J. exp. Biol. ***176****, 103–116 (1993)*
Printed in Great Britain © The Company of Biologists Limited 1993

THE *IN VITRO* EFFECT OF HYPOXIA ON THE TROUT ERYTHROCYTE β-ADRENERGIC SIGNAL TRANSDUCTION SYSTEM

SCOTT D. REID*, Y. LEBRAS† and S. F. PERRY

Department of Biology, University of Ottawa, 160 Louis Pasteur, Ottawa, Ontario, K1N 6N5 Canada

Accepted 7 October 1992

Summary

We have investigated the effects of acute *in vitro* hypoxia on trout (*Oncorhynchus mykiss*) erythrocytes in order to elucidate the mechanism(s) by which increased catecholamine responsiveness of the Na^+/H^+ antiporter is achieved. Blood was withdrawn from cannulated trout and maintained *in vitro* under normoxic (P_{O_2}=17.9±0.2kPa, N=12) or hypoxic (P_{O_2}=2.8±0.1kPa, N=12) conditions for 30min prior to exposure to concentrations of noradrenaline, forskolin or 8-bromo-cyclic AMP ranging from 0 to 10^{-6} mol l^{-1}, 10^{-7} to 10^{-5} mol l^{-1} or 10^{-4} to 10^{-2} mol l^{-1}, respectively. Na^+/H^+ exchange activity was quantified as the maximal reduction in whole-blood pH (pHe) after addition