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THE EFFECTS OF REPEATED PHYSICAL STRESS ON THE β-ADRENERGIC RESPONSE OF THE RAINBOW TROUT RED BLOOD CELL

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Summary

The effects of a 7-day period of daily physical stress (chasing until exhaustion) on the β-adrenergic response of the rainbow trout (*Oncorhynchus mykiss*) red blood cell (rbc) were examined *in vitro*. Physical stress was associated with pronounced increases in the circulating levels of the catecholamine hormones (adrenaline and noradrenaline) measured on days 1, 3 and 7 of the stress regime. After 7 days, the numbers of high-affinity cell surface β-adrenoceptors were reduced in the physically stressed fish when measured *in vitro* under conditions of normoxia (20 % reduction) or hypoxia (30 % reduction). Under hypoxic conditions, the binding affinity of the rbc β-adrenoceptor was significantly higher in the stressed fish.

Although the stressed fish had fewer β-adrenoceptors, rbc adrenergic responsiveness was enhanced after 7 days of physical stress as determined from dose–response curves relating noradrenaline concentration to water and Na^+ accumulation (indices of rbc adrenergic Na^+/H^+ exchange activity). The EC_{50} values (concentrations yielding half-maximal responses) for noradrenaline were lowered significantly by 1.7- to 3.9-fold in the blood from physically stressed fish. The enhanced adrenergic responsiveness of the rbcs appeared to be unrelated to changes in the initial steps of the β-adrenergic signal transduction pathway leading to cyclic AMP production because physical stress was without effect on the magnitude or the dose-dependency of rbc cyclic AMP accumulation.

To determine whether post-cyclic-AMP events were affected by physical stress, water and Na^+ accumulation were measured in rbcs that had been incubated with the permeable cyclic AMP analogue 8-bromo cyclic AMP. The EC_{50} values for 8-bromo cyclic AMP were lowered by 1.6- to 1.7-fold in the blood from stressed fish.

These experiments demonstrate that repeated physical stress significantly enhances the adrenergic responsiveness of the rainbow trout rbc, presumably by modifying the sensitivity of the Na^+/H^+ exchanger (or the steps immediately preceding exchanger activation) to cyclic AMP. The results are discussed with respect to the interrelationships between chronic and acute stress responses in fish.

Key words: *Oncorhynchus mykiss*, red blood cell, stress, cortisol, catecholamines, cyclic AMP, β-adrenoceptors, Na^+/H^+ antiporter, rainbow trout.

Introduction

In response to severe acute stress, many teleost fish, including the rainbow trout (*Oncorhynchus mykiss*), release the catecholamine hormones adrenaline and noradrenaline into their circulation (reviewed by Randall and Perry, 1992). The elevation of circulating catecholamine levels is generally thought to promote an array of compensatory physiological and biochemical responses aimed at alleviating many of the deleterious consequences of acute stress. In rainbow trout, one of the most significant responses is the β-adrenergic activation of a red blood cell (rbc) Na^+/H^+ exchanger (reviewed by Nikinmaa and Tufts, 1989; Fiévet and Motais, 1991; Nikinmaa, 1992; Thomas and Perry, 1992). This response promotes the regulation of blood oxygen transport owing to the effects of Na^+/H^+ exchange activation on the intracellular levels of allosteric modifiers of haemoglobin oxygen-binding (Nikinmaa, 1992).

Activation of rbc Na^+/H^+ exchange commences with the binding of catecholamines to β-adrenoceptors on the cell surface. This interaction triggers, *via* the β-adrenergic signal transduction pathway, the formation of the second messenger cyclic AMP. The elevation of intracellular cyclic AMP levels ultimately initiates activation of the Na^+/H^+ exchanger *via* phosphorylation by cyclic-AMP-dependent protein kinase A (Guizouarn *et al.* 1993). The β-adrenergic response can be modified both by the degree of blood oxygenation (Motais *et al.* 1987) and by changes in the acid–base status of the blood (Nikinmaa *et al.* 1987; Borgese *et al.* 1987; Salama and Nikinmaa, 1989; Cossins and Kilbey, 1989). One well-documented mechanism underlying the acute modification of the β-adrenergic signal transduction pathway in fish is the rapid insertion/removal of β-adrenoceptors from the rbc

plasma membrane. But, since β-adrenergic cell swelling is also enhanced by hypoxic conditions *in vitro* independently of intracellular cyclic AMP levels, regulation of the rbc adrenergic response is also likely to occur at the level of the Na^+/H^+ exchanger itself (Reid and Perry, 1994*a*).

Recent work in this laboratory has shown that elevation of plasma cortisol levels in rainbow trout can increase the size of the cytosolic receptor pool and that these additional receptors can be mobilized to the cell surface during an appropriate acute stress (e.g. hypoxia) to increase rbc adrenergic responsiveness (Reid and Perry, 1991). These observations were the basis for a model (Perry and Reid, 1993) in which, under chronic stress, elevated levels of cortisol (Schreck, 1990) 'pre-adapt' the rbc to respond to adrenergic stimulation during acute stress. However, chronic stress may also be associated with persistently elevated levels of catecholamines, especially if the stress consists of repetitive acute disturbances over the long term. In direct contrast to the effects of cortisol, increases in plasma catecholamine levels are known to reduce β-adrenoceptor numbers in rainbow trout (Gilmour *et al.* 1994) by a process termed down-regulation (e.g. Hausdorff *et al.* 1990). Therefore, during long-term repetitive stress in which *both* plasma cortisol and catecholamine levels are habitually elevated, rbc adrenergic responsiveness is likely to be influenced by the simultaneous and opposite effects of cortisol (up-regulation) and catecholamines (down-regulation). Thus, with this general background, the goal of the present study was to evaluate the effects of a 7-day period of daily physical disturbance on (i) the levels of plasma catecholamine hormones (adrenaline, noradrenaline), (ii) the initial steps of the rbc β-adrenergic signal transduction pathway (cell surface β-adrenoceptor numbers, cyclic AMP accumulation) and (iii) Na^+/H^+ exchange activity (as estimated by cell water and Na^+ accumulation).

Materials and methods

Experimental animals

Freshwater rainbow trout *Oncorhynchus mykiss* (Walbaum) were obtained from a local hatchery (Linwood Acres Trout Farm) and transported to the University of Ottawa, where they were maintained indoors in large rectangular tanks supplied with flowing and aerated City of Ottawa dechlorinated tap water at 10 °C (see Perry *et al.* 1992, for the chemical composition of the water). The fish used in these experiments consisted of individuals approximately 2 years of age weighing between 200 and 300 g (experimental N=124). All fish were fed daily to satiation with commercial salmonid pellets. Photoperiod was kept constant at 12 h:12 h light:dark. Fish were acclimated to these conditions for approximately 2 weeks prior to experimentation.

Animal preparation

Fish were anaesthetised in a 0.1 g l^{-1} solution of ethyl-*m*-aminobenzoate (MS 222; Sigma Chemical Company), adjusted to pH 7.5 with $NaHCO_3$, and placed onto an operating table to allow continuous retrograde irrigation of the gills with anaesthetic solution. To permit blood sampling, an indwelling cannula was implanted into the dorsal aorta (Soivio *et al.* 1975) using flexible polyethylene tubing (Clay-Adams PE 50; internal diameter 0.580 mm, external diameter 0.965 mm). Rainbow trout were revived on the operating table by irrigation of the gills with aerated water, then transferred to individual opaque acrylic experimental chambers (volume 3 l) supplied with aerated, flowing water. Cannulae were flushed daily with freshwater teleost saline (Wolf, 1963) containing 50 i.u. ml^{-1} ammonium heparin (Sigma Chemical Company).

Experimental protocol

Stress regime

Fish were divided into two groups and held under identical conditions. One group served as the controls while the other group was subjected to daily physical stress (Reid *et al.* 1994) for a period of 7 days. Two fish were removed from the holding tank and placed into a separate tank where they were chased with a net for a period of 5–6 min until they were visibly exhausted and could be handled without resistance. This procedure was repeated until all fish had been chased until exhaustion. This stress regime was performed each morning for 7 days. Owing to poor feeding in the stressed fish (see also Reid *et al.* 1994), neither group was fed during the 7-day experimental period.

Plasma stress hormone levels

The levels of plasma catecholamines were measured in cannulated fish after 1, 3 and 7 days of chasing, as follows. Fish were cannulated 18 h prior to chasing (i.e. on days 0, 2 and 6) and then placed into opaque acrylic individual holding boxes for recovery. Previous work in this laboratory has shown that plasma catecholamine levels are restored to normal within 4 h of recovery from dorsal aortic cannulation (S. F. Perry and A. Tsay, unpublished results; see also Fig. 1). The next morning, a pre-stress blood sample (300 μl) was removed and the fish then was transferred to the chasing tank and chased until exhaustion. A blood sample was withdrawn immediately upon exhaustion and, after returning the fish to its holding box, additional blood samples were taken at 0.25, 0.5, 1, 4, 8, 12 and 24 h. Plasma was obtained by centrifugation (12 000 ***g*** for 30 s), quick-frozen in liquid N_2 and then stored for less than 2 weeks at −80 °C for subsequent determination of plasma adrenaline and noradrenaline levels.

