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THE RELATIONSHIP BETWEEN β-ADRENOCEPTORS AND ADRENERGIC RESPONSIVENESS IN TROUT (*ONCORHYNCHUS MYKISS*) AND EEL (*ANGUILLA ROSTRATA*) ERYTHROCYTES

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Summary

Experiments were performed *in vitro* specifically to elucidate the underlying mechanism(s) of the attenuated adrenergic responses of eel (*Anguilla rostrata*) erythrocytes. This was achieved by comparing β-adrenoceptor numbers and affinities in addition to (i) Na^+/H^+ exchange activity, (ii) cell swelling and (iii) cyclic AMP formation mediated by catecholamines in eel and trout (*Oncorhynchus mykiss*) erythrocytes under normoxic and hypoxic conditions.

Under normoxic conditions, eel erythrocytes displayed a total absence of Na^+/H^+ exchange activity (as determined from measurements of extracellular pH) after addition of noradrenaline (50–1000 nmol l^{-1}) in contrast to a pronounced dose-dependent response in trout. Incubation of the blood under hypoxic conditions, to achieve approximately 50 % haemoglobin O_2-saturation, further increased the extent of Na^+/H^+ exchange activation in trout and elicited a statistically significant, although physiologically small (10 % of the response in trout), activation of H^+ extrusion activity in eel. Catecholamine-mediated cell swelling, although obvious in trout, was absent in eel when estimated under hypoxic conditions.

Eel erythrocytes possessed approximately 50 % fewer surface β-adrenoceptors than did trout erythrocytes, although the dissociation constants (K_D) of these receptors did not differ between eel and trout. The numbers and affinities of the erythrocyte β-adrenoceptors were not significantly affected by the hypoxic incubation.

Both eel and trout erythrocytes displayed a dose-dependent elevation of cyclic AMP concentration in response to noradrenaline that was further increased by hypoxia. Surprisingly, eel erythrocytes produced larger quantities of cyclic AMP despite the lower numbers of surface β-adrenoceptors. Thus, the absence of adrenergic swelling and the attenuated H^+ extrusion response in eel erythrocytes cannot be attributed to insufficient numbers of β-adrenoceptors or to functional uncoupling of these receptors from adenylate cyclase. Instead, the differences between trout and eel may reflect differing numbers of Na^+/H^+ exchangers or

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fundamental differences in the manner by which these exchangers are activated by cyclic AMP.

Introduction

The erythrocytes of rainbow trout and several other teleost species possess a plasma membrane Na^+/H^+ exchanger that is activated by elevated levels of the circulating catecholamines adrenaline or noradrenaline (Baroin *et al.* 1984; see also reviews by Nikinmaa, 1986; Boutilier and Ferguson, 1989; Tufts and Randall, 1989; Nikinmaa and Tufts, 1989; Motais *et al.* 1990; Fievet and Motais, 1991). Adrenergic stimulation of the rainbow trout erythrocyte Na^+/H^+ exchanger either *in vitro* (Nikinmaa, 1983; Cossins and Richardson, 1985) or *in vivo* (Nikinmaa, 1982; Primmett *et al.* 1986; Milligan and Wood, 1987; Tetens and Christensen, 1987; Vermette and Perry, 1988*a*,*b*; Perry and Vermette, 1987; Claireaux *et al.* 1988; Perry and Kinkead, 1989) initiates a complex series of physiological responses within the erythrocyte that ultimately serve to enhance blood oxygen-transport by increasing the affinity or the capacity of haemoglobin to bind oxygen (see reviews by Perry and Wood, 1989; Thomas and Motais, 1990; Thomas and Perry, 1992). These responses include elevation of intracellular pH (pHi) (e.g. Nikinmaa, 1983; Cossins and Richardson, 1985; Nikinmaa *et al.* 1987), cell swelling (see review by Motais and Garcia-Romeu, 1986) and reductions in cellular nucleoside triphosphate (NTP) levels (Nikinmaa, 1983; Milligan and Wood, 1987; Ferguson *et al.* 1989; Ferguson and Boutilier, 1989). The adrenergic decrease in intracellular NTP levels arises primarily as a result of a pronounced increase in ATP utilization by the plasma membrane sodium/potassium pump (Tufts and Boutilier, 1991) owing to its stimulation by intracellular Na^+ accumulation (see review by Motais and Garcia-Romeu, 1986).

The adrenergic sensitivity of the erythrocyte Na^+/H^+ exchanger in several teleosts varies according to the pH and/or the oxygen status of the blood. In rainbow trout, the responsiveness of erythrocytes to catecholamines *in vitro* is enhanced by extracellular acidosis, with maximal activity of the Na^+/H^+ exchanger occurring between pH 7.2 and 7.3 (Nikinmaa *et al.* 1987; Borgese *et al.* 1987; Cossins and Kilbey, 1989). Similarly, adrenergic responsiveness in trout erythrocytes is enhanced by equilibrating the blood under hypoxic conditions (Motais *et al.* 1987; Reid and Perry, 1991). In addition to these intraspecific variations in the responses of trout erythrocytes to catecholamines, there are pronounced interspecific variations. Several teleost species display markedly reduced responses to catecholamines (carp, *Cyprinus carpio*; Salama and Nikinmaa, 1988, 1989; Fuchs and Albers, 1988; starry flounder, *Platichthys stellatus*; Milligan and Wood, 1987; pikeperch, *Stizostedion lucioperca*; Salama and Nikinmaa, 1989) or apparently total insensitivity to catecholamines (tench, *Tinca tinca*; Jensen, 1987; American eel, *Anguilla rostrata*; Hyde and Perry, 1989, 1990) in comparison to the salmonid species. The erythrocytes of the eel are particularly intriguing because they appear to lack adrenergic Na^+/H^+ exchange *in vivo* or *in*

