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CATECHOLAMINE-ACTIVATED SODIUM/PROTON EXCHANGE IN THE RED BLOOD CELLS OF THE MARINE TELEOST *GADUS MORHUA*

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Summary

The effects of catecholamines on the pH and the cellular ion and water content were investigated in red blood cells from the Atlantic cod (*Gadus morhua*). Noradrenaline induced a rapid decrease in the extracellular pH (pHe) of red blood cells suspended in a CO_2/bicarbonate or in a CO_2/bicarbonate-free buffer system. The noradrenaline-induced changes in pHe were a saturable function of the external sodium ion concentration and were inhibited by amiloride but not by DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, final concentration of both 10^{-4} mol l^{-1}). The catecholamine-induced extracellular acidification was accompanied by an intracellular alkalization and protons were moved from their electrochemical equilibrium. Proton extrusion was associated with an increase in the red blood cell sodium and chloride concentrations. In the presence of DIDS, the chloride movements were blocked and the net proton efflux under these conditions matched the net sodium influx. The results strongly suggested the activation of a sodium/proton exchanger by catecholamines in the red blood cells of the Atlantic cod. The red blood cell receptor affinity for adrenaline was three times higher than that for noradrenaline. Comparison with data in the literature for *in vivo* catecholamine concentrations indicated that adrenaline was more effective than noradrenaline in activating the red blood cell sodium/proton exchanger in the Atlantic cod *in vivo*.

Introduction

Protons are usually passively distributed across the red blood cell membranes of both freshwater and marine teleost fishes, as shown for carp (Albers and Goetz, 1985), rainbow trout (Heming *et al.* 1986) and Atlantic cod (Berenbrink and Bridges, 1994). However, in the red blood cells of the rainbow trout, catecholamines activate a sodium/proton exchanger (Nikinmaa and Huestis, 1984; Baroin *et al.* 1984; Cossins and Richardson, 1985). The resulting net proton efflux shifts the equilibrium between

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carbonic acid and bicarbonate inside the red blood cell towards the latter, bicarbonate leaving the red blood cell in exchange for chloride. As a result of the intracellular sodium and chloride accumulation, the red blood cells swell due to osmotically induced water uptake (Borgese *et al.* 1987). The activation of the sodium/proton exchanger has important physiological consequences. Facing extracellular acidosis, the catecholamine-induced proton efflux and the resulting intracellular alkalization will increase haemoglobin O_2-affinity and offset the potentially detrimental Bohr and Root effects associated with decreased pHe. Catecholamine-induced changes in red blood cell ion and water content as well as changes in pHi, pHe and P_{50} have been reported in many teleosts other than rainbow trout (Nikinmaa and Huestis, 1984; Powers *et al.* 1986; Ferguson and Boutilier, 1988; Salama and Nikinmaa, 1988, 1989; Fuchs and Albers, 1988; Tufts and Randall, 1989; Cossins and Kilbey, 1991). However, none of these numerous studies has shown whether the changes observed on catecholamine addition were due to the same mechanism as in the rainbow trout. In addition, most of the studies have been carried out on freshwater or anadromous fish species. Recently, we reported a sodium-dependent pHi regulatory mechanism in the red blood cells of the marine Atlantic cod (Berenbrink and Bridges, 1994). This mechanism has not yet been described for teleost red blood cells and was not through sodium/proton exchange. It was, therefore, of particular interest to determine whether Atlantic cod red blood cells also possess a catecholamine-activated sodium/proton exchanger.

The present study examined the influence of catecholamines on the red blood cells from a fully marine teleost, the Atlantic cod. The effects of catecholamines on pHe, pHi and red blood cell ion and water content have been studied and, in order to elucidate the mechanisms of catecholamine action, the sodium-dependence and the influence of DIDS and amiloride on the catecholamine-induced processes have been investigated. The red blood cell receptor affinities for adrenaline and noradrenaline were determined and are discussed in view of the elevated *in vivo* plasma catecholamine concentrations in Atlantic cod under stressful conditions (Butler *et al.* 1989; Fritsche and Nilsson, 1990; Perry *et al.* 1991).

Materials and methods

Animal collection and preparation of red blood cells

Atlantic cod, *Gadus morhua* (200–1000 g), were caught in February/March (winter animals) in the German Bight near Helgoland and kept in running, aerated sea water at 8–10 ˚C at the Marine Biological Station, Helgoland. Animals were held in large aquaria for between 1 and 3 weeks before they were used in the experiments. They were offered small pieces of fish but, during winter, feeding behaviour was at a minimum. Summer animals were collected and maintained as described by Berenbrink and Bridges (1994). Blood sampling and the preparation of red blood cells was carried out as described earlier (Berenbrink and Bridges, 1994).