Determination of red blood cell β-adrenoceptors

The numbers (B_{max}) and binding affinities ($1/K_D$) of rbc surface β-adrenoceptors were determined *in vitro* in control fish and in fish subjected to 7 days of physical stress. After 7 days, a cannula was implanted into the dorsal aorta (see above) and the fish were allowed to recover in individual holding boxes for 24 h. A 2.5 ml blood sample was withdrawn from the dorsal aortic cannula and divided equally between two tonometry flasks (Eschweiler; 5 ml volume) containing 50 i.u. ml^{-1} ammonium heparin. The blood was equilibrated

for 45 min at 10 °C either with normoxic (P_{CO_2}=0.25 kPa; P_{O_2}=20.0 kPa, remainder N_2) or with hypoxic (P_{CO_2}=0.25 kPa; P_{O_2}=1.1 kPa, remainder N_2) gas mixtures.

The protocol used to determine B_{max} and K_D was similar to that employed by Reid *et al.* (1991). Briefly, this technique uses a hydrophilic radioligand binding assay (Marttila and Nikinmaa, 1988) that specifically targets high-affinity cell surface β-adrenoceptors. Radioligand binding was initiated by the addition of 40 μl of blood to 160 μl of physiological teleost saline (Wolf, 1963) containing 100 mg ml^{-1} ascorbic acid to which the radioligand (±)-3,4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one (^{3}H-CGP 12177; 5–40 nmol l^{-1}) had been added alone or in combination with 200 μmol l^{-1} (−)-isoproterenol. Incubations were performed in triplicate. The number of rbcs added to the incubation solution was determined by diluting 10 μl of blood in 10 ml of saline and then counting the number of rbcs present using a haemocytometer.

Incubations were stopped, after 45 min, by transferring the rbcs to borosilicate filters (no. 32, Mandel Scientific) using a cell membrane harvester (Brandell 24R) and five subsequent washings with 5 ml of ice-cold saline (0.9 % w/v NaCl). The filters were placed into glass scintillation vials containing 8 ml of fluor (ACS II, Amersham) and then stored in the dark for 24 h before being counted. The sample radioactivity was determined using a liquid scintillation counter (Canberra Packard model 2500 TR) with all counts automatically corrected to disints min^{-1} using an external standard technique. The maximal number of isoproterenol-displaceable binding sites (B_{max}, in disints min^{-1}) and the apparent dissociation constants (K_D) were determined using Scatchard plot analysis (Scatchard, 1949). Binding site density was then converted to and expressed as binding sites per rbc by multiplying B_{max} (disints min^{-1} cell^{-1}) by the specific activity of the radioligand and Avogadro's number. In accordance with previously reported findings (Reid *et al.* 1991), the isoproterenol-displaceable CGP 12177 binding sites are hereafter referred to as rbc surface β-adrenoceptors.

The effects of noradrenaline on levels of water, Na^+ and cyclic AMP in red blood cells

Blood was withdrawn from the dorsal aortic cannula of control or stressed fish and pooled to obtain a sufficient volume for a single experiment (i.e. N=1). In practice, 6.0 ml was obtained from two fish and this was diluted with 2.0 ml of teleost saline to yield the volume (8.0 ml) required for an experiment. Blood sampling was halted immediately at the first sign of agitation or struggling. Blood was stored on ice in 25 ml round-bottomed tonometer flasks (final heparin concentration 50 i.u. ml^{-1}) for 2 h prior to experimentation. Plasma catecholamine levels were measured immediately in the pooled blood, and the noradrenaline levels were found to be 5.6±1.2 nmol l^{-1} and 4.1±0.7 nmol l^{-1} (means ± S.E.M., N=8) in the control and chased fish, respectively. Although plasma catecholamine levels were not determined after the 2 h waiting period, previous studies in this laboratory have shown that the concentration of noradrenaline decreases at a rate of 2 nmol l^{-1} h^{-1} in resting blood samples over such a time period (S. G. Reid, unpublished results). Furthermore, Tetens *et al.* (1988) have reported that catecholamines decay with a half-life of 24 min when stored at 15 °C under conditions of continuous tonometry and that this decay is insensitive to oxygenation status. Thus, in the present study, we have assumed noradrenaline levels of 1 nmol l^{-1} in the blood prior to experimentation.

Adrenergic cell swelling, Na^+ accumulation and cyclic AMP accumulation were determined at 10 °C in normoxic (P_{CO_2}=0.25 kPa, P_{O_2}=20.0 kPa, remainder N_2, pHe=7.8; 30 min pre-incubation) and hypoxic (P_{CO_2}=0.25 kPa, P_{O_2}=1.1 kPa, remainder N_2, pHe=7.8; 30 min pre-incubation) blood samples (500 μl) after exposure to saline and seven doses of noradrenaline to yield final nominal concentrations of 10^{-9} mol l^{-1} (saline addition) and 5×10^{-9} to 10^{-5} mol l^{-1}. After 5 min, a sample (100 μl) was removed to determine rbc cyclic AMP levels and cell solids, and after 30 min the remaining blood (400 μl) was removed to determine rbc water and Na^+ contents. These sampling times were selected on the basis of previous studies showing that cyclic AMP accumulation was maximal 5 min after addition of agonist and that changes in cell water levels were detectable 30 min after addition of agonist (Salama and Nikinmaa, 1990; Salama, 1993).

To determine cyclic AMP levels, 50 μl of the 100 μl blood sample was deproteinized with 50 μl of 0.6 mol l^{-1} perchloric acid and then neutralized with 1.0 mol l^{-1} $KHCO_3$. A supernatant was obtained by centrifugation (10 000 ***g*** for 2 min) and then stored at −80 °C (for 2–4 weeks) prior to analysis. Red cell dry mass was determined on the remaining 50 μl of sample by drying the pellet to a constant mass.

To determine cell water content, half of the 400 μl blood sample was centrifuged (10 000 ***g*** for 2 min) and the supernatant and upper layer of cells were discarded. The red cell pellet was weighed, dried to a constant mass overnight and re-weighed. The water content of the cells (%H_2O) was calculated as the percentage water of the total mass and was not corrected for any water contained within trapped plasma. To determine rbc [Na^+], the remaining 200 μl of blood was centrifuged and the cell pellet was weighed, deproteinized with 0.6 mol l^{-1} perchloric acid and stored at 4 °C until analysis a few days later.

The effects of 8-bromo cyclic AMP on red blood cell water and Na^+ contents

Blood from control or stressed fish was sampled, stored and equilibrated under normoxic and hypoxic conditions as described above. Red cell water and Na^+ contents were determined after a 30 min exposure to saline and to a range of 8-bromo cyclic AMP concentrations (10^{-4} to 10^{-2} mol l^{-1} final nominal concentrations).

Analytical procedures

Plasma catecholamine levels were determined on alumina-

extracted samples (200 μl) using HPLC in conjunction with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine was used as an internal reference standard in all analyses. Red cell cyclic AMP levels were measured according to the protocol of a commercial kit (Amersham catalogue no. TRK.432). Red cell [Na^+] was determined by flame emission spectrophotometry (Varian Spectraa 250 Plus).

Calculation of EC_{50} values

The EC_{50} values for noradrenaline-evoked and 8-bromo-cyclic-AMP-evoked responses were calculated in three different ways. First, sigmoidal dose–response curves of averaged data were constructed using iterative curve-fitting software (Sigmaplot Windows ver. 2.0) in which the EC_{50} values were generated automatically. Second, Hill plots were constructed from each individual dose–response curve, using responses between 5 and 95 %, to yield an EC_{50} value from each experiment. These EC_{50} values were averaged and an appropriate statistical analysis was performed on the means (see below). Third, Hill plots were constructed using data from the averaged dose–response curves. Finally, an EC_{50} value was calculated by averaging the results of the three techniques.

Statistical analyses

Data in tables and figures are presented as mean values ±1 standard error of the mean (S.E.M.). The data were statistically analysed by parametric analysis of variance (ANOVA) followed by a Fisher's least squares difference (LSD) multiple-comparison test (comparison of all points) or Bonferroni's comparison with a single control point. When parametric test assumptions were violated, the data was analysed using Kruskal–Wallis ANOVA followed by Dunn's multiple-comparison test (comparison of all points) or Dunnett's comparison with a single control point. All statistical testing, including determinations of normality and variance, was performed using commercial software (Sigmastat Windows). The fiducial limit of significance was set at 5 %.

Results

Plasma catecholamine levels

Plasma catecholamine levels were elevated immediately upon exhaustion of the fish and remained significantly elevated for 15–30 min (Fig. 1). The post-exhaustion levels of catecholamines were statistically greater on day 1 when compared with levels after 3 and 7 days of chasing. The predominant circulating catecholamine after each acute stress episode was adrenaline. Typically, adrenaline accounted for more than 85 % of the total circulating catecholamine concentration; the peak values for adrenaline ranged between 50 and 275 nmol l^{-1}, whereas the peak values for noradrenaline ranged between 20 and 40 nmol l^{-1}. Serial blood sampling, in itself, did not contribute to a rise in plasma catecholamine levels as indicated by the stable and low (less than 5 nmol l^{-1}) levels of adrenaline and noradrenaline in the control fish (Fig. 1A).