vitro even under conditions normally expected to maximize teleost erythrocyte adrenergic responses (i.e. acidosis and/or hypoxia; Hyde and Perry, 1989, 1990).

The underlying mechanisms for the interspecific differences in the adrenergic responses of teleost erythrocytes are unknown. Previous studies have demonstrated (i) that accumulation of cyclic AMP within the carp erythrocyte after adrenergic stimulation is a prerequisite for activation of the Na^+/H^+ exchanger (Salama and Nikinmaa, 1990) and (ii) that the sensitivity of trout erythrocytes to catecholamines is directly proportional to the number of high-affinity β-adrenoceptors present on the plasma membrane (Reid and Perry, 1991). In view of the accepted relationship between cell surface β-adrenoceptors and cyclic AMP formation, a primary goal of the present study was to determine whether the interspecific variability between the adrenergic responses of trout and eel erythrocytes is related to differences in the number and/or affinity of cell surface β-adrenoceptors in the two species or whether it results from functional dissimilarities in the coupling of these receptors to cyclic AMP formation.

Materials and methods

Experimental animals and holding conditions

American eels (*Anguilla rostrata* LeSueur) weighing approximately 200–300 g (experimental $N=59$) were obtained from an eel ladder associated with the Saunders Hydroelectric Dam in Cornwall, Ontario, and were transported on ice to the University of Ottawa. Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex weighing approximately 175–250 g (experimental $N=66$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported in oxygenated water to the University of Ottawa.

All fish were maintained on a 12 h:12 h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechlorinated City of Ottawa tap water ($[Na^+]=0.12\,mmol\,l^{-1}$, $[Cl^-]=0.15\,mmol\,l^{-1}$, $[Ca^{2+}]=0.40–0.45\,mmol\,l^{-1}$, $[K^+]=0.03\,mmol\,l^{-1}$, pH=7.5–8.0) for at least 1 month prior to experimentation. Water temperature in the holding and experimental facilities varied between 8 and 12 °C during the course of experiments (July–October). Trout were fed to satiation on alternate days with a commercial salmonid diet that was withheld 48 h prior to commencing experiments. Eels were not fed throughout the period of this study.

Surgical procedures

Eels were anaesthetized in a 0.2 % (w/v) solution of ethyl-*m*-aminobenzoate (MS 222, Sigma) adjusted to pH 7.5–8.0 with Tris buffer (Trizma Base, Sigma). To permit blood collection for *in vitro* experiments, indwelling cannulae were implanted into the pneumogastric artery as previously described in detail (Hyde and Perry, 1989). The eel was then transferred to an opaque fibreglass chamber (volume 3 l) where it was allowed to recover from the effects of anaesthesia and surgery for at least 48 h prior to withdrawal of blood. The cannulae were flushed daily with freshwater teleost physiological saline (Wolf, 1963).

Trout were anaesthetized in a 0.01% (w/v) solution of MS 222 adjusted to pH 7.5–8.0 with $NaHCO_3$ and placed onto an operating table that allowed continuous irrigation of the gills with anaesthetic solution. Indwelling cannulae were implanted into the dorsal aorta (Soivio *et al.* 1975) with flexible polyethylene tubing (Clay Adams PE 50; internal diameter 0.580 mm, outer diameter 0.965 mm). Trout were revived on the operating table by irrigation of the gills with aerated water, then transferred to opaque fibreglass chambers where they were allowed to recover for at least 48 h prior to blood withdrawal. Cannulae were flushed daily with freshwater teleost physiological saline (Wolf, 1963). All saline contained 10 i.u. ml^{-1} ammonium heparin (Sigma).

Blood sampling and storage

After at least 48 h of recovery from surgery, approximately 2 ml of blood was withdrawn from the cannulae of individual fish into pre-heparinized syringes and then pooled in round-bottomed flasks to yield final volumes of 6 ml (i.e. $N=1$) for adrenergic sensitivity experiments (series 1) or 4 ml (i.e. $N=1$) for β-adrenoceptor determinations (series 2). Thus, six fish were required to achieve $N=1$ in series 1 and four fish were required in series 2. The blood sampling was terminated immediately if fish struggled or became agitated. In series 1, the pooled blood was gassed with oxygen for approximately 30 s and then maintained on ice for approximately 4 h before adrenergic responsiveness was determined (Thomas *et al.* 1991). In series 2, the pooled blood was used either immediately or after 4 h as in series 1.