Experimental protocol

Washed red blood cells (1.6–3.2 ml, haematocrit 20 %) were equilibrated for 20 min at

15 °C with a humidified 1 %CO_2/99 % air mixture (P_{CO_2} 1.0 kPa) supplied by a gas-mixing pump (2M303/a-F, Wösthoff KG, Bochum, Germany) in an intermittently rotating tonometer (Zentralwerkststatt für Biologie, Universität Düsseldorf). An 800 μl blood sample was then taken to measure control values for pHe, pHi and red blood cell ion and water content. (−)Noradrenaline was then added (final concentration 10^{-5} mol l^{-1}) as the (+)bitartrate salt dissolved in water (Sigma Chemie, München, Germany) and another 800 μl sample of the red blood cell suspension was sampled 3, 10 or 20 min following the addition of noradrenaline.

Studies with ion transport inhibitors

The effects of the ion transport inhibitor DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, Sigma Chemie, München, Germany; dissolved in dimethylsulphoxide, final concentration 10^{-4} mol l^{-1}) were studied in the following manner. 4 ml samples of the red blood cell suspension (haematocrit 20 %) were pre-equilibrated for 60 min with a 1 % CO_2/99 % air mixture and then divided into four equal subsamples. The first subsample served as a control and the other three were incubated for another 10 min in the presence of DIDS, noradrenaline (10^{-5} mol l^{-1}) or noradrenaline plus DIDS. To test the effects of amiloride (Sigma Chemie, München, Germany; dissolved in dimethylsulphoxide), 2 ml samples of a red blood cell suspension were equilibrated as described above either in the presence or in the absence of amiloride (final concentration 10^{-4} mol l^{-1}). After 20 min of equilibration, the respective control samples were taken and noradrenaline was added (10^{-5} mol l^{-1}). Following another 10 min incubation period, samples of stimulated red blood cells were taken.

The influence of sodium

The influence of extracellular sodium on catecholamine-induced proton extrusion was tested in bicarbonate-free solutions to inhibit sodium-, chloride- and bicarbonate-dependent acid extrusion (Berenbrink and Bridges, 1994). Red blood cells were washed three times with salines in which different amounts of sodium chloride had been replaced by choline chloride. The composition of the saline was: (in mmol l^{-1}) NaCl, 0–144; choline chloride, 144–0; KCl, 6; $CaCl_2$, 5; $MgSO_4$, 1; D-glucose, 5; and Hepes, 10; adjusted to pH 7.7 with KOH. After adjusting the haematocrit value to 20 %, 3 ml samples were first equilibrated with air for 30 min followed by equilibration with pure nitrogen for 45 min to achieve nominally CO_2/bicarbonate-free conditions. As soon as the pHe was constant, noradrenaline (10^{-5} mol l^{-1}) was added and the pHe was measured every 2–3 min for up to 20 min.

Dose–response curves

The dose–response curves for the effect of adrenaline and noradrenaline on pHe were obtained as follows. 4–6 ml of the red blood cell suspension (haematocrit 20 %) was equilibrated with a 1 % CO_2/99 % air mixture for 45 min. From this suspension, 480 μl samples were transferred to a second tonometer vessel with the same equilibration conditions and incubated for a further 15 min. At this time, 20 μl of distilled water (control) or a freshly prepared (−)adrenaline [(+) bitartrate salt, dissolved in water] or

noradrenaline solution was added to give final nominal concentrations ranging from 10^{-10} to 10^{-4} mol l^{-1}. The changes in the pHe of the suspension were monitored over 15 min.

Analytical procedures

Determinations of haematocrit, pHe, pHi and red blood cell ion and water content were carried out as described previously (Berenbrink and Bridges, 1994). The red blood cell water content is expressed as a percentage, and intracellular ion concentrations in mmol l^{-1} red blood cell water. Net fluxes are expressed as μmol g^{-1} dry cell solids min^{-1}.

Calculations and statistics

The transmembrane distribution ratio for protons (rH^+) was calculated from pHe and pHi: $rH^+=[H^+]_e/[H^+]_i=10^{(pHi-pHe)}$. Lines were fitted to the data sets using a curve-fitting programme (SigmaPlot 4.1, Jandel Scientific, Corte Madera, USA) and appropriate polynomial, hyperbolic and exponential functions. The concentrations giving 50 % responses (the EC_{50} values) of adrenaline and noradrenaline were calculated from the log-transformed data of the dose–response curves using a Hill equation as described by Tetens *et al.* (1988). Differences between two linear regression lines were evaluated by analysis of covariance and the corresponding *F*-test. Unless otherwise indicated, all values are given as the mean ± S.D. Differences between means were statistically evaluated with Student's *t*-test for independent samples. $P<0.05$ was taken as the significance level for both the *F*-test and the Student's *t*-test.