Red blood cell β*-adrenoceptors*

The number of high-affinity cell surface β-adrenoceptors was reduced significantly by 20 % after 7 days of physical stress, when measured under normoxic incubation conditions (Fig. 2A). Incubation of the blood under hypoxic conditions did not cause a statistically significant increase in the number

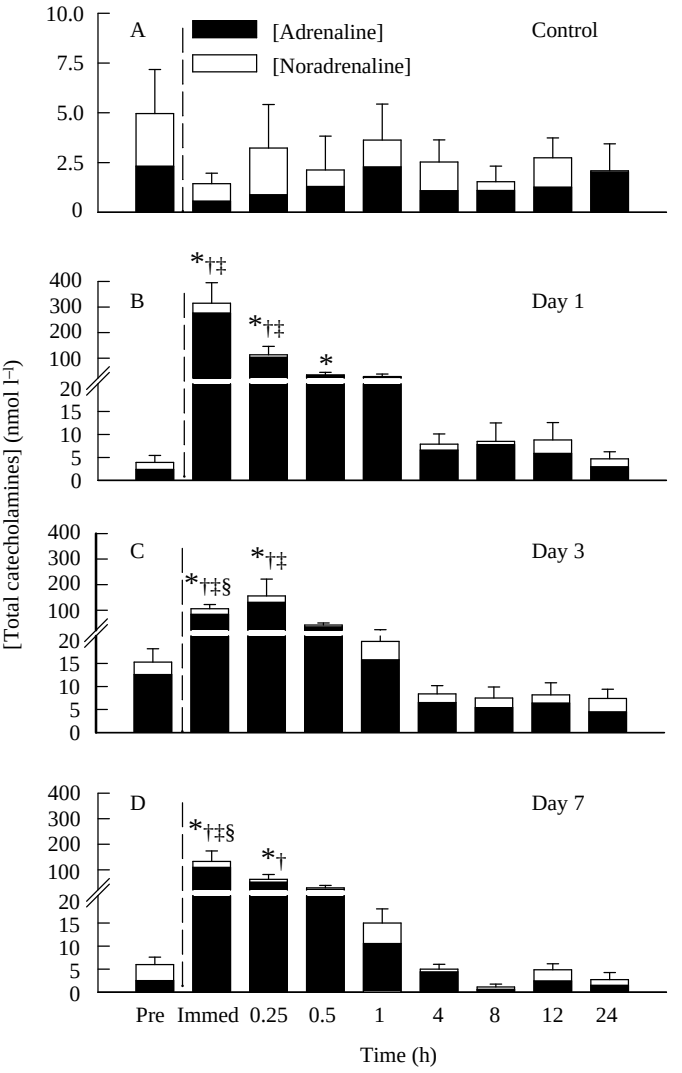

Fig. 1. Plasma catecholamine levels over a 24 h period in control fish (A; *N*=6) and in fish that had been physically stressed (chased until exhaustion) on day 1 (B; *N*=6), day 3 (C; *N*=6) and day 7 (D; *N*=6) of a 7-day repeated stress regime. Plasma adrenaline levels are indicated by the filled bars while noradrenaline levels are indicated by the open bars. † denotes a statistical difference in adrenaline concentration from the pre-stress (Pre) value; ‡ denotes a statistical difference in noradrenaline concentration from the pre-stress (Pre) value; * denotes a statistical difference in total catecholamine concentration from the pre-stress (Pre) value. § denotes a statistical difference from the corresponding total catecholamine value on day 1 (P<0.05). Immed, value immediately after chasing. Values are means + S.E.M.

of surface β-adrenoceptors in either group of fish, but further widened the difference in numbers of receptor between the control and stressed fish (30 % difference; Fig. 2A).

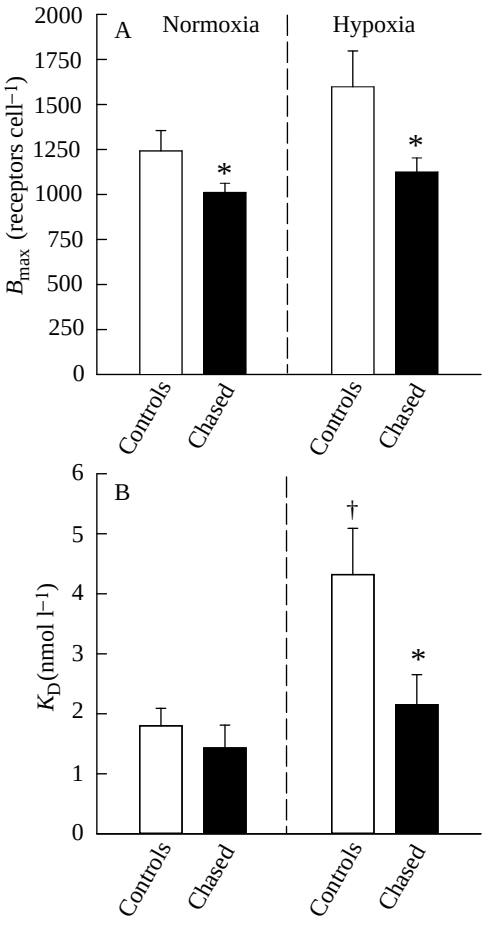

Fig. 2. The effects of 7 days of repeated physical stress on (A) the numbers of red blood cell surface β-adrenoceptors (B_{max}) and (B) their dissociation constant (K_D) determined *in vitro* under normoxic or hypoxic conditions. The open bars represent blood from control fish (N=8) while the filled bars represent blood taken from stressed fish (N=8). * denotes a statistically significant difference from the corresponding control blood; † denotes a statistically significant difference from the corresponding value under normoxic conditions (P<0.05). Values are means + S.E.M.

The apparent receptor affinity was unaltered by repeated physical stress when measured under normoxic conditions (Fig. 2B). Under hypoxic conditions, however, the receptor affinity was significantly decreased (K_D increased), but only in the rbcs from control fish. Thus, during hypoxia, the receptor affinity was higher in the rbcs obtained from stressed fish (K_D values of 4.2 and 2.1 nmol l^{-1} in the control and stressed groups, respectively; Fig. 2B).

Red cell responses to noradrenaline

Cell swelling

Red cells exhibited a dose-dependent rise in water content (Fig. 3A,D) in response to noradrenaline, which is a characteristic of β-adrenergic cell swelling induced by activation of rbc Na^+/H^+ exchange. Resting rbc water content and maximal β-adrenergic cell swelling were greatest under hypoxic conditions (compare Fig. 3A,D) and, moreover, there was an obvious leftward shift in the dose–response curve caused by hypoxia (compare Fig. 3C,F). Table 1 shows that, in the control fish, the average EC_{50} value was lowered from 2.85×10^{-7} mol l^{-1} (normoxia) to 2.55×10^{-8} mol l^{-1} (hypoxia). In the chased fish, hypoxia was associated with a

Table 1. *EC_{50} values for noradrenaline-induced cell swelling in control and stressed fish determined under normoxic or hypoxic conditions*

Method	EC_{50} (mol l^{-1}) Control fish (N = 15)	EC_{50} (mol l^{-1}) Chased fish (N = 15)	ΔEC_{50}
Normoxia			
Hill plot of mean DRCs	1.69×10^{-7}	7.78×10^{-8}	2.2
Hill plots of individual DRCs	$4.47\times10^{-7}\pm1.61\times10^{-7}$	$1.15\times10^{-7}\pm2.51\times10^{-8}$*	3.9
Iterative curve-fitting of mean DRCs	2.39×10^{-7}	5.00×10^{-8}	4.8
Average	2.85×10^{-7}	8.09×10^{-8}	3.5
Hypoxia			
Hill Plot of mean DRCs	2.71×10^{-8}	1.80×10^{-8}	1.5
Hill plots of individual DRCs	$3.07\times10^{-8}\pm3.3\times10^{-9}$†	$1.85\times10^{-8}\pm2.48\times10^{-9}$†,*	1.7
Iterative curve-fitting of mean DRCs	1.86×10^{-8}	1.21×10^{-8}	1.5
Average	2.55×10^{-8}	1.62×10^{-8}	1.6

EC_{50} values from the dose–response curves (DRCs) were calculated in three different ways (see Materials and methods) and then averaged. Statistical analyses were performed solely on the mean EC_{50} values obtained from individual DRCs.

† indicates a significant difference between normoxic and hypoxic blood; * indicates a significant difference between blood from control and chased fish.

Values are means ± S.E.M.

ΔEC_{50}, value for control fish/value for chased fish.