Experimental protocol

Series 1. Adrenergic responsiveness of erythrocytes

The responsiveness of eel and trout erythrocytes to catecholamines was determined *in vitro* by (i) estimating the intensity of Na^+/H^+ exchange by assessing the extent of extracellular (plasma) acidification or cell swelling and (ii) monitoring the intracellular accumulation of cyclic AMP. The experiments were performed under both normoxic (except cell swelling estimates) and hypoxic conditions because of the marked stimulatory effects of acute hypoxia on teleost erythrocyte adrenergic responses (Motais *et al.* 1987; Salama and Nikinmaa, 1988; Reid and Perry, 1991).

Normoxia. The intensity of Na^+/H^+ exchange was quantified by monitoring the peak changes in whole-blood pH (extracellular pH or pHe) after addition of catecholamine. This technique, previously discussed in detail (Thomas *et al.* 1991), essentially determines the extent of the pHe disequilibrium arising from the extrusion of H^+ from the erythrocyte. The standard protocol was to measure the changes in pHe after the addition of 10 μl of noradrenaline (L-noradrenaline bitartrate dissolved in saline) or saline (controls) to 400 μl of blood, contained within round-bottomed tonometer flasks, to yield final nominal concentrations ranging from 50 to 1000 nmol l^{-1}. The blood was maintained in a shaking water

bath (8–12°C) and gassed continuously with a humidified normoxic gas mixture (0.5 % CO_2, 20.0 % O_2, 79.5 % N_2) supplied by a gas-mixing pump (Wösthoff, model M301-A/F). Whole-blood pH was measured on 50 μl samples immediately prior to additions of noradrenaline and at 5, 6 and 7 min thereafter. This was accomplished by withdrawing blood from the tonometer flasks 3 min after addition of noradrenaline. A single 50 μl sample was aspirated into a pH electrode (see below) and readings were taken at the appropriate times. Preliminary experiments showed that the peak changes in pHe occurred within 5–7 min of the addition of catecholamine. Cyclic AMP content was determined using 200 μl of blood sampled 5 min after the addition of noradrenaline or saline. A previous study (Salama and Nikinmaa, 1990) demonstrated that, in carp erythrocytes, cyclic AMP levels were maximally elevated within 5 min of addition of catecholamines *in vitro* and thereafter remained stable for at least 30 min. We did not establish similar relationships for trout and eel erythrocytes because it was considered essential that cyclic AMP levels were measured at the exact time that Na^+/H^+ exchange activity was being assessed (5 min).

In addition, a separate sample of the total blood pool was equilibrated with normoxic gas and subsequently sampled to determine the oxygen partial pressure (P_{O_2}), the oxygen content and haemoglobin levels.

Hypoxia. Eel or trout blood was equilibrated with hypoxic gas mixtures for 30 min to achieve approximately 50 % haemoglobin O_2-saturation (see Table 1). For eel, this was accomplished using a mixture of 0.24 % CO_2, 1.76 % O_2 and 98 % N_2 while for trout a mixture of 0.24 % CO_2, 2.76 % O_2 and 97 % N_2 was used; the mixtures were provided by Wösthoff gas-mixing pumps. The choice of these levels of hypoxia was based on the results of a study (Perry and Reid, 1992) which demonstrated that both eel and trout release catecholamines into the circulation during acute hypoxia when haemoglobin O_2-saturation decreases to about 50 % saturation *in vivo*. The intensity of catecholamine-stimulated Na^+/H^+ exchange and cyclic AMP accumulation were measured in an identical fashion to normoxia experiments (see above). In addition, the extent of adrenergic cell swelling was assessed in a separate series of experiments by determining the ratio of erythrocyte water content to dry mass (Motais *et al.* 1987) in control erythrocytes and after addition of 50 or 500 nmol l^{-1} noradrenaline (final nominal concentrations).

Series 2. Characterization of β-adrenoceptors

The goal of these experiments was to determine whether there were differences in the numbers and/or affinities of erythrocyte surface β-adrenoceptors between eel and trout and to determine the impact of acute hypoxic incubation of the blood in each species.

The characterization of erythrocyte β-adrenoceptors was accomplished on whole blood using a hydrophilic radioligand binding assay (Marttila and Nikinmaa, 1988) that can distinguish high-affinity cell surface receptors from low-affinity cytosolic receptors (André *et al.* 1981; Reid *et al.* 1991).

In one set of experiments, the characterization of β-adrenoceptors was

performed immediately after removal of the blood from the animal. In another, the characterization of β-adrenoceptors was performed after the blood had been handled, maintained and gassed in an identical way (normoxia or hypoxia) to the blood used for the determination of erythrocyte adrenergic responsiveness (series 1).

Analytical procedures

Series 1

Blood P_{O_2} and pH were measured with Radiometer oxygen (model E5406) and pH (model G299A) electrodes housed in cuvettes at ambient temperature and utilized in conjunction with a PHM-71 acid–base analyzer (Radiometer). Total oxygen content was determined on 20 μl samples using the method of Tucker (Tucker, 1967). Blood haemoglobin levels were measured on 20 μl samples using a commercial spectrophotometric assay (Sigma).

