to exposure duration to normoxia and hypoxia (Pw_{O_2}=1.33 or 4.00 kPa)*

Experimental condition	Time (min)	pHe	pHi	pHe–pHi	Hct (%)	[Hb] ($g\,dl^{-1}$)	MCHC ($g\,dl^{-1}$)
Normoxic sham	0	7.96±0.02	7.13±0.02	0.82±0.03	14.3±1.1	3.1±0.3	21.8±0.7
	10	7.97±0.02	7.14±0.02	0.83±0.02	13.8±1.2	2.9±0.2	21.4±0.7
	30	7.96±0.03	7.15±0.01	0.81±0.03	13.6±1.4	3.0±0.3	21.7±0.8
	60	7.94±0.03	7.11±0.01	0.83±0.03	12.5±1.5	2.8±0.3	22.9±0.5
Normoxic theophylline	0	7.95±0.04	7.15±0.02	0.80±0.02	15.0±1.2	3.3±0.1	22.4±1.2
	10	7.98±0.03	7.15±0.01	0.83±0.03	14.9±1.1	3.1±0.2	20.6±0.5
	30	7.95±0.02	7.16±0.01	0.80±0.02	14.6±0.9	3.1±0.2	21.7±1.0
	60	7.94±0.02	7.15±0.01	0.80±0.02	14.1±1.2	3.0±0.2	21.2±0.5
Hypoxic sham,	0	7.93±0.02	7.18±0.02	0.75±0.02	12.9±0.9	2.7±0.3	20.4±0.8
Pw_{O_2}=4.00 kPa	10	7.95±0.01	7.18±0.02	0.77±0.02	12.3±0.7	2.6±0.2	21.2±1.0
	30	7.97±0.01	7.19±0.02	0.78±0.03	12.0±0.7	2.5±0.2	21.0±0.8
	60	7.94±0.01	7.18±0.02	0.77±0.03	12.1±1.0	2.5±0.2	20.7±0.8
Hypoxic theophylline,	0	7.94±0.02	7.13±0.01	0.81±0.02	14.0±0.9	3.0±0.2	21.4±0.3
Pw_{O_2}=4.00 kPa	10	7.98±0.02	7.14±0.02	0.84±0.02	12.7±0.7	2.6±0.1	20.8±0.4
	30	7.98±0.01	7.14±0.02	0.84±0.02	12.3±0.6*	2.5±0.1*	20.8±0.7
	60	7.98±0.01	7.14±0.02	0.83±0.02	11.7±0.7*	2.5±0.1*	12.7±0.6
Hypoxic sham,	0	7.93±0.02	7.16±0.02	0.77±0.02	16.3±1.3	3.3±0.2	20.8±0.9
Pw_{O_2}=1.33 kPa	10	7.93±0.02	7.16±0.02	0.77±0.01	16.1±1.1	3.2±0.2	20.3±0.9
	30	7.92±0.03	7.16±0.02	0.76±0.02	14.6±1.0	3.0±0.2	20.8±0.8
	60	7.85±0.03	7.11±0.02	0.74±0.02	12.3±0.7*	2.6±0.2*	21.6±1.1
Hypoxic theophylline,	0	7.93±0.02	7.13±0.00	0.80±0.02	15.8±1.5	3.1±0.3	19.9±1.2
Pw_{O_2}=1.33 kPa	10	7.91±0.02	7.13±0.01	0.78±0.02	16.9±1.5	3.4±0.3	20.0±0.6
	30	7.82±0.04*	7.10±0.01	0.72±0.03	16.9±1.3	3.4±0.3	20.1±0.5
	60	7.77±0.02*	7.07±0.01*	0.70±0.03*	15.3±1.4	3.0±0.3	19.7±0.7

Animals were infused with either saline or theophylline.

Values are means ± 1 S.E.M., values of *N* are given n Materials and methods.

*Significantly different from control time 0 value ($P<0.05$).

pHe, whole-blood pH; pHi, red blood cell pH; Hct, haematocrit; [Hb], haemoglobin concentration; MCHC, mean cellular haemoglobin concentration.

normoxic theophylline, 4.00 kPa Pw_{O_2} hypoxic sham and 4.00 kPa Pw_{O_2} hypoxic theophylline groups, respectively.