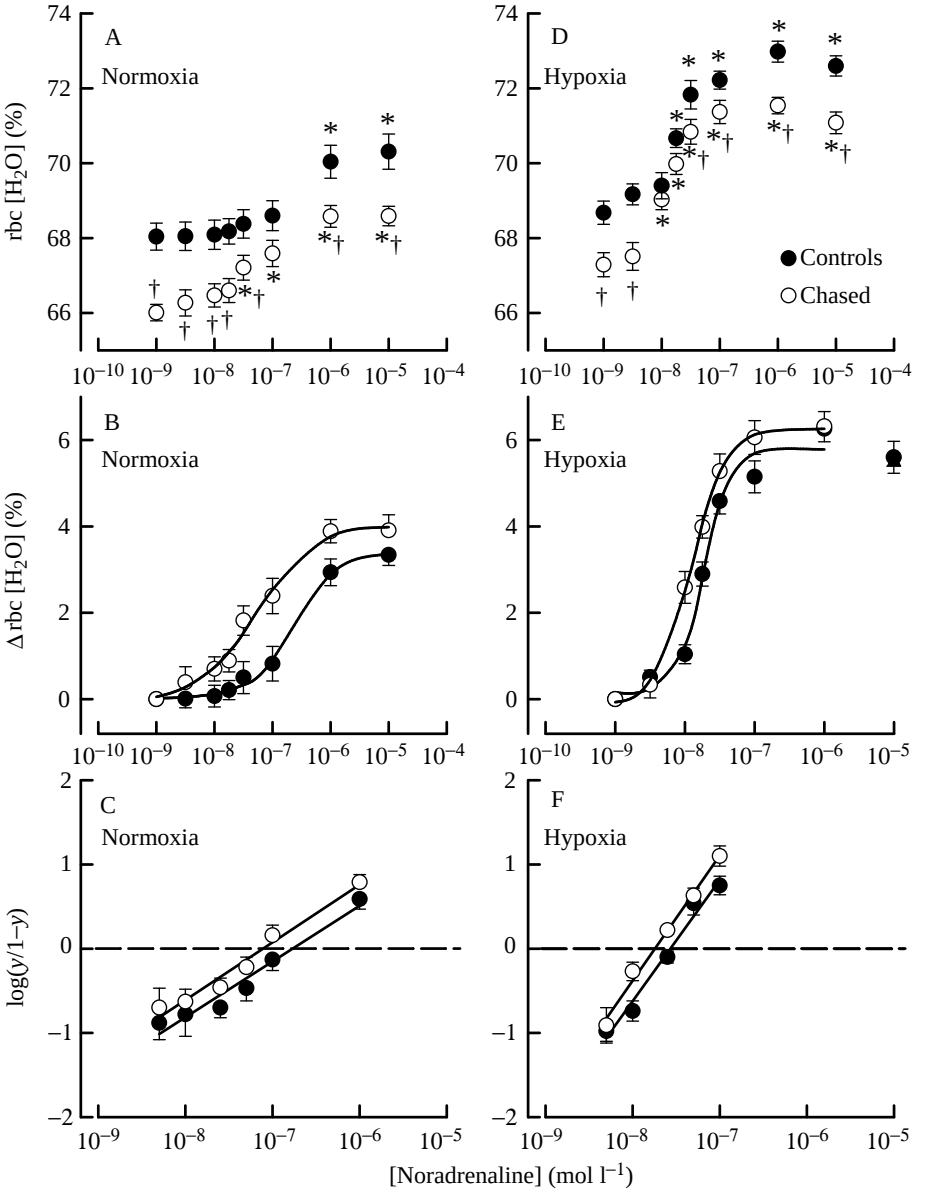

Fig. 3. The effects of noradrenaline concentration on β-adrenergic red blood cell (rbc) swelling determined by measuring the percentage of cell water (rbc [H_2O]; A,D) and the change in rbc water content (Δrbc [H_2O]; B,E). Experiments were performed *in vitro* under conditions of normoxia (A–C) and hypoxia (D–F) using blood from control (filled symbols; N=15) and physically stressed (open symbols; N=15) fish. The sigmoidal dose–response curves were drawn using iterative curve-fitting software (Sigmaplot). The mean data constituting the dose–response curves in B and E were transformed to generate the Hill plots given in C and F. The following linear regressions were calculated. (1) Normoxia control: y=0.66x+4.48; r^2=0.96. (2) Normoxia stressed: y=0.69x+4.87; r^2=0.97. (3) Hypoxia control: y=1.44x+10.90; r^2=0.98. (4) Hypoxia stressed: y=1.49x+11.52; r^2=0.99. For all regressions, P<0.05. * denotes a statistically significant difference from the value at the lowest noradrenaline concentration (10^{-9} mol l^{-1}); † denotes a statistically significant difference between the control and chased fish (P<0.05). Values are means ± S.E.M.

lowering of the average EC_{50} from 8.09×10^{-8} mol l^{-1} to 1.62×10^{-8} mol l^{-1} (Table 1).

Repeated chasing had a marked impact on both the resting levels of rbc water content (Fig. 3A,D) and β-adrenergic cell swelling (Fig. 3B–F; Table 1). The water content of the rbcs from chased fish was significantly lower at nearly all concentrations of noradrenaline under normoxic or hypoxic conditions. Furthermore, repeated chasing caused an obvious leftward shift of the dose–response curve (Fig. 3B–F). Under normoxic conditions, the average EC_{50} was reduced from 2.85×10^{-7} mol l^{-1} to 8.09×10^{-8} mol l^{-1}, a statistically significant difference based on the analysis of individual dose–response curves (Table 1). Under hypoxic conditions, the reduction in the EC_{50} caused by repeated stress was smaller but, nevertheless, there was a significant lowering (statistical analysis performed on individual dose–response curves) of the average EC_{50} from 2.55×10^{-8} mol l^{-1} to 1.62×10^{-8} mol l^{-1} (Table 1).

Accumulation of Na^+

The interactive effects of hypoxia and repeated stress on β-adrenergic rbc Na^+ accumulation were similar to the changes in rbc water content (see Fig. 4; Table 2). In both the control and the stressed fish, hypoxia elicited an increase in maximal Na^+ accumulation (Fig. 4B,E) and a leftward shift of the dose–response curves (Fig. 4C,F). Hypoxia lowered the EC_{50} values from 2.67×10^{-7} mol l^{-1} to 6.77×10^{-8} mol l^{-1} in the control fish and from 1.49×10^{-7} mol l^{-1} to 4.01×10^{-8} mol l^{-1} in the stressed fish (Table 2).

At low concentrations of noradrenaline (5×10^{-8} to 10^{-9} mol l^{-1}), the intracellular [Na^+] was lower in the stressed fish under normoxic conditions only (Fig. 4A,D). Maximal Na^+ accumulation was unaffected by chasing, but the EC_{50} was significantly reduced in the normoxic blood (2.67×10^{-7} mol l^{-1} *versus* 1.49×10^{-7} mol l^{-1}; Table 2) although not significantly in the hypoxic blood (P=0.07, Table 2) by chasing.

Table 2. *EC_{50} values for noradrenaline-induced cell Na^+ accumulation in control and stressed fish determined under normoxic or hypoxic conditions*

Method	EC_{50} ($mol\,l^{-1}$) Control fish (N = 15)	Chased fish (N = 15)	ΔEC_{50}
Normoxia			
Hill plot of mean DRCs	2.31×10^{-7}	1.49×10^{-7}	1.6
Hill plots of individual DRCs	$2.94\times10^{-7}\pm5.44\times10^{-8}$	$1.46\times10^{-7}\pm1.70\times10^{-8}$*	2.0
Iterative curve-fitting of mean DRCs	2.76×10^{-7}	1.52×10^{-7}	1.8
Average	2.67×10^{-7}	1.49×10^{-7}	1.8
Hypoxia			
Hill Plot of mean DRCs	7.03×10^{-8}	4.54×10^{-8}	1.5
Hill plots of individual DRCs	$8.50\times10^{-8}\pm1.7\times10^{-8}$†	$4.72\times10^{-8}\pm5.0\times10^{-9}$	1.8
Iterative curve-fitting of mean DRCs	4.79×10^{-8}	2.77×10^{-8}	1.7
Average	6.77×10^{-8}	4.01×10^{-8}	1.7

EC_{50} values from the dose–response curves (DRCs) were calculated in three different ways (see Materials and methods) and then averaged. Statistical analyses were performed solely on the mean EC_{50} values obtained from individual DRCs.

† indicates a significant difference between normoxic and hypoxic blood; * indicates a significant difference between blood from control and chased fish.

Values are means ± S.E.M.

ΔEC_{50}, value for control fish/value for chased fish.

Table 3. *EC_{50} values for 8-bromo-cyclic-AMP-induced cell swelling in control and stressed fish determined under hypoxic conditions*

Method	EC_{50} ($mol\,l^{-1}$) Control fish (N = 6)	Chased fish (N = 6)	ΔEC_{50}
Hypoxia			
Hill plot of mean DRCs	3.19×10^{-4}	2.15×10^{-4}	1.5
Hill plots of individual DRCs	$3.61\times10^{-4}\pm4.0\times10^{-5}$	$2.13\times10^{-4}\pm3.96\times10^{-5}$*	1.7
Iterative curve-fitting of mean DRCs	2.12×10^{-4}	1.39×10^{-4}	1.5
Average	2.97×10^{-4}	2.01×10^{-4}	1.5

EC_{50} values from the dose–response curves (DRCs) were calculated in three different ways (see Materials and methods) and then averaged. Statistical analyses were performed solely on the mean EC_{50} values obtained from individual DRCs.

* indicates a significant difference between blood from control and chased fish.

Values are means ± S.E.M.

ΔEC_{50}, value for control fish/value for chased fish.