The change in pHe (ΔpHe) elicited by addition of noradrenaline was determined as the difference between pHe prior to noradrenaline addition and the maximal reduction in pHe following stimulation. The measured values of ΔpHe were corrected for differences in blood haemoglobin concentration according to Thomas *et al.* (1991). Thus, all results have been expressed on the basis of a blood haemoglobin concentration of 10 g 100 ml^{-1}.

Erythrocyte cyclic AMP content was determined on 40 μl of packed erythrocytes, obtained by centrifugation (12 000 *g*, 2 min), according to the protocol of a commercially available radioimmunoassay (Amersham).

Erythrocyte swelling was determined by measuring the changes in cell water content using the method of Motais *et al.* (1987). Blood was collected, stored and gassed as described above. Samples (300 μl) were taken from a pool and placed in round-bottomed flasks to which noradrenaline (20 μl) was then added to achieve final nominal concentrations of zero (additions of saline), 50 and 500 nmol l^{-1}. As erythrocyte swelling is a progressive process following activation of Na^+/H^+ exchange, an exposure time of 30 min was chosen to improve the resolution of the assay. After 30 min, a 200 μl sample was centrifuged (20 000 *g*, 10 min; Sorval RC2B centrifuge) in a pre-dried centrifuge tube (Eppendorf) and the plasma discarded. The packed erythrocytes were then weighed, dried to a constant mass and re-weighed. Cell swelling was calculated and expressed as grams of erythrocyte water per gram of erythrocyte solid. Samples were made in triplicate from six different pools of blood (N=6).

Series 2

Blood samples were placed within heparinized round-bottomed flasks (100 i.u. ml^{-1} blood; ammonium heparin) and placed on ice. The blood was frequently swirled prior to and during radioligand assays to prevent erythrocytes from settling. Radioligand binding was initiated by the addition of 40 μl of blood to 160 μl of physiological saline (Wolf, 1963) to which the radioligand (±)-3,4-(3-*t*-

butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one (^{3}CGP 12177; hereafter referred to as CGP; 5–40 nmol l^{-1}) had been added alone or in combination with 200 μmol l^{-1} (−)-isoproterenol (Sigma). The number of erythrocytes added to the incubation solution was determined by diluting 10 μl of blood in 10 ml saline and then counting the numbers of erythrocytes present using a haemocytometer (American Optical).

Incubations were stopped, after a 45 min radioligand incubation, by transferring the erythrocytes to borosilicate filters (no. 32, Mandel Scientific), using a cell membrane harvester (Brandell 24R) and four subsequent washings with 5 ml of ice-cold saline. The filters were placed into glass vials containing 8 ml of fluor (ACS II, Amersham) and allowed to stand in the dark for at least 24 h before being counted. The sample radioactivity was then 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 (B_{max}) was then converted to, and expressed on, a binding site per erythrocyte basis by multiplying the maximal number of specific binding sites (disints min^{-1} $cell^{-1}$) by the radioligand specific activity and Avogadro's number. In accordance with our previously reported findings (Reid *et al.* 1991), isoproterenol-displaceable CGP binding sites are hereafter referred to as erythrocyte *surface* β-adrenoceptors.

Statistical analysis

Values shown in tables and figures are means ±1 standard error of the mean (S.E.M.). Statistical differences between means were determined by analysis of variance (ANOVA) followed by Fisher's LSD multiple comparison test, using a commercial statistical software package (Statview 512$^+$); 95 % was accepted as the level of confidence.

Results

Series 1. Adrenergic responsiveness of erythrocytes

Under normoxic conditions, erythrocytes from rainbow trout displayed a pronounced adrenergic activation of Na^+/H^+ exchange *in vitro* at physiologically relevant levels of noradrenaline, as indicated by the peak reductions in pHe after addition of catecholamine (Fig. 1A). Stimulation of the Na^+/H^+ exchanger was apparent at the lowest concentration of noradrenaline utilized (50 nmol l^{-1}). The activation of the Na^+/H^+ exchanger demonstrated dose-dependency between 50 and 250 nmol l^{-1}. At higher concentrations of noradrenaline (500–1000 nmol l^{-1}), no further changes in pHe were recorded (Fig. 1A). In contrast to the marked responsiveness of trout erythrocytes, red cells from American eel displayed a total lack of response to catecholamine under normoxic conditions, even at the highest concentration of noradrenaline employed (Fig. 1B).