Results

When compared with control values, addition of noradrenaline to Atlantic cod red blood cells caused an immediate (within the first minute) and significant decrease in pHe (Fig. 1). A minimum pHe value of 7.52 was reached 7 min after the injection and this remained stable during the remaining incubation period. Adrenaline also elicited a pronounced acidification of the external medium. The effects of increasing doses on the relative proton extrusion were similar for both catecholamines (Fig. 2A). Changes in catecholamine concentrations between 10^{-8} mol l^{-1} and 10^{-6} mol l^{-1} caused correspondingly increased extracellular acid–base perturbation (Fig. 2A). Above this range, no further stimulation was achieved. The Hill plot (for responses in the range 5–95 %) yielded different regression lines for adrenaline and noradrenaline with the line for adrenaline being significantly transposed to the left, to a lower hormone concentration. The concentrations for a 50 % response, EC_{50}, were 4.7×10^{-8} mol l^{-1} for adrenaline and 1.44×10^{-7} mol l^{-1} for noradrenaline, indicating a threefold higher affinity of the red blood cell receptors for adrenaline than for noradrenaline.

The effect of catecholamines on pHe was also seen in red blood cells obtained from summer Atlantic cod. Under the same experimental conditions, incubation with noradrenaline (10^{-5} mol l^{-1}) caused an extracellular acidification of a similar magnitude to that for red blood cells obtained from winter animals (after 10 min, $\Delta pHe=-0.072\pm0.009$, $N=9$, in summer and $\Delta pHe=-0.075\pm0.011$, $N=5$, in winter).

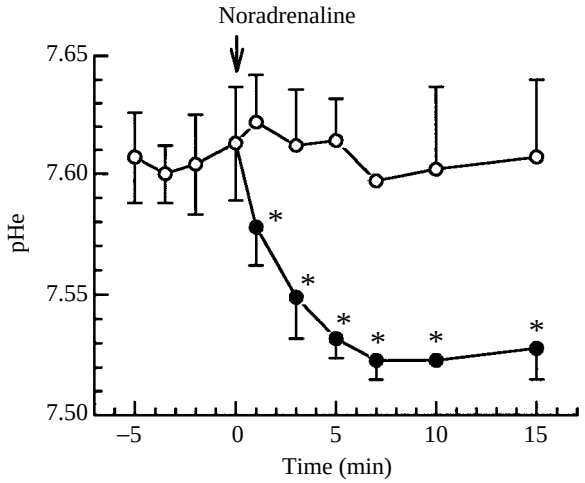

Fig. 1. Time course of changes in pHe of Atlantic cod red blood cells incubated in the absence (open circles) or presence (filled circles) of saturating doses of noradrenaline (10^{-4} to 10^{-6} mol l^{-1} final concentration, added at time zero). Each symbol represents the mean ± S.D. of 3–7 equilibrations. Data are from 10 individual animals; an asterisk denotes a significant difference between noradrenaline-treated cells and control cells.

The effect of external sodium on the proton extrusion observed under catecholamine stimulation was tested by substituting choline for sodium. At constant catecholamine concentration, decreasing extracellular sodium concentrations led to gradually smaller changes in the pHe (Fig. 3A). No changes in pHe could be elicited by noradrenaline with sodium nominally absent from the external medium. The noradrenaline-induced acidification increased as a function of the external sodium concentration and approached an asymptote at the highest external sodium concentrations tested (Fig. 3B). A half-maximal response was achieved at an extracellular sodium concentration of 25 mmol l^{-1}.

The sodium-dependence of the catecholamine-induced extracellular acidification was further investigated using amiloride as an inhibitor of sodium transport. Addition of noradrenaline to control red blood cells elicited a pHe decrease with $\Delta pHe = -0.075 \pm 0.011$ pH units (N=5). In the presence of amiloride (10^{-4} mol l^{-1}), the same dose of noradrenaline had no significant effect on pHe, $\Delta pHe = -0.024 \pm 0.26$ (N=5). Incubation with amiloride alone had no significant effect on pHe. Thus, the catecholamine-induced acidification of the extracellular medium was strongly dependent upon extracellular sodium and was blocked by amiloride.

The addition of catecholamines to red blood cell suspensions resulted in intracellular alkalization (Fig. 4A). Three minutes after the addition of noradrenaline, pHe decreased significantly below the initial value, whereas pHi increased only slightly. After 10 min, the red blood cell pH was significantly elevated above the corresponding initial value. As a result of the pH changes, the proton distribution ratio across the red blood cell membrane increased significantly and approached a plateau within 3 min following the addition of catecholamine (Fig. 4B).