Accumulation of cyclic AMP

Red cell cyclic AMP accumulated in a dose-dependent manner with significant increases first observed at 10^{-7} and $10^{-6}\,mol\,l^{-1}$ in normoxic and hypoxic blood, respectively (Fig. 5). Neither hypoxia nor repeated chasing significantly affected the pattern of cyclic AMP accumulation during adrenergic stimulation (Fig. 5B,D). Because maximal responses were not observed in these experiments, Hill plots were not constructed and EC_{50} values were not calculated.

Red cell responses to 8-bromo cyclic AMP

To investigate further whether repeated physical stress was influencing the responsiveness of the Na^+/H^+ exchanger to cyclic AMP, rbcs were incubated with the permeable analogue 8-bromo cyclic AMP under normoxic or hypoxic conditions. Hypoxia alone caused a marked increase in the maximal cell swelling (compare Fig. 6B,D) and appeared to induce a leftward shift of the dose–response curve. Although it was not possible to calculate EC_{50} values in the normoxic blood owing to the lack of a typical sigmoidal dose–response relationship (Fig. 6B), the data shown in Fig. 6A,C clearly indicate that cell swelling commenced at lower concentrations of 8-bromo cyclic AMP in the hypoxic blood. Under hypoxic conditions, the blood from chased fish displayed a significantly lower EC_{50} compared with that of control fish (Fig. 6E; Table 3).

The changes in rbc [Na^+] during incubation with 8-bromo cyclic AMP are illustrated in Fig. 7 and Table 4. Hypoxia, by itself, increased maximal Na^+ accumulation (Fig. 7A–E) and

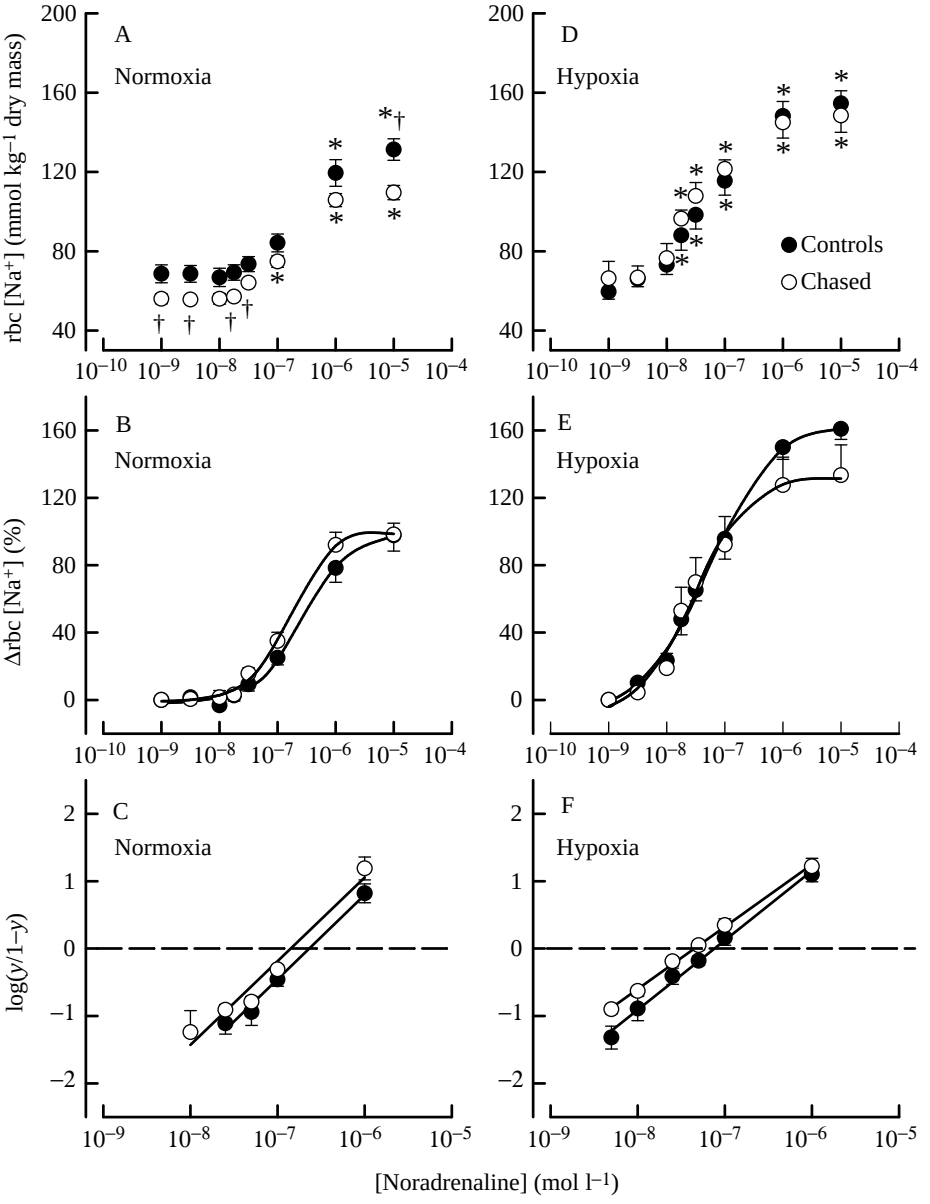

Fig. 4. The effects of noradrenaline concentration on β-adrenergic red blood cell (rbc) Na^+ accumulation determined by measuring the concentration of cell Na^+ (rbc $[Na^+]$; A,D) and the change in rbc Na^+ concentration (Δrbc $[Na^+]$; B,E). Experiments were performed *in vitro* under conditions of normoxia (A–C) and hypoxia (D–F) using blood from control (filled symbols; N=15) and physically stressed (open symbols; N=15) fish. The sigmoidal dose–response curves were drawn using iterative curve-fitting software (Sigmaplot). The mean data constituting the dose–response curves in B and E were transformed to generate the Hill plots given in C and F. The following linear regressions were calculated. (1) Normoxia control: y=1.25x+8.30; r^2=0.99. (2) Normoxia stressed: y=1.24x+8.51; r^2=0.97. (3) Hypoxia control: y=1.03x+7.35; r^2=0.99. (4) Hypoxia stressed: y=0.92x+6.80; r^2=0.99. For all regressions, P<0.05. * denotes a statistically significant difference from the value at the lowest noradrenaline concentration (10^{-9} mol l^{-1}); † denotes a statistically significant difference between the control and chased fish (P<0.05). Values are means ± S.E.M.

evoked a leftward shift of the dose–response curve in both the control and the chased fish (Fig. 7C,F). Repeated stress did not alter the maximal accumulation of Na^+ but did cause a leftward shift in the dose–response curve in the hypoxic blood (Fig. 7F); the average EC_{50} was lowered 1.6-fold from 5.16×10^{-4} to 3.20×10^{-4} mol l^{-1} (Table 4). In the normoxic blood, the average EC_{50} values were 1.23×10^{-3} and 8.34×10^{-4} mol l^{-1} in the control and stressed fish, respectively (P=0.06; Table 4).

Discussion

Plasma stress hormones

The pattern of elevation of plasma catecholamine levels observed in fish after physical stress was similar to that observed in other studies on rainbow trout employing violent exhaustive exercise as the mode of stress (e.g. Tang and Boutilier, 1988; Van Dijk and Wood, 1988; Wood *et al.* 1990; see also review by Gamperl *et al.* 1994). It is clear from these studies that maximal catecholamine levels are achieved immediately upon exhaustion and the levels then decline rapidly to reach baseline values within 0.5–2 h. The rapid decline in plasma catecholamine levels presumably arises from the combined effects of tissue accumulation and metabolic degradation (Nekvasil and Olson, 1986*a*,*b*; for a review, see Randall and Perry, 1992). Indeed, Nekvasil and Olson (1986*a*) have estimated the biological half-life of an injected catecholamine dose to be less than 10 min.

In the present study, plasma adrenaline was by far the predominant circulating catecholamine after stress. This also suggests that adrenaline was the primary catecholamine released from its site of storage, the chromaffin cells associated with the wall of the posterior cardinal vein (Nandi, 1961; Nilsson, 1983; Randall and Perry, 1992; Reid and Perry, 1994*b*; Reid *et al.* 1994). The prevalence of adrenaline in the