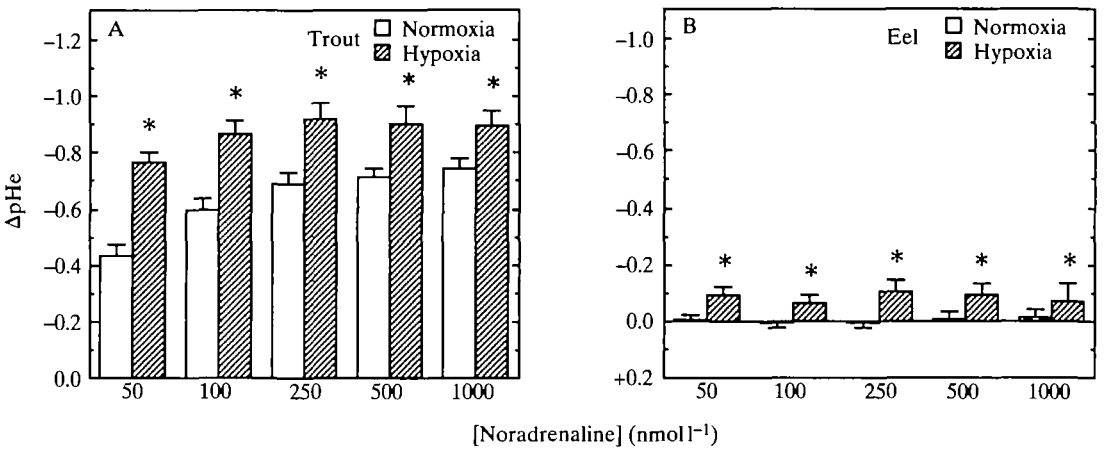

Fig. 1. A comparison of adrenergic activation of erythrocyte Na^+/H^+ exchange in (A) rainbow trout (*Oncorhynchus mykiss*) and (B) American eel (*Anguilla rostrata*). Na^+/H^+ exchange activity was assessed by monitoring the reductions in whole-blood pH (ΔpHe) *in vitro* 5 min after addition of noradrenaline (50–1000 nmol l^{-1} final nominal concentrations) to whole blood equilibrated either under normoxic (open histograms) or hypoxic (hatched histograms) conditions. All values shown are means±1 S.E.M. (N=6); * indicates a significant difference (P<0.05) from the corresponding normoxic value.

Table 1. *A summary of selected respiratory and acid–base variables in blood pooled from either American eel (*Anguilla rostrata*) or rainbow trout (*Oncorhynchus mykiss*) stored on ice for 4 h and subsequently gassed with either normoxic or hypoxic gas mixtures*

	Normoxia		Hypoxia	
	American eel	Rainbow trout	American eel	Rainbow trout
pHe	8.12±0.02	7.99±0.02	8.04±0.03	8.01±0.03
P_{O_2} (kPa)	17.9±0.2	18.1±0.5	1.9±0.1	3.1±0.5
Total O_2 (ml 100 ml^{-1})	6.54±0.6	6.79±0.7	3.40±0.4	3.20±0.3
[Haemoglobin] (g 100 ml^{-1})	5.53±0.4	6.01±0.4	5.57±0.4	5.98±0.4
[Total O_2]/[Haemoglobin] (ml g^{-1})	1.21±0.05	1.13±0.06	0.60±0.04	0.54±0.06

Values shown are means±1 S.E.M.; N=6.

Equilibration of blood with hypoxic gas mixtures, to reduce haemoglobin O_2-saturation to approximately 50 % as indicated by the [total O_2]/[haemoglobin] ratio (Table 1), increased the responsiveness of the Na^+/H^+ exchanger of trout erythrocytes to noradrenaline (especially at the lower concentrations of noradrenaline; Fig. 1A) and initiated adrenergic activation of H^+ extrusion in eel erythrocytes (Fig. 1B). Under hypoxic conditions, the adrenergic activation of

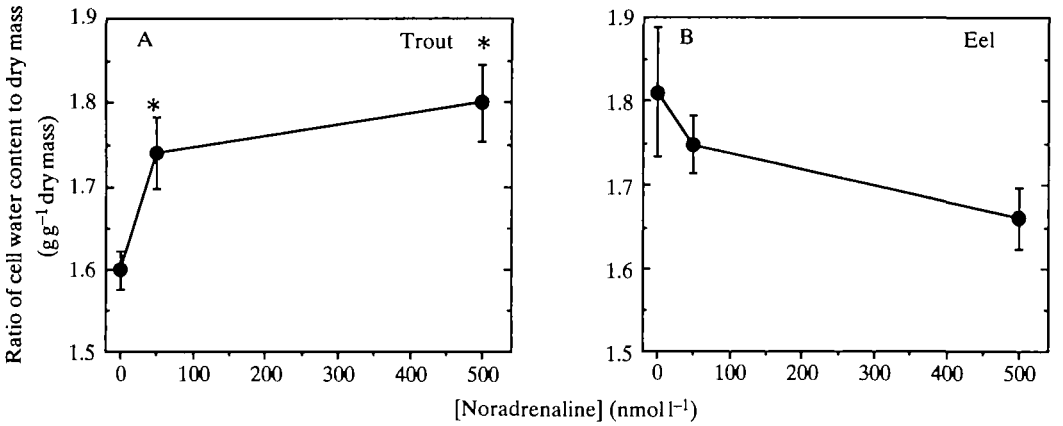

Fig. 2. A comparison of adrenergic erythrocyte swelling in (A) rainbow trout (*Oncorhynchus mykiss*) and (B) American eel (*Anguilla rostrata*). Erythrocyte swelling was assessed by monitoring the changes in the ratio of cell water content ($g H_2O$) to dry mass (g) *in vitro* after addition of either 50 or 500 nmol l^{-1} noradrenaline final nominal concentration) to whole blood equilibrated under hypoxic conditions. All values shown are means±1 S.E.M. (N=6); * indicates a significant difference (P<0.05) from the corresponding control value (zero nominal noradrenaline concentration).