Fig. 5 shows the changes in red blood cell ion and water content under various conditions. Apart from its effects on pHe and pHi (Fig. 5A,B), noradrenaline also significantly increased the red blood cell sodium and chloride concentrations

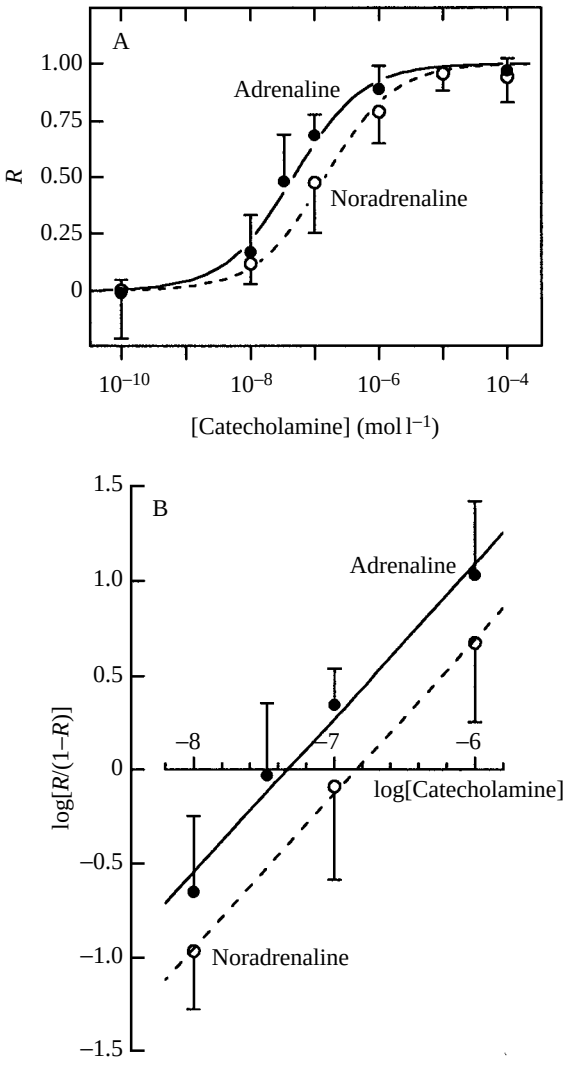

Fig. 2. (A) Dose–response curves for adrenaline (filled circles) and noradrenaline (open circles). The red blood cell response (R) is the change in extracellular proton concentration elicited by a particular dose of catecholamine relative to the maximal change in extracellular proton concentration obtained (assessed after 10 min). Each symbol represents the mean ± S.D. of 3–7 equilibrations, N=5 for both hormones. The lines were calculated from Hill plots of the data for adrenaline (solid line) and noradrenaline (dashed line). (B) Hill plot of the dose–response curves for adrenaline (solid line) and noradrenaline (dashed line). Onl data between 5 and 95 % response levels were included. Equations for the regression lines are: $\log[R/(1-R)]=0.818\log[\text{adrenaline}]+6.00$ (r=0.89, n=17, N=5) and $\log[R/(1-R)]=0.820\log[\text{noradrenaline}]+5.61$ (r=0.88, n=17, N=5), respectively, where n is the number of equilibrations and N the number of individual Atlantic cod.

(Fig. 5D,F). Simultaneously, the red blood cell water content was slightly elevated, indicating cell swelling (Fig. 5C). To test whether the increase in cellular chloride concentration was a necessary component of the catecholamine-activated acid extrusion or whether it was only secondary in nature, the effect of noradrenaline was also assessed