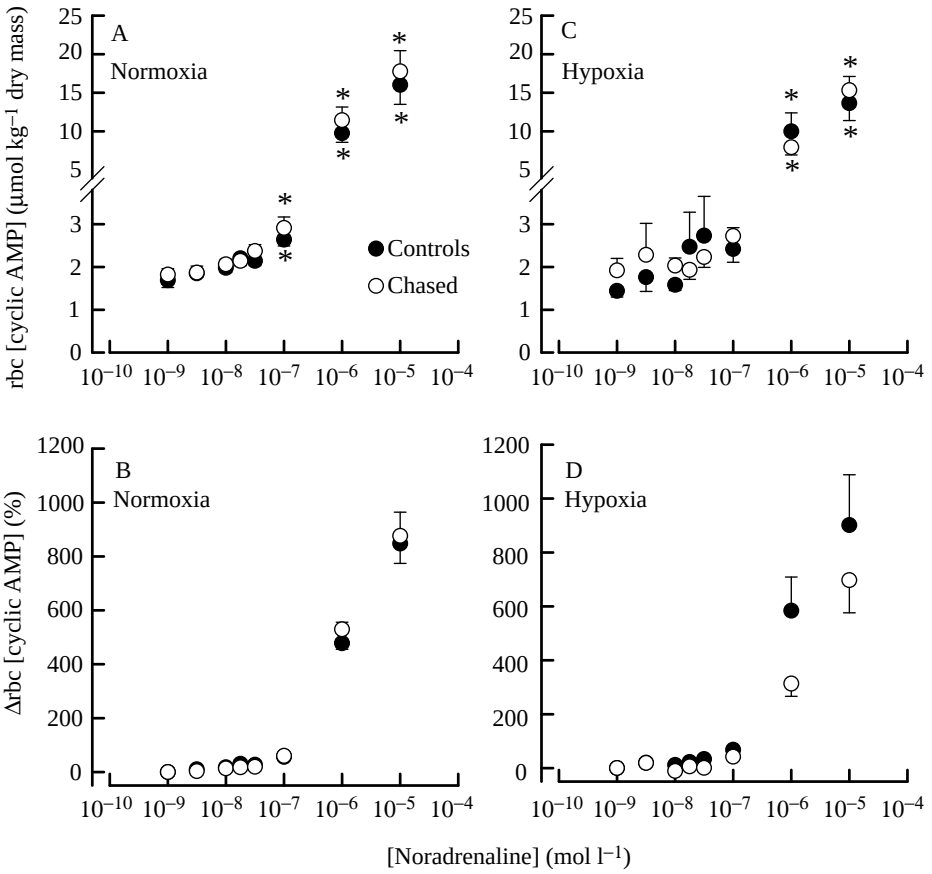

Fig. 5. The effects of noradrenaline concentration on β-adrenergic red blood cell (rbc) cyclic AMP accumulation determined by measuring the concentration of cell cyclic AMP (rbc [cyclic AMP]; A,C) and the change in rbc cyclic AMP concentration (Δrbc [cyclic AMP]; B,D). Experiments were performed *in vitro* under conditions of normoxia (A,B) and hypoxia (C,D) using blood from control (filled symbols; *N*=15) and physically stressed (open symbols; *N*=15) fish. The data in B and D did not conform with usual sigmoidal dose–response curves and thus iterative curve-fitting was not attempted and Hill plots were not constructed. * denotes a statistically significant difference (P<0.05) from the lowest noradrenaline concentration (10^{-9} mol l^{-1}). Values are means ± S.E.M.

Table 4. *EC_{50} values for 8-bromo-cyclic-AMP-induced cell Na^+ accumulation in control and stressed fish determined under normoxic or hypoxic conditions*

Method	EC_{50} (mol l^{-1}) Control fish (N = 6)	EC_{50} (mol l^{-1}) Chased fish (N = 6)	ΔEC_{50}
Normoxia			
Hill plot of mean DRCs	1.11×10^{-3}	8.80×10^{-4}	1.3
Hill plots of individual DRCs	$1.23\times10^{-3}\pm2.44\times10^{-4}$	$7.73\times10^{-4}\pm8.38\times10^{-5}$	1.6
Iterative curve-fitting of mean DRCs	1.35×10^{-3}	8.50×10^{-4}	1.5
Average	1.23×10^{-3}	8.34×10^{-4}	1.5
Hypoxia			
Hill Plot of mean DRCs	5.51×10^{-4}	3.23×10^{-4}	1.7
Hill plots of individual DRCs	$5.33\times10^{-4}\pm4.56\times10^{-5}$†	$3.40\times10^{-4}\pm5.91\times10^{-5}$†,*	1.6
Iterative curve-fitting of mean DRCs	4.64×10^{-4}	2.96×10^{-4}	1.6
Average	5.16×10^{-4}	3.20×10^{-4}	1.6

EC_{50} values from the dose–response curves (DRCs) were calculated in three different ways (see Materials and methods) and then averaged. Statistical analyses were performed solely on the mean EC_{50} values obtained from individual DRC.

† indicates a significant difference between normoxic and hypoxic blood; * indicates a significant difference between blood from control and chased fish.

Values are means ± S.E.M.

ΔEC_{50}, value for control fish/value for chased fish.

plasma is also consistent with the results of *in situ* perfusion studies on rainbow trout showing that adrenaline is the principal catecholamine released from the posterior cardinal vein during stimulation with the chlolinoceptor agonist carbachol (Fritsche *et al.* 1993; Reid and Perry, 1994*b*). With a few exceptions, the results of previous studies investigating the effects of acute stress on teleost fish also demonstrate that plasma adrenaline is the primary circulating catecholamine

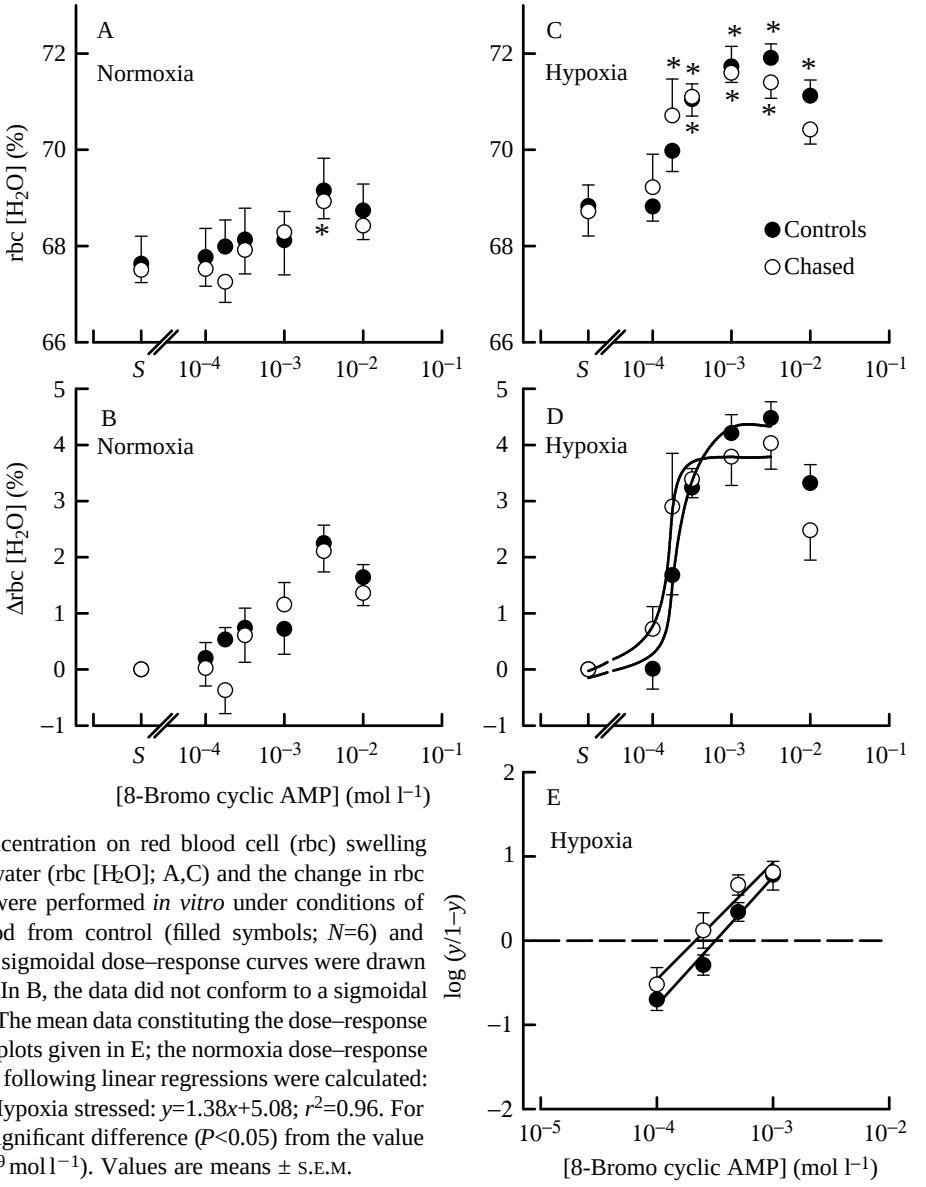

Fig. 6. The effects of 8-bromo cyclic AMP concentration on red blood cell (rbc) swelling determined by measuring the percentage of cell water (rbc [H_2O]; A,C) and the change in rbc water content (Δrbc [H_2O]; B,D). Experiments were performed *in vitro* under conditions of normoxia (A,B) and hypoxia (C–E) using blood from control (filled symbols; N=6) and physically stressed (open symbols; N=6) fish. The sigmoidal dose–response curves were drawn using iterative curve-fitting software (Sigmaplot). In B, the data did not conform to a sigmoidal function and thus curve-fitting was not employed. The mean data constituting the dose–response curves in D were transformed to generate the Hill plots given in E; the normoxia dose–response data were inappropriate to generate Hill plots. The following linear regressions were calculated: (1) Hypoxia control: y=1.52x+5.32; r^2=0.99. (2) Hypoxia stressed: y=1.38x+5.08; r^2=0.96. For all regressions, P<0.05. * denotes a statistically significant difference (P<0.05) from the value at the lowest noradrenaline concentration (S, 10^{-9} mol l^{-1}). Values are means ± S.E.M.