Na^+/H^+ exchange in eel was approximately 10-fold less than in trout, when compared by using the extent of the pHe disequilibrium as an indicator (compare Fig. 1A and B).

Catecholamine-mediated cell swelling was estimated by determining the ratio of erythrocyte water content to dry mass using concentrations of noradrenaline previously shown to evoke activation of Na^+/H^+ exchange (Fig. 1). Under hypoxic conditions, rainbow trout erythrocytes underwent significant swelling after the addition of either 50 or 500 nmol l^{-1} noradrenaline (Fig. 2A). Eel erythrocytes, in contrast, did not exhibit swelling and indeed there was a trend (not statistically significant) towards a reduction in cell volume after the addition of noradrenaline (Fig. 2B).

Both eel and trout erythrocytes displayed increased levels of cyclic AMP after the addition of increasing concentrations of noradrenaline (Fig. 3). In trout, a statistically significant response was observed at concentrations above 100 nmol l^{-1} (Fig. 3A) whereas in eel a significant response was detected at a concentration of only 50 nmol l^{-1} (Fig. 3B). In both eel and trout, equilibration of the blood under hypoxic conditions provoked larger adrenergic increases in intracellular cyclic AMP levels in comparison to the normoxic situation (Fig. 3). Surprisingly, eel erythrocytes accumulated significantly larger quantities of cyclic AMP than did trout erythrocytes at all concentrations of noradrenaline employed.

Series 2. Characterization of β-adrenoceptors

Erythrocytes from American eel possessed 1780±310 surface β-adrenoceptors

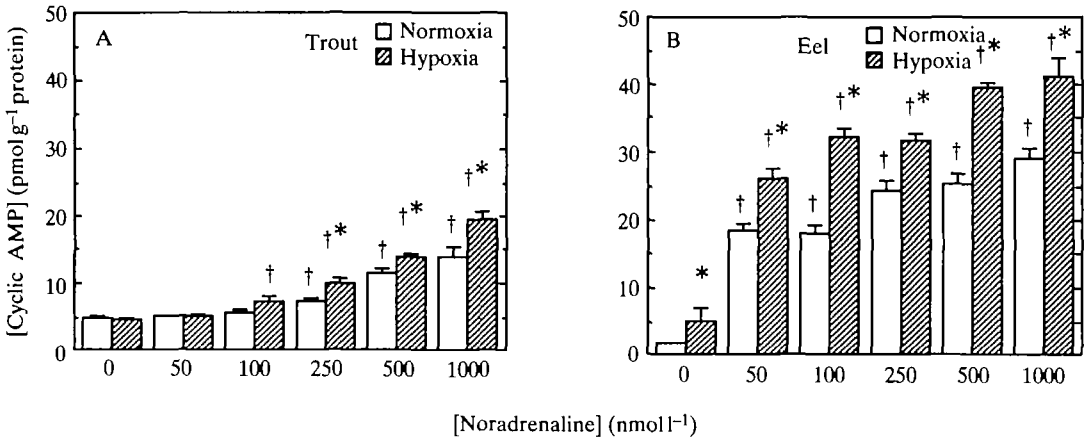

Fig. 3. A comparison of adrenergic accumulation of cyclic AMP *in vitro* in erythrocytes of (A) rainbow trout (*Oncorhynchus mykiss*) and (B) American eel (*Anguilla rostrata*). Cyclic AMP levels were monitored 5 min after the addition of noradrenaline (50–1000 nmol l^{-1} final nominal concentration) to whole blood equilibrated either under normoxic (open histograms) or hypoxic (hatched histograms) conditions. All values shown are means±1 S.E.M. (N=6); * indicates a significant difference (P<0.05) from the corresponding normoxic value. † indicates a significant difference (P<0.05) from the corresponding control value (zero final nominal noradrenaline concentration).

(isoproterenol-displaceable CGP binding sites) per cell or approximately 50 % of the numbers detected on the surface of rainbow trout erythrocytes (3713±627). The β-adrenoceptors from eel and trout displayed similar affinities: 12.3±2.8 nmol l^{-1} for eel (N=6) and 10.6±2.7 nmol l^{-1} for trout (N=6).

In a separate series of experiments, the blood was treated in an identical manner to that in series 1 in order to compare the adrenergic responsiveness of the erythrocytes with the numbers and affinities of surface β-adrenoceptors. These experiments revealed (i) that storage of the blood on ice for 4 h reduced the numbers of β-adrenoceptors in both eel and trout erythrocytes, (ii) that erythrocytes from trout possessed greater numbers of β-adrenoceptors (although this was statistically significant only under hypoxic conditions; Table 2) and (iii) that hypoxia did not significantly influence the numbers or affinities of the receptors.