Discussion

AR blockade and erythrocytes

Relative to the sham groups, AR blockers had no effect during normoxic conditions on any of the parameters measured in this study, indicating no action of adenosine in normoxia. With hypoxic exposure in rainbow trout, the rbc transmembrane pH difference was significantly smaller in the groups treated with the adenosine receptor blockers than in the sham groups. This difference may reflect direct or indirect effects of AR blockade on the rbcs of rainbow trout. Although adenosine has been shown to play a role in the regulation of glycolysis in vertebrate erythrocytes (Fievet *et al.* 1987; Kaloyianni *et al.* 1993), and fish rbcs possess a nucleoside transport mechanism which may make adenosine available for rbc energy metabolism (Fincham *et al.* 1991), there is no evidence that these processes are mediated *via* adenosine receptors. Therefore, a direct mechanism to explain the effects of AR blockade on pH regulation does not appear likely.

Indirect effects of AR blockade on rainbow trout erythrocytes may arise through changes in plasma pH and circulating [catecholamines] following hypoxic exposure. Theophylline and enprofylline treatments enhance the metabolic acidosis that develops with hypoxic exposure (Bernier *et al.* 1996). Since, at a given sampling time, there was no difference in rbc pH between the different hypoxic groups, the greater decrease in pHe with AR blockade will result in a smaller rbc transmembrane pH difference in those treatments. Lower pHe values can also increase the alkalization of rbcs observed in hypoxia, because the sensitivity of the rbc Na^+/H^+ exchanger to adrenergic stimulation increases as a function of decreasing pHe (Borgese *et al.* 1987). Given the concentrations of both catecholamines in this study, and the

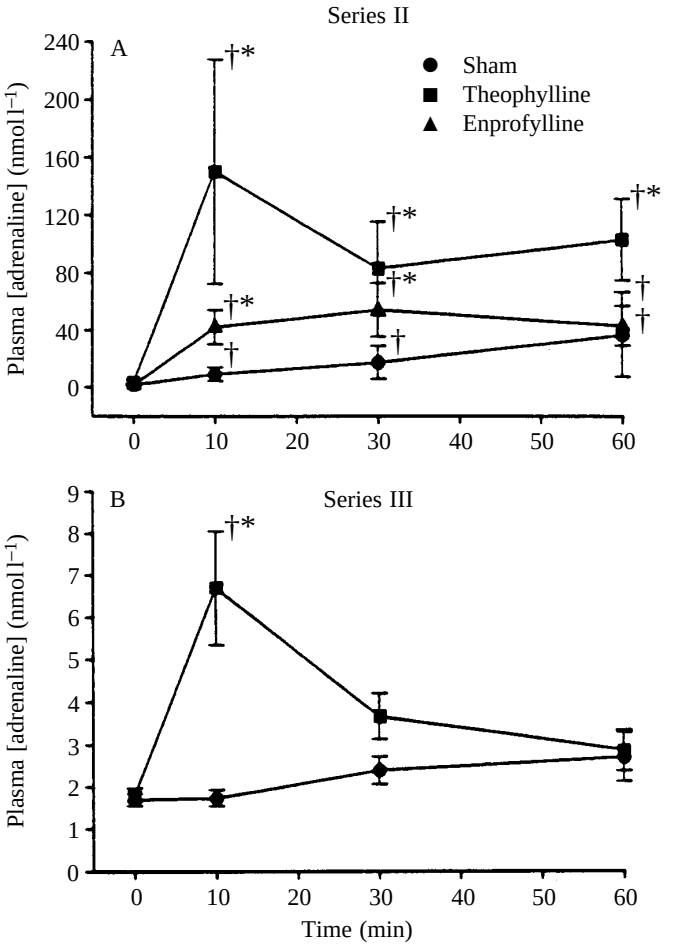

Fig. 2. Plasma [adrenaline] of rainbow trout (A, series II) and Pacific hagfish (B, series III) in relation to exposure duration to hypoxia (P_{WO_2}=3.33 kPa in series II and 1.33 kPa in series III). Animals were injected with saline (circles), theophylline (squares) or enprofylline (triangles). Time 0 values are controls. † indicates a significant difference from the control value in a given treatment. * indicates a significant difference from the value for the hypoxic sham treatment at a given sampling time (P<0.05). Values are means ±1 S.E.M.; values of N are given in the Materials and methods section.