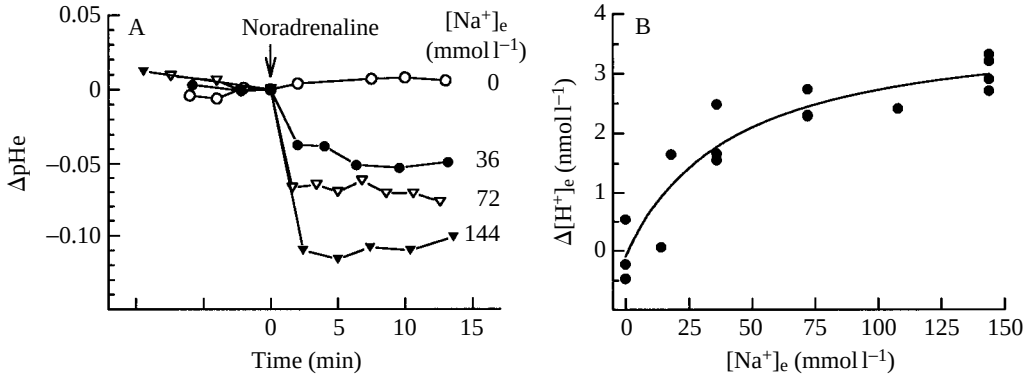

Fig. 3. Sodium-dependence of the noradrenaline-induced acid extrusion in red blood cells of the Atlantic cod (bicarbonate-free media, Hepes buffer under 100 % nitrogen atmosphere). (A) Example of the time course of noradrenaline-induced pHe changes in incubations in which different amounts of sodium have been replaced by choline. Different symbols denote different extracellular sodium concentrations (shown at the right of each curve). (B) Noradrenaline-induced proton extrusion $\Delta[H^+]_e$ after 10 min of incubation as a function of the external sodium concentration $[Na^+]_e$. The data (16 equilibrations, blood of four individuals) were fitted to a hyperbolical saturation curve. At 72 mmol l^{-1} external $[Na^+]$ two values are very close together so that they appear as one symbol (the lower symbol).

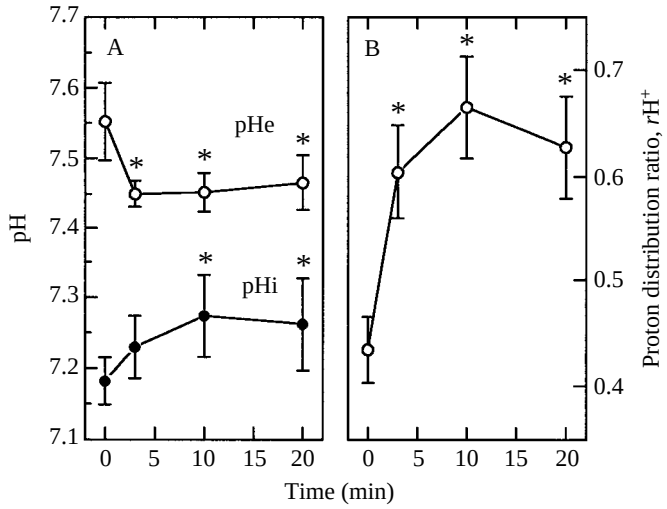

Fig. 4. (A) Noradrenaline-induced changes in pHe and pHi plotted against time. Noradrenaline was added at time zero. Mean values that differ significantly from the value at time zero are denoted by an asterisk; data are from five individuals. (B) Time course of the changes in the proton distribution ratio, rH^+, in the same experiments as in A.