(e.g. see Table 2 in Gamperl *et al.* 1994). However, this does not necessarily exclude an important role for noradrenaline in mediating adrenergic physiological responses. For example, thorough analyses of the dose-dependency of rbc Na^+/H^+ exchange in rainbow trout blood *in vitro* have revealed a much higher sensitivity to noradrenaline than to adrenaline (Tetens *et al.* 1988; Salama, 1993). The higher sensitivity of the rainbow trout rbc to noradrenaline has led to speculation that the rbc β-adrenoceptor in this species is of the β_1 subtype (Tetens *et al.* 1988). In the present study, the maximal plasma concentrations of noradrenaline achieved immediately after stress were approximately 20–40 nmol l^{-1}. On the basis of the *in vitro* dose–response curves for cell swelling and Na^+ accumulation and a previous study (Tetens *et al.* 1988), significant activation of Na^+/H^+ exchange probably did occur in the chased fish upon release of catecholamines, despite the fact that the release occurred under normoxic conditions when adrenergic responsiveness is presumably lower (e.g. see Fig. 3B). Chasing the fish, however, probably caused a significant depression of blood pH (Wood *et al.* 1990) and this is known to enhance the adrenergic responsiveness of rainbow trout rbcs *in vitro* under normoxic conditions (e.g. Nikinmaa *et al.* 1987). An important *caveat* is that the dose–response curves were constructed after 7 days of repeated chasing, at which time the EC_{50} values for adrenergic Na^+/H^+ exchange were markedly lowered. It is uncertain when exactly during the 7-day stress regime this leftward shift of the dose–response curve occurred. Although EC_{50} values for adrenaline were not determined, the results of previous studies documenting high EC_{50} values (e.g. 7.6×10^{-7} mol l^{-1}, Tetens *et al.* 1988; 2×10^{-6} mol l^{-1}, Salama, 1993) do not support a significant role for adrenaline in the activation of rbc Na^+/H^+ exchange in rainbow trout, at least under normoxic conditions. Interestingly, Berenbrink and Bridges (1994) showed that the rbcs of Atlantic cod (*Gadus morhua*) possess β-adrenoceptors that appear to have a higher affinity for adrenaline than for

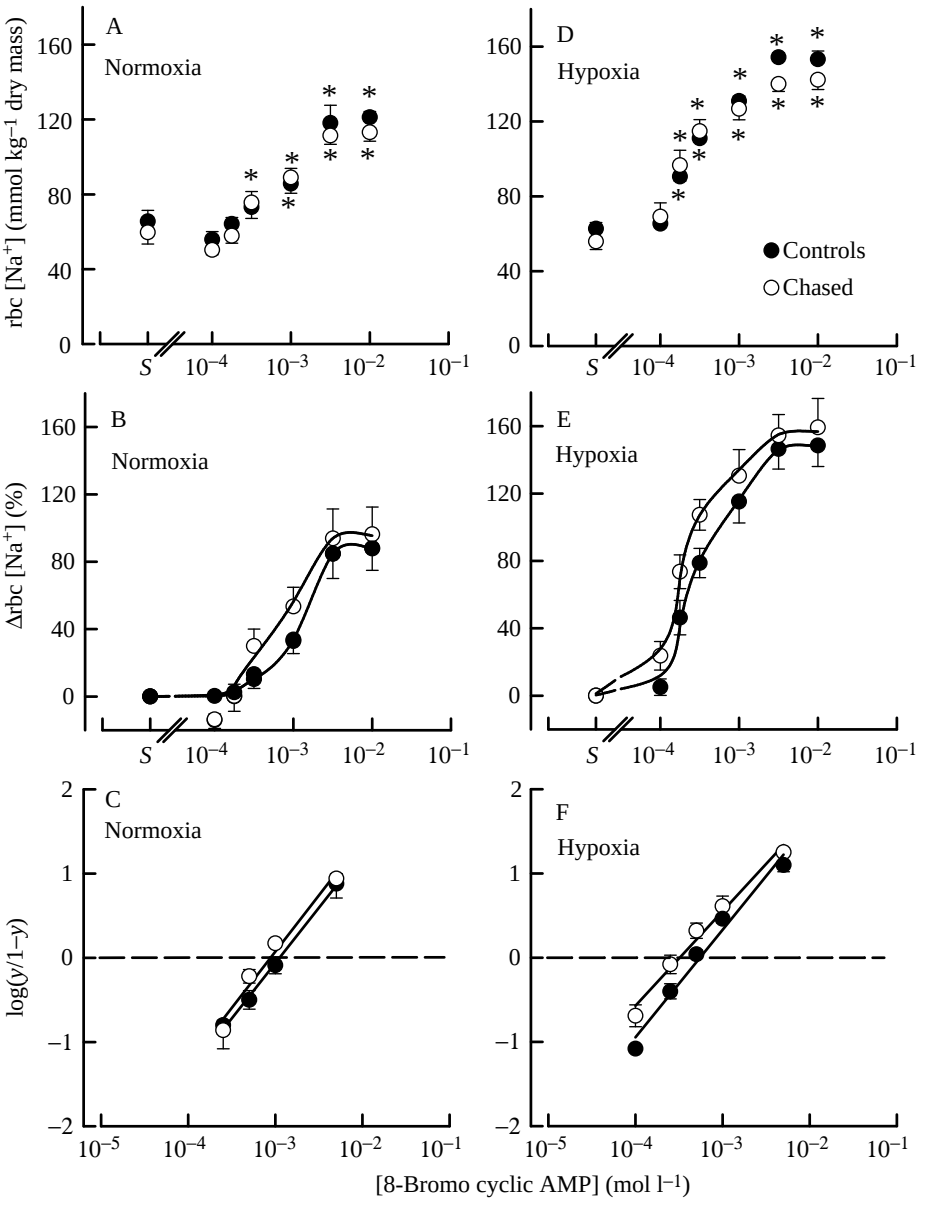

Fig. 7. The effects of 8-bromo cyclic AMP concentration on red blood cell (rbc) Na^+ accumulation determined by measuring the concentration of cell Na^+ (rbc [Na^+]; A,D) and the change in rbc Na^+ concentration (Δrbc [Na^+]; B,E). Experiments were performed *in vitro* under conditions of normoxia (A–C) and hypoxia (D–F) using blood from control (filled symbols; N=6) and physically stressed (open symbols; N=6) fish. The sigmoidal dose–response curves were drawn using iterative curve-fitting software (Sigmaplot). The mean data constituting the dose–response curves in B and E were transformed to generate the Hill plots given in C and F. The following linear regressions were calculated: (1) Normoxia control: y=1.31x+3.88; r^2=0.99. (2) Normoxia stressed: y=1.33x+4.08; r^2=0.97. (3) Hypoxia control: y=1.28$x\times$+4.16; r^2=0.97. (4) Hypoxia stressed: y=1.12x+3.92; r^2=0.98. For all regressions, P<0.05. * denotes a statistically significant difference (P<0.05) from the value at the lowest noradrenaline concentration (S, 10^{-9} mol l^{-1}). Values are means ± S.E.M.

noradrenaline, and thus that adrenaline may play a more important role in this species.

An important observation of the present study was an attenuation of the catecholamine release response on days 3 and 7 of the repetitive stress protocol; the maximal levels of plasma catecholamines were significantly higher on day 1 (Fig. 1). These data imply a desensitization of the catecholamine release process with repeated stress and thus confirm the results of a previous study (Reid *et al.* 1994) on catecholamine secretion in an *in situ* perfused posterior vein preparation. In that study, a pronounced desensitization of carbachol-evoked catecholamine release was noted following 5 days of repeated physical stress. Because the sensitivity of release, rather than the maximal quantities of catecholamines released, was lowered, it was suggested by Reid *et al.* (1994) that the desensitization was caused by a reduced affinity of chromaffin cell cholinoceptors for cholinergic agonists. Catecholamine release *in vivo* is probably not caused exclusively by stimulation of cholinoceptors, but is presumably the net result of numerous factors including a variety of neural inputs (Reid *et al.* 1995), humoral influences (Opdyke *et al.* 1981; Fritsche *et al.* 1993; Kloas *et al.* 1994; Epple *et al.* 1994) and localized chemical changes (Opdyke *et al.* 1983; Perry *et al.* 1991). Thus, desensitization of the catecholamine release process, which was apparent in the present study, could have arisen in many ways. It is unlikely that the reduced levels of catecholamines immediately post-exhaustion on days 3 and 7 were caused by accelerated catecholamine degradation or clearance, given that the blood samples were taken within seconds after the stress period. The physiological significance of reduced levels of circulating catecholamines with repeated stress may be to prevent excessive stimulation of target cells. This idea is consistent with general models of desensitization of G-protein-coupled receptors (Collins *et al.* 1990; Hausdorff *et al.* 1990; Gershengorn, 1994).