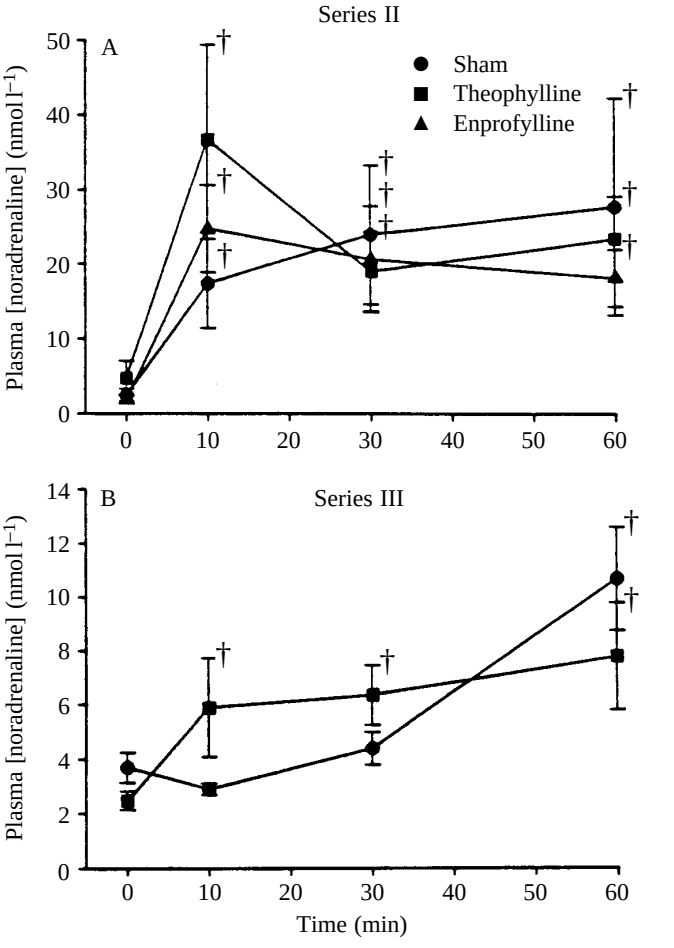

Fig. 3. Plasma [noradrenaline] of rainbow trout (A, series II) and Pacific hagfish (B, series III) in relation to exposure duration to hypoxia (P_{WO_2}=3.33 kPa in series II, P_{WO_2}=1.33 kPa in series III). Animals were injected with saline (circles), theophylline (squares) or enprofylline (triangles). Time 0 values are controls. † indicates a significant difference from the control value in a given treatment (P<0.05). Values are means ±1 S.E.M.; values of N are given in the Materials and methods section.

much greater affinity of the red blood cell β_1-adrenoceptors for noradrenaline, the red cell β-adrenergic responses were probably elicited by noradrenaline (Tetens *et al.* 1988). Although AR blockade during hypoxia initially resulted in higher circulating concentrations of noradrenaline, the differences between the three hypoxic groups were not significant. Thus, the most probable effect of adenosine on trout erythrocytes is indirect and *via* changes in plasma pH.

The possibility that AR blockade indirectly enhances the activity of the rbc Na^+/H^+ exchanger in rainbow trout may also explain the greater decrease in MCHC values observed after treatment with the blockers than in the sham groups. The greater Na^+ influx resulting from an enhanced rbc Na^+/H^+ exchanger activity would cause more osmotic swelling of the cells (Nikinmaa, 1992) and further decrease MCHC values. One possible consequence of greater erythrocyte swelling with AR blockade under hypoxic conditions is a small increase in blood oxygen-affinity (Holk and Lykkeboe, 1995).

No significant regulation of rbc pH or changes in MCHC values were observed in the hagfish experimental series, although plasma [noradrenaline] increased significantly in both 1.33 kPa P_{WO_2} hypoxic exposure groups and [adrenaline] increased significantly in the theophylline-treated group, Although the [catecholamine] may not have been sufficiently high to activate a β-adrenergic-sensitive Na^+/H^+ exchanger, *in vitro* noradrenaline concentrations much smaller than the ones recorded in this study for the hagfish result in adrenergic rbc pH regulation in rainbow trout (Tetens *et al.* 1988). One possible explanation of these results is the absence of an rbc Na^+/H^+ exchange transport pathway in hagfish rbcs. This is supported by the inability of hagfish rbcs to respond to intracellular acidification, or to osmotic swelling when exposed to a hypotonic medium (Nikinmaa *et al.* 1993). Hagfish

erythrocytes are also unusual in virtually lacking the anion-exchange pathway (Ellory *et al.* 1987); they do, however, possess other ion-transport systems (Ellory and Wolowyk, 1991).