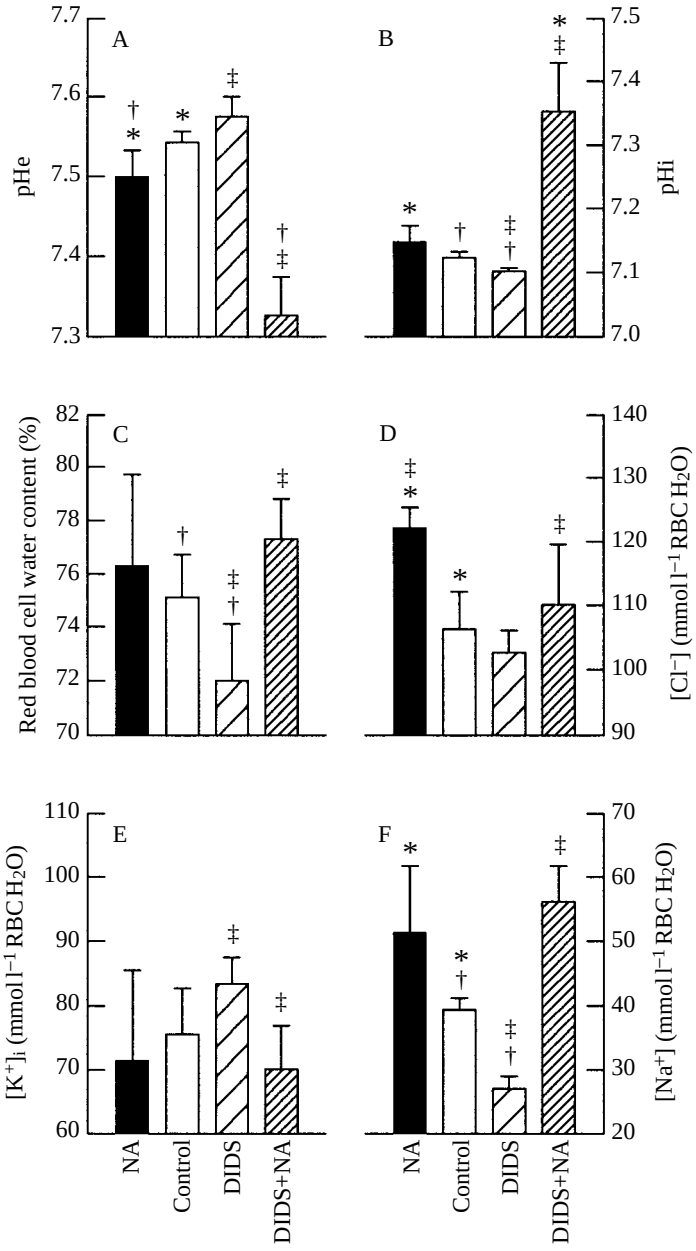

Fig. 5. Effect of noradrenaline, DIDS and DIDS plus noradrenaline on pHe (A), pHi (B), water content (C) and intracellular chloride (D), potassium (E) and sodium (F) concentrations of Atlantic cod red blood cells (RBC). The bars indicate control red blood cells (open) and red blood cells treated with noradrenaline (NA, filled), DIDS (coarse cross-hatched) and DIDS+noradrenaline (fine cross-hatched). In each panel, bars marked by the same symbol differed significantly from each other. Only red blood cells that differed in just one treatment were compared with each other. Data from four individual Atlantic cod.

or whether it was only secondary in nature, the effect of noradrenaline was also assessed in the presence of DIDS.

The values for pHi as well as for the red blood cell water and sodium content were significantly lowered in the presence of DIDS (Fig. 5B,C,F). This was indicative of a DIDS-sensitive mechanism that had already elevated pHi and the red blood cell sodium and water content in the untreated control red blood cells. The inhibitory effect of DIDS excludes this component as residual sodium/proton exchange activity.

DIDS markedly enhanced the noradrenaline-induced changes in pHe and pHi (Fig. 5A,B). At the same time, the combination of DIDS+noradrenaline abolished the net flux of chloride into the red blood cells that was normally observed in the presence of noradrenaline (Fig. 5D). In contrast, similar intracellular sodium and potassium concentrations were found in red blood cells treated with noradrenaline and with DIDS+noradrenaline (Fig. 5E,F). The red blood cell potassium concentration was the reverse of the red blood cell water content under all conditions studied (Fig. 5C,E). The changes in red blood cell potassium concentration are therefore readily explained by the diluting/concentrating effects of the changes in red blood cell water content. This was not, however, the case for the intracellular sodium concentration (Fig. 5F). Noradrenaline stimulated a net sodium influx into control and DIDS-treated red blood cells. The overall noradrenaline-induced increase in intracellular sodium concentration was higher in DIDS-treated cells, since the red blood cell sodium concentration was already decreased in the presence of DIDS. The marked accentuation of the noradrenaline-induced pHe and pHi changes in the presence of DIDS could be explained by the blockage of the red blood cell anion exchanger. This inhibition would hinder rapid bicarbonate and hydroxide movements across the red blood cell membrane, thereby preventing the cycling of proton equivalents across the cell membrane and leading to enhanced changes in pHi and pHe.

In DIDS-treated red blood cells, the backflux of proton equivalents into the red blood cells is thought to be minimal and the magnitude of the catecholamine-induced proton extrusion could be estimated. Taking the extracellular non-bicarbonate buffer into account, the amount of protons extruded was calculated using the Henderson–Hasselbalch equation and the appropriate pK value (Boutilier *et al.* 1985). The proton extrusion was compared with the respective increase in intracellular sodium concentration. Over the limited timescale of 10 min used in these experiments, the influence of the sodium pump on red blood cell sodium concentration was assumed to be negligible. The calculated net proton efflux and the measured net sodium influx after catecholamine addition were 7.5±2.4 and 12.3±3.4 μmol g^{-1} dry cell solids min^{-1}, respectively (N=4). The similar size of these two fluxes suggests a coupled 1:1 movement of these ions across the red blood cell membrane *via* a catecholamine-induced sodium/proton exchanger.

Discussion

The catecholamines noradrenaline and adrenaline activate a proton extrusion mechanism in the red blood cell membrane of the Atlantic cod. The transporter shows

Table 1. *Plasma adrenaline and noradrenaline levels and their effect on the red blood cells of the Atlantic cod,* Gadus morhua *under various experimental conditions*