In a preliminary study (S. F. Perry and S. G. Reid, unpublished observations), plasma cortisol levels were measured on uncannulated juvenile trout (mass 20–115 g; N=124) after 1, 3 and 7 days of chasing. Plasma levels rose from 11.6±6.3 nmol l^{-1} to peak levels of approximately 415 nmol l^{-1} and remained elevated for 1–12 h depending upon the number of days of prior chasing. Although these data obtained from juvenile fish may differ from the pattern of cortisol elevation in the larger fish that were used in the *in vitro* experiments, they are consistent with the hypothesis that acute physical disturbance causes pronounced and prolonged elevation of plasma cortisol levels in trout (see Gamperl *et al.* 1994). Thus, increases in plasma cortisol levels in the stressed fish could conceivably have influenced rbc β-adrenoceptors (see below), as demonstrated previously (Reid and Perry, 1991).

Red blood cell β-*adrenoceptors*

Elevated levels of stress hormones have been shown to influence rbc β-adrenoceptors in rainbow trout, although in opposing ways. Increased levels of cortisol *in vivo* or *in vitro* are known to increase the numbers of internalized β-adrenoceptors and can result in an increase in the numbers of cell surface β-adrenoceptors when these cells are subsequently exposed to acute hypoxia (Reid and Perry, 1991). Additionally, the number of surface β-adrenoceptors in rainbow trout hepatocytes was markedly increased after *in vivo* elevation of plasma cortisol levels (Reid *et al.* 1992). In contrast, increased levels of catecholamines are known to lower the numbers of rbc surface β-adrenoceptors in rainbow trout (Gilmour *et al.* 1994).

In the present study, the number of rbc surface β-adrenoceptors measured in the blood of the repeatedly stressed fish was lower than in the control fish when assayed under both normoxic and hypoxic conditions. These results suggest that the effects of cortisol on increasing rbc β-adrenoceptor numbers were more than offset by the down-regulation caused by the elevated catecholamine levels and any other potential negative modifiers of rbc β-adrenoceptors that were not measured in these experiments. Thus, the model of Perry and Reid (1993), which proposes that chronic stress may 'pre-adapt' the teleost rbc to acute stress, is tenable only if plasma catecholamine levels do not rise concurrently with cortisol levels.

The protocol employed in this study required that all fish be cannulated 18 h prior to blood withdrawal. Thus, even the non-stressed group probably experienced post-surgery elevations of plasma cortisol and catecholamine levels (Gingerich and Drottar, 1989). Typically, in this laboratory, plasma catecholamine levels remain elevated for less than 4 h (S. F. Perry and A. Tsay, unpublished data) and, although the cortisol levels probably remained high in the 24 h following surgery, there were nevertheless pronounced differences (also see below) between the repeatedly stressed and non-stressed groups.

Previous studies have demonstrated varied results with respect to the effects of acute hypoxia *in vitro* on the numbers of rbc β-adrenoceptors. Although there are several reports of increased numbers of β-adrenoceptors during acute hypoxia (Marttila and Nikinmaa, 1988; Reid and Perry, 1991; Reid *et al.* 1993), this study and a previous study (Perry and Reid, 1992) were unable to demonstrate statistically significant increases. The inability of hypoxia to modify the rbc β-adrenoceptor population in the present study is consistent with the identical pattern of cyclic AMP accumulation that was observed in normoxic and hypoxic blood (see below). Thus, while increased number of rbc surface β-adrenoceptors may contribute to enhanced adrenergic responsiveness under certain conditions (Reid and Perry, 1991; Reid *et al.* 1993; Perry and Reid, 1993), the relatively small magnitude of the response and its inconsistency raise doubts as to its physiological importance. Indeed, the stimulatory effects of hypoxia observed in the present study can be explained solely on the basis of post-adrenoceptor modifications (see below). Although, the apparent affinity of the rbc β-adrenoceptors decreased in the control fish during hypoxia, this did not appear to influence the pattern of cyclic AMP accumulation during adrenergic stimulation.

Effects of acute hypoxia on adrenergic responses

In this study, rbc Na^+ and water accumulation were used as indices of β-adrenergic Na^+/H^+ exchange activity. It is important to point out that the net accumulation of Na^+ and water within the cell after application of catecholamines will also be influenced by secondary mechanisms, including Na^+ pump activity and volume regulatory responses. Thus, while we believe that the predominant factor determining rbc Na^+ and water levels is activity of the Na^+/H^+ exchanger, we cannot entirely exclude involvement of these other factors.

Although several previous studies have demonstrated a stimulatory effect of acute hypoxia on adrenergic responsiveness in rainbow trout (e.g. Motais *et al.* 1987; Reid and Perry, 1991; Salama, 1993), this is the first study to show a clear positive influence on both the maximal activity of the adrenergic response and the sensitivity of this response to applied catecholamines. These results were obtained by a rigorous analysis of dose–response curves. Owing to the importance of obtaining reliable EC_{50} values, three separate methods of determination were employed and the results were then averaged (see Materials and methods). Statistical evaluation, however, could be performed only on the EC_{50} values obtained from averaging individual dose–response curves. Thus, the statistical differences noted in this paper are based on such values.

The lowering of the EC_{50} values (increased response affinity) and the increase in the apparent maximal Na^+/H^+ exchange activity during hypoxia were clearly unrelated to modification of the cell surface β-adrenoceptors because the B_{max} of these receptors was unchanged during hypoxia and the K_D values were either unchanged (chased fish) or actually increased (control fish). Consequently, the pattern of cyclic AMP accumulation was also unaltered by hypoxia (see also Salama, 1993). Thus, unlike in previous studies (e.g. Reid *et al.* 1993), there was no evidence of hypoxia-mediated modification of the β-adrenergic signal transduction pathway leading to cyclic AMP accumulation. Instead, the dual effects of hypoxia on increasing the affinity and the maximal activity of Na^+/H^+ exchange are presumably related to regulation at the level of the exchanger itself, as proposed

initially by Motais *et al.* (1987). This could involve a direct effect of hypoxia on the numbers and/or affinities of the Na^+/H^+ exchanger (Reid and Perry, 1994*a*) or an indirect effect mediated by the oxygenation state of haemoglobin (Motais *et al.* 1987; Nikinmaa and Jensen, 1992). The results of experiments using 8-bromo cyclic AMP revealed that the responsiveness of the Na^+/H^+ exchanger to cyclic AMP was modified by hypoxia since there were significant increases in maximal Na^+/H^+ exchange activity (Figs 6, 7) and reductions in EC_{50} values (Table 4).

We are unable to explain the apparent lack of an effect of hypoxia on cyclic AMP accumulation in the present study, given the previous results obtained from this laboratory (Reid and Perry, 1991; Reid *et al.* 1993). Salama (1993) also did not observe an effect of hypoxia on cyclic AMP formation in rainbow trout rbcs, yet a previous study from that same laboratory (Salama and Nikinmaa, 1990) demonstrated a marked stimulatory effect of hypoxia on cyclic AMP formation in carp (*Cyprinus carpio*) rbcs. Thus, increases in adrenergic responsiveness during acute hypoxia in teleost rbcs may, or may not, involve modification of the β-adrenergic signal transduction pathway leading to cyclic AMP formation, while regulation at the level of the Na^+/H^+ exchanger appears to be a more pronounced and prevalent response.

Effects of repeated physical stress on adrenergic responses

The rbcs from the physically stressed fish had lower water contents under resting conditions *in vitro* compared with those of the control fish. The underlying explanation for the difference is unclear but it may reflect overcompensation of the rbc volume regulatory response in the stressed fish, which probaby experienced daily adrenergic rbc swelling in response to the chasing.

This is the first study to demonstrate that chronic repeated stress can markedly influence the responsiveness of the rainbow trout rbc to acute adrenergic stimulation. Specifically, chronic stress caused significant decreases in the noradrenaline EC_{50} values without affecting maximal Na^+/H^+ exchange activity. The increased affinity of the Na^+/H^+ exchanger for exogenous noradrenaline appeared to be unrelated to changes in the β-adrenergic signal transduction pathway because the pattern of cyclic AMP accumulation during adrenergic stimulation was unaltered. Thus, the significant reduction in the numbers of cell surface β-adrenoceptors associated with repeated stress clearly had no negative impact on signal transduction. Under hypoxic incubation conditions, the rbc β-adrenoceptors from the stressed fish displayed a higher binding affinity, yet this also did not affect the pattern of cyclic AMP formation. Thus, the enhanced rbc adrenergic responsiveness in the stressed fish must reflect post-cyclic-AMP modifications. This conclusion is supported by the results of the experiments demonstrating a lowering of the 8-bromo cyclic AMP EC_{50} values in hypoxic stressed fish (Tables 3, 4). The mechanisms underlying the increased sensitivity of the Na^+/H^+ exchanger to adrenergic stimulation following repeated stress are unknown. Regardless of the underlying mechanisms, the results reveal that the prior history of a fish can markedly influence rbc adrenergic responses *in vitro*, even over a relatively short period (e.g. 7 days). Furthermore, the impact of prior stress on the rbc adrenergic response may vary according to the nature of the stress. For example, Cossins and Kilbey (1989) documented that stressful episodes (e.g. rain storms or rapid temperature changes) at a commercial fish farm were associated with a reduced rbc adrenergic response. Therefore, in the light of these results, experiments designed to assess rbc adrenergic responses must carefully control for the potential effects of chronic or prior stress on the acute adrenergic stress response.

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