AR blockade and [Hb] of the blood

The increase in [Hb] observed in the hypoxic sham rainbow trout group of series II has been observed in several studies with similar acute hypoxic exposure (Tetens and Lykkeboe, 1985; Boutilier *et al.* 1988; Claireaux *et al.* 1988). With severe hypoxia, [Hb] may increase by haemoconcentration because of fluid shifting from the blood to lactate-loaded muscles (Milligan and Wood, 1986*a*,*b*). Increasing catecholamine concentrations with hypoxic exposure can also lead to an elevation in [Hb] *via* an adrenaline-induced diuresis (Vermette and Perry, 1987). *In vitro* and *in vivo* studies have also shown that recruitment of red blood cells can result from contraction of the spleen by stimulation of β-adrenoceptors (Nilsson and Grove, 1974; Vermette and Perry, 1988; Perry and Kinkead, 1989; Kita and Itazawa, 1989). Contraction of the spleen is probably the dominant response causing an increase in [Hb] following an increase in plasma catecholamine levels, since adrenergic elevation of blood [Hb] is absent in splenectomized trout (Perry and Kinkead, 1989).

Relative to the hypoxic sham groups of series I and II, adenosine receptor blockade negated the effects of acute hypoxia on [Hb]. While AR blockade resulted in significantly higher plasma [adrenaline] than the sham treatment in series II, and a dose-dependent relationship between plasma [adrenaline] and arterial blood [Hb] has been observed in rainbow trout (Perry and Kinkead, 1989), no change in [Hb] was observed following AR blockade. Changes in [Hb] resulting from water influx into tissues and haemoconcentration cannot explain these results, since hypoxic trout treated with theophylline have a significantly higher tissue [lactate] than their hypoxic sham counterparts (Bernier *et al.* 1996), a situation favouring osmotic swelling of the tissues. Given the relative importance of the adrenoceptor-sensitive recruitment of rbcs from the spleen to the final [Hb] (Pearson and Stevens, 1991), these results indicate that adenosine receptor blockade may prevent splenic release of rbcs during hypoxia. This hypothesis is supported by *in vitro* studies demonstrating mediation of rat splenic contraction by adenosine A_1 receptor activation (Fozard and Milavec-Krizman, 1993). However, the effects of adenosine receptor agonists on the rat isolated spleen are not the result of direct or indirect interactions with the α_1-adrenoceptors of that tissue (Fozard and Milavec-Krizman, 1993). Therefore, whereas contraction of the spleen is activated independently by adenosine and catecholamines in the rat, a somewhat different mechanism involving an interaction between adenosine and catecholamine receptors may be present in rainbow trout.

In hagfish, there was no increase in [Hb] in any experimental group, irrespective of the degree of hypoxia or [catecholamine]. In fact, in two groups we observed a decrease in [Hb]. Treatment with the adenosine receptor blocker theophylline was also without any effect on the changes in blood [Hb]. These results are consistent with the absence of a spleen in hagfish.

AR blockade and catecholamine release

The control catecholamine values for rainbow trout (series II) are in accordance with reported values (Randall and Perry, 1992) and, although catecholamine levels have not been previously reported for Pacific hagfish, the control values of series III are similar to the concentrations reported in Atlantic hagfish (Perry *et al.* 1993). Adenosine receptor blockade with methylxanthines had no effect on the concentrations of catecholamines in the normoxic animals. These results are similar to those observed in several mammalian studies (Andersson *et al.* 1984; Whyte *et al.* 1987; Ishizaki *et al.* 1988; Schwertschlag *et al.* 1993), but also contrast with others (Vestal *et al.* 1983; Higbee *et al.* 1987).

In response to severe hypoxic stress, rainbow trout and Pacific hagfish showed significant increases in catecholamine levels. This hormonal response to hypoxia has been well documented in fish (Ristori and Laurent, 1989; Perry *et al.* 1993). Theophylline treatment, in all the hypoxic groups, resulted in greater circulating concentrations of these hormones.

In fish, hypoxaemia is the dominant factor initiating the release of catecholamines (Perry *et al.* 1991; Randall and Perry, 1992). Hence, differences in the oxygen-carrying capacity between the various hypoxic treatments could have accounted for the observed differences in circulating catecholamine concentrations. Oxygen content measurements in series II do not support this hypothesis, since all three hypoxic groups were shown to have similar oxygen content values throughout the hypoxic exposure (see Bernier *et al.* 1996).

In mammals, high [theophylline] has been shown to stimulate the release of catecholamines, particularly adrenaline, from isolated perfused adrenal glands (Peach, 1972; Poisner, 1973). The ability of theophylline to inhibit phosphodiesterase (PDE), and thereby elevate tissue cyclic AMP concentrations, was originally considered as a possible mechanism. However, PDE inhibition is achieved at theophylline concentrations that have toxic *in vivo* effects in mammals (Aronson *et al.* 1992; Schwertschlag *et al.* 1993) and cannot explain the stimulation of catecholamine release.