State of the fish during blood sampling	Measured plasma adrenaline level (nmol l^{-1})	Calculated effect on red blood cell H^+ extrusion (%)*	Measured plasma noradrenaline level (nmol l^{-1})	Calculated effect on red blood cell H^+ extrusion (%)*	Reference
Resting	2.6 (7)	9	3.4 (7)	5	Axelsson and Nilsson (1986)
	4.5±1.55 (8)	13	7.6±1.9 (8)	8	Fritsche and Nilsson (1990)
	2.7 (9)	9	2 (9)	3	Axelsson and Fritsche (1991)
	4.0±1.55 (8)	12	5.0±1.48 (8)	6	Butler *et al*. (1989)
	3 (7)	10	6 (7)	7	Perry *et al*. (1991)
Swimming					
12–15 min at 0.67 body length s^{-1}	3.1 (7)	10	4.5 (7)	6	Axelsson and Nilsson (1986)
10 min at 0.67 body length s^{-1}	5 (9)	14	7 (9)	8	Axelsson and Fritsche (1991)
At U_{crit}, 1 body length s^{-1}	46.0±8.9 (9)	50	25.8±5.5 (9)	20	Butler *et al*. (1989)
Acute hypoxia					
P_{O_2} gradually lowered					
30 min, Pw_{O_2} = 10.0 kPa	123 (8)	69	37 (8)	25	Perry *et al*. (1991)
30 min, Pw_{O_2} = 7.3 kPa	37 (8)	45	26 (8)	20	Perry *et al*. (1991)
30 min, Pw_{O_2} = 5.3 kPa	110 (8)	67	49 (8)	29	Perry *et al*. (1991)
P_{O_2} rapidly reduced					
2 min, Pw_{O_2} = 5.3-4.0 kPa	7 (8)	18	24.9±7.0 (8)	19	Fritsche and Nilsson (1990)
6 min, Pw_{O_2} = 5.3-4.0 kPa	25.5±7.0 (8)	38	33.5±9.0 (8)	23	Fritsche and Nilsson (1990)
8 min, Pw_{O_2} = 5.3-4.0 kPa	54 (8)	53	28 (8)	21	Axelsson and Fritsche (1991)
30 min, Pw_{O_2} = 5.3 kPa	32 (7)	42	31 (7)	22	Perry *et al*. (1991)

Table 1. *Continued*

State of the fish during blood sampling	Measured plasma adrenaline level (nmol l^{-1})	Calculated effect on red blood cell H^+ extrusion (%)*	Measured plasma noradrenaline level (nmol l^{-1})	Calculated effect on red blood cell H^+ extrusion (%)*	Reference
'Resting'					
Head kidney sham-operated, ⩾1 h recovery	29.8±6.8 (8)	41	13.2±1.8 (8)	12	Wahlqvist and Nilsson (1980)
'grab and stab' blood sampling	21 (9)	34	3 (9)	4	Nilsson *et al*. (1976)
Animals killed immediately before blood sampling	21.4±3.8 (9)	35	33.2±5.5 (9)	23	M. Berenbrink and C. R. Bridges (unpublished results)
Severe stress					
3 min air exposure, head kidney sham operated, ⩾24 h recovery	120 (7)	68	63 (7)	34	Perry *et al*. (1991)
15 min air exposure, 'grab and stab' blood sampling	120 (8)	68	30 (8)	22	Nilsson *et al*. (1976)
10 min struggling induced by lowered water level	292.5±90.6 (8)	82	32.1±7.4 (8)	23	Wahlqvist and Nilsson (1980)
30 min trawling, animals killed immediately before blood sampling	999±437 (7)	93	177±81 (7)	54	M. Berenbrink, S. Ulmer and C. R. Bridges (unpublished results)

*Red blood cell responses were calculated from the log-transformed dose–response curves for adrenaline and noradrenaline from Fig. 2 and are expressed as a percentage of the maximal response. Only responses calculated from mean catecholamine concentrations are shown, assuming independent action of the two hormones on the red blood cells.

Concentrations are given as means ± S.E.M. or as means only when they were calculated from literature data.

Unless otherwise stated, blood samples were taken *via* implanted catheters; number of animals are given in parentheses.

similar characteristics to that of the catecholamine-activated Na^+/H^+ exchanger described for rainbow trout red blood cells (Nikinmaa and Huestis, 1984; Baroin *et al.* 1984; Cossins and Richardson, 1985). These are as follows. (1) Noradrenaline and adrenaline induce a rapid and dose-dependent extracellular acidification (Figs 1, 2), which is inhibited by amiloride. This acidification is also a saturable function of the external sodium concentration (Fig. 3). (2) The decrease in pHe results in an increase in pHi, which contrasts with a passive, Donnan-like distribution of protons across the red blood cell membrane (Fig. 4). (3) In the presence of DIDS (Fig. 5), when both the rapid proton equilibration *via* the chloride/bicarbonate exchanger and the sodium-dependent chloride/bicarbonate exchanger are blocked (Boron and Russel, 1983), the net proton efflux matches the net sodium influx. This provides evidence for the coupled movement of sodium and protons with a stoichiometry of 1:1. (4) The effects of catecholamines on the red blood cells involve an increase in the red blood cell chloride and sodium concentrations, which leads to red blood cell swelling (Fig. 5). The striking similarities between the Na^+/H^+ exchanger of rainbow trout red blood cells and the catecholamine-activated acid extrusion in Atlantic cod red blood cells suggest that the same mechanisms are present in the red blood cells of the two species. However, some differences must be considered.