Discussion

The results of previous studies have shown that β-adrenoceptor-mediated accumulation of intracellular cyclic AMP is a prerequisite for adrenergic activation of teleost erythrocyte Na^+/H^+ exchange (Mahe *et al.* 1985; Salama and Nikinmaa, 1990; Reid and Perry, 1991). In the present study, we have clearly demonstrated that eel erythrocytes possess significantly fewer surface β-adrenoceptors yet they accumulate greater quantities of cyclic AMP than do trout erythrocytes after exposure to physiologically relevant concentrations of the naturally occurring

Table 2. *A comparison of the numbers (B_{max}) and dissociation constants (K_D) of erythrocyte surface β-adrenoceptors (isoproterenol-displaceable CGP binding sites) in blood withdrawn from American eel (*Anguilla rostrata*) and rainbow trout (*Oncorhynchus mykiss*), kept on ice for 4 h, and then equilibrated with either normoxic or hypoxic gas mixtures*

	Normoxia		Hypoxia	
	American eel	Rainbow trout	American eel	Rainbow trout
B_{max} (receptors erythrocyte^{-1})	980±296	1620±724	752±198*	2465±993
K_D (nmol l^{-1})	8.1±1.5	7.3±0.9	6.9±1.3	7.4±0.9

All values shown are means±1 S.E.M.; N=6.
* indicates a significant difference from the value for trout (P<0.05).

catecholamine noradrenaline. Thus, the absence of adrenergic Na^+/H^+ exchange during normoxia and the attenuated response (in comparison to rainbow trout) under hypoxic conditions is probably not the result of insufficient numbers of β-adrenoceptors or of the uncoupling of these receptors from adenylate cyclase, as we had originally proposed (see Introduction). Instead, the differences between eel and trout may arise from differences in the number of the Na^+/H^+ exchangers or differences in the manner by which these exchangers are activated by cyclic AMP.

This is only the second direct assessment of erythrocyte β-adrenoceptor numbers and affinities in eel. Bennett and Rankin (1985) first established the presence of erythrocyte β-adrenoceptors in a related species (European eel; *Anguilla anguilla*). However, owing to differences in methodology and choice of radioligand (Bennett and Rankin, 1985, used [^{3}H]dihydroalprenolol on membrane preparations), comparisons between the two studies would be inappropriate. The eel erythrocyte β-adrenoceptors identified in the present study possessed similar affinities to the trout erythrocyte receptors although there were significantly fewer of them. In both species, these receptors were apparently coupled to adenylate cyclase because significant quantities of cyclic AMP were formed after incubation with noradrenaline. Interestingly, the results of the study of Bennett and Rankin (1985) suggested that the β-adrenoceptors of European eel erythrocytes were of the β_2 subtype, based on comparisons of the binding of radiolabelled adrenaline and noradrenaline. In rainbow trout, it is generally accepted that the erythrocytes possess adrenoceptors of the β_1 subtype (Tetens *et al.* 1988). To explain the lack of responsiveness of eel erythrocytes, Hyde and Perry (1990) proposed that only the β_1 adrenoceptors are capable of modulating the erythrocyte pH regulatory response. This explanation, however, cannot account for the attenuated adrenergic activation of the Na^+/H^+ exchanger in eel because stimulation of the β-adrenoceptors (regardless of their subtype) provoked marked increases in the levels of cyclic AMP.

The observation that eel erythrocytes accumulated greater quantities of cyclic AMP after adrenergic stimulation than did trout erythrocytes, despite smaller numbers of surface β-adrenoceptors, was unexpected given the direct relationship between the numbers of surface β-adrenoceptors and erythrocyte adrenergic responsiveness previously established for rainbow trout (Reid and Perry, 1991). This finding may reflect fundamental interspecific differences at various steps in the β-adrenergic signal transduction pathway. The possibilities include differences in the activities of adenylate cyclase, the enzyme catalyzing the conversion of ATP to cyclic AMP, or phosphodiesterase, the enzyme that catalyses the hydrolysis of cyclic AMP to AMP.

The primary technique used in the present study to assess activity of the Na^+/H^+ exchanger was to monitor the changes in pHe after addition of catecholamine. The peak change in pHe is a reliable indicator of the extent of the pH disequilibrium occurring in the plasma as a result of the abrupt and massive extrusion of H^+ from the erythrocyte (see discussion in Thomas *et al.* 1991). Owing to the absence of extracellular carbonic anhydrase, a disequilibrium develops because the extruded protons combine slowly with plasma HCO_3^- at the uncatalyzed rate. The absolute magnitude of the pHe disequilibrium will depend on several factors including (i) plasma buffering capacity, (ii) the activity of the Cl^-/HCO_3^- exchanger on the erythrocyte membrane, and (iii) the degree of activation of the Na^+/H^+ exchanger. Hyde *et al.* (1987) determined plasma buffering capacity in American eel to be $-2.73\,mmol\,l^{-1}$, a value similar to that found in most teleosts. Thus, unusually high plasma buffering capacity cannot explain the absence of a significant pHe disequilibrium in the plasma after adrenergic stimulation of the erythrocytes. We are unaware of any studies that have compared the activities of the Cl^-/HCO_3^- exchanger between trout and eel erythrocytes. An unusually low activity of the Cl^-/HCO_3^- exchanger would be expected to increase the extent of the pHe disequilibrium after adrenergic activation of Na^+/H^+ exchange, as previously shown in trout erythrocytes pretreated with the anionic exchange blockers 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) (Perry *et al.* 1991) or 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) (Cossins and Kilbey, 1989). This is because the usual titration of H^+ (originating from Na^+/H^+ exchange) by HCO_3^- (originating from Cl^-/HCO_3^- exchange) would be reduced. In contrast, an unusually high activity of Cl^-/HCO_3^- exchange would be expected to reduce the magnitude of the pHe disequilibrium after adrenergic stimulation although activation of the Na^+/H^+ exchanger would be sustained for longer periods owing to CO_2 recycling between the plasma and the erythrocyte (Motais *et al.* 1989). Thus, low Cl^-/HCO_3^- exchange activity would clearly reduce the extent of adrenergic cell swelling (the net result of inward H_2O movement owing to sustained Na^+ and Cl^- entry into the cell) after stimulation of Na^+/H^+ exchange, as was experimentally demonstrated for trout erythrocytes by Nikinmaa *et al.* (1987) using anionic exchange inhibitors. Conversely, unusually high Cl^-/HCO_3^- exchange activity would enhance adrenergic erythrocyte swelling. Obviously, the inability to detect significant Na^+/H^+