At low levels, theophylline blocks all adenosine receptor subtypes. Since adenosine has the capacity to depress synaptic transmission (Stone and Bartrup, 1991) and to suppress the release of many neurotransmitters in the peripheral nervous system (Fredholm and Dunwiddie, 1988), it is possible that adenosine receptor blockade may modulate regulation of the sympathoadrenal system. Given that the chromaffin tissues are by far the most important source of circulating catecholamines in fish (Randall and Perry, 1992), modulation of the sympathetic innervation to this tissue may have some significance in the control of levels of circulating catecholamines. Experiments on the Atlantic cod have shown that neural innervation of the chromaffin tissue may be a

requirement for noradrenaline secretion, but not for adrenaline secretion (Perry *et al.* 1991). Hagfish chromaffin cells receive no extrinsic innervation (Nilsson and Holmgren, 1993), so, while the modulation of synaptic transmission by adenosine may be a useful hypothesis for the control of noradrenaline release in rainbow trout, some other mechanism must be involved to explain adrenaline secretion in the rainbow trout theophylline treatment and in the hagfish experiment.

Similarities in the response profile of catecholamines in the hypoxic rainbow trout and Pacific hagfish treated with theophylline point to a possibly similar mode of action by this adenosine receptor blocker in both species. The profile for adrenaline is characterized by an early peak, with a subsequent return to the basal concentration. By contrast, the increase in [noradrenaline] is more gradual and smaller, and levels remain elevated throughout the treatment. In studies involving man, [adrenaline] is also elevated to a greater degree over basal levels than is [noradrenaline] following theophylline treatment (Vestal *et al.* 1983; Higbee *et al.* 1987; Ishizaki *et al.* 1988), and [adrenaline] may also peak prior to termination of the treatment (Ishizaki *et al.* 1988). These differences between the pattern in the circulating concentrations of adrenaline and noradrenaline in fish treated with theophylline may be further evidence for the presence of two populations of chromaffin cells containing predominantly one of the two catecholamines and responsive to different release stimuli (Perry *et al.* 1991; Reid and Perry, 1994).

Adenosine receptor blockade may prevent an inhibitory feedback mechanism of catecholamine secretion. Adenosine, formed from ATP which is released in parallel with catecholamines from chromaffin cell vesicles, inhibits catecholamine secretion from bovine adrenal medulla cells by inhibiting calcium flux (Chern *et al.* 1987, 1992). Hence, in this system, adenosine may act as a negative feedback regulator of catecholamine secretion. Support for this modulatory effect of endogenous adenosine also comes from an *in vivo* study where the adenosine receptor antagonist 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine (DPSPX) inhibited adrenaline release from the adrenal medulla and suppressed plasma noradrenaline levels in hydralazine-induced hypotensive rats (Tseng *et al.* 1994). Experiments on adrenalectomized rats have led to the conclusion that the increase in [adrenaline] following DPSPX treatment resulted from release from the adrenal medulla, whereas the increase in [noradrenaline] was mainly from sympathetic nerve ending spill-over (Tseng *et al.* 1994).

Although these modulatory actions of adenosine have not yet been investigated in fish, results obtained in this study support a mechanism based on the involvement of adenosine receptors. In series II, differences in the [adrenaline] in the three hypoxic groups reflect the degree to which adenosine receptors are antagonized by the treatments. Whereas fish in the saline treatment had the lowest [adrenaline], the levels were intermediate after treatment with the weak adenosine receptor blocker enprofylline, and the increases were most significant after higher-affinity receptor blocker by theophylline. The lack of an effect of the adenosine receptor blockers in all the normoxic treatments is also consistent with the hypothesis that methylxanthines will impair the regulation of catecholamine levels under conditions that may result in the formation of adenosine. However, the possibility that tissue uptake and metabolic degradation of catecholamines are inhibited by methylxanthines cannot be excluded.

Although there are marked differences between rainbow trout and Pacific hagfish in their tolerance to hypoxia, and in the strategy utilized to resist periods of oxygen shortage, the results obtained throughout this study indicate that adenosine has an important modulatory role in both species. Given that hagfish may be an early offshoot from the primitive vertebrate stock, literally hundreds of millions of years ago (Hardisty, 1979; Barback, 1991), it is rather surprising to obtain this degree of similarity between the two species in their response to adenosine receptor blockade. This long conservative history in the possible physiological actions of adenosine lends support to the original idea of Newby (1984) of a primitive and ubiquitous life-preserving function for adenosine.

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