In rainbow trout and carp, the red blood cell receptor affinity for noradrenaline is 60- to 80-fold higher than that for adrenaline (Tetens *et al.* 1988; Salama and Nikinmaa, 1990; Salama, 1992). This led to the conclusion that noradrenaline is the predominant catecholamine affecting fish red blood cells at physiological concentrations of catecholamines (Tetens *et al.* 1988). Atlantic cod red blood cells clearly deviate from this scheme. The receptor affinities for adrenaline (EC_{50}=47 nmol l^{-1}) and noradrenaline (EC_{50}=144 nmol l^{-1}) differ by a factor of only three, indicating a less specific discrimination between the two catecholamines (Fig. 2). Moreover, adrenaline rather than noradrenaline is preferentially bound by the adrenergic receptor of Atlantic cod red blood cells. The higher receptor affinity for adrenaline, taken together with the *in vivo* concentration of this drug reported in the literature (Table 1), suggests that adrenaline, rather than noradrenaline, is the major catecholamine effecting the red blood cell response in Atlantic cod *in vivo*. Resting values for the plasma noradrenaline concentration slightly exceed the respective values for adrenaline (Axelsson and Nilsson, 1986; Fritsche and Nilsson, 1990; Perry *et al.* 1991), but both catecholamines can evoke 4–13 % of the maximal catecholamine-induced proton extrusion even in resting animals, as calculated from the log-transformed dose–response curve (Fig. 2B). Various types of stress result in elevated plasma adrenaline levels corresponding to a stimulation of Na^+/H^+ exchange between 20 % and 90 % (Table 1). The concomitant increase in noradrenaline concentration is usually smaller, but would be sufficient to elicit red blood cell responses of about 20–50 %, assuming adrenaline-independent binding. Similar or higher stress levels of noradrenaline compared with adrenaline are only observed when the P_{O_2} of the water is reduced within a few minutes and, even then, the calculated effect of adrenaline on the red blood cells is larger (Table 1). Thus, in Atlantic cod red blood cells under physiological conditions, adrenaline is more important in activating the red blood cell Na^+/H^+ exchanger than is noradrenaline.

It is interesting to note that, in Atlantic cod red blood cells in winter, hypercapnic acidification *per se* leads to active proton extrusion, which is probably sodium-dependent chloride/bicarbonate exchange (Berenbrink and Bridges, 1994). The present study provides further evidence for a catecholamine-independent pHi regulatory mechanism in Atlantic cod red blood cells at 1 % CO_2 (Fig. 5). The extrusion of protons and the concomitant increase in intracellular sodium concentration as well as the DIDS-sensitivity of this mechanism are in line with the action of a sodium-dependent chloride/bicarbonate exchanger. Additionally, in the presence of catecholamines, the low specificity between noradrenaline and adrenaline in Atlantic cod red blood cells allows both catecholamines to play a part in activating Na^+/H^+ exchange under physiological conditions (Table 1). In contrast to Atlantic cod red blood cells, rainbow trout red blood cells seem to depend solely on catecholamines and specifically on noradrenaline to achieve active pHi regulation *in vivo* (Tetens *et al.* 1988). A general evolutionary trend seems to lead from continuous active pHi regulation, such as that found in red blood cells from primitive vertebrates like the lamprey (Nikinmaa, 1986), to predominantly passive proton distribution, such as that found in rainbow trout red blood cells, where active pH regulation is restricted to more narrow physiological conditions. The broad range of physiological conditions over which pHi is actively regulated in Atlantic cod red blood cells might therefore represent an intermediate step in red blood cell evolution.

In Atlantic cod, the presence of three acid–base transport systems in a single cell type (chloride/bicarbonate exchange, sodium/proton exchange and sodium-dependent chloride/bicarbonate exchange) may seem unlikely; however, a similar set of pHi regulatory transporters has previously been described for renal mesanglial cells of the rat (Ganz *et al.* 1989). The apparent absence of sodium-dependent chloride/bicarbonate exchange in Atlantic cod red blood cells in summer and the occurrence of all three transporters in winter (Berenbrink and Bridges, 1994) indicate a changing requirement for red blood cell pHi regulation during the year. This is supported by the general observation of strong seasonal changes in the blood variables of many fish species (Woodhead and Woodhead, 1959; Denton and Yousef, 1975; Lane, 1979; Härdig and Höglund, 1984).

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