exchange activity in eel erythrocytes, using the extent of the pHe disequilibrium or cell swelling as indices, cannot be attributed to abnormal activity of the Cl^-/HCO_3^- exchanger because the rate of Cl^-/HCO_3^- exchange affects these indices in the opposite direction. Thus, we are confident of the conclusion from this and from previous studies (Hyde and Perry, 1989, 1990) that the erythrocytes of American eel display extremely reduced (if not entirely absent) activity of Na^+/H^+ exchange after adrenergic stimulation, despite the pronounced increases in cyclic AMP levels.

The total absence or marked attenuation of adrenergic activation of Na^+/H^+ exchange in eel erythrocytes is unlikely to be a consequence of insufficient cell surface β-adrenoceptors, despite their lower numbers, because of the pronounced accumulation of cyclic AMP at all the levels of noradrenaline utilized (Fig. 3B). We suggest, therefore, that eel erythrocytes lack appreciable numbers of the Na^+/H^+ exchangers themselves, or that these exchangers require much larger quantities of cyclic AMP (in comparison to trout) for their activation.

Adrenergic activation of erythrocyte Na^+/H^+ exchange was enhanced under hypoxic conditions in trout and initiated to a slight degree in eel (Fig. 1). These results confirm previous studies that have demonstrated a positive influence of hypoxia on catecholamine-mediated erythrocyte responses (Motais *et al.* 1987; Salama and Nikinmaa, 1988, 1990; Reid and Perry, 1991). Several mechanisms are believed to be responsible for the enhancement of Na^+/H^+ exchange during acute hypoxia *in vitro* including (i) increased numbers of cell surface β-adrenoceptors (Marttila and Nikinmaa, 1988; Reid and Perry, 1991) as a result of rapid mobilization of nascent cytosolic receptors (Reid and Perry, 1991), (ii) increased responsiveness of the Na^+/H^+ exchanger to intracellular cyclic AMP (S. D. Reid, Y. LeBras and S. F. Perry, unpublished observations), and (iii) a possible linkage between haemoglobin structure, which is affected by oxygenation, and the Na^+/H^+ exchanger (Motais *et al.* 1987). In the present study, both trout and eel erythrocytes produced significantly greater quantities of cyclic AMP during hypoxia yet neither the number nor the affinity of the cell surface β-adrenoceptors was altered. In trout there was a trend towards increased numbers of β-adrenoceptors (Table 2) and it would seem that the unusually high variability of the data obscured any statistically significant differences that were apparent in previous studies (Marttila and Nikinmaa, 1988; Reid and Perry, 1991). However, there was clearly no trend towards increasing numbers or altered affinities of β-adrenoceptors in eel during hypoxia (Table 2), so an alternative explanation must be sought for the increased adrenergic accumulation of cyclic AMP during hypoxia (Fig. 3). An obvious possibility is that the activity of adenylate cyclase is specifically increased during hypoxia. However, our recent studies on rainbow trout erythrocytes do not support this contention because application of the adenylate cyclase activator forskolin caused identical elevations of intracellular cyclic AMP levels (S. D. Reid, Y. LeBras and S. F. Perry; unpublished observations) under normoxic and hypoxic conditions. Alternative possibilities are (i) that hypoxia in some way affects the β-adrenoceptor/G-protein interaction

so as to increase the activity of adenylate cyclase indirectly and/or (ii) that the metabolic degradation of cyclic AMP is reduced by hypoxia.

An obvious question that arises from this study pertains to the physiological significance of the β-adrenoceptor-mediated rise in cyclic AMP levels in eel erythrocytes, since there appears to be little, if any, coupling of this response to activation of Na^+/H^+ exchange. Indeed, there is no evidence to support adrenergic regulation of erythrocyte intracellular pH or blood oxygen content during periods of catecholamine mobilization *in vivo* (Hyde and Perry, 1989, 1990). Nevertheless, the presence of a β-adrenergic signal transduction system in erythrocytes of American eel is clearly indicated by the present study and its physiological role remains to be elucidated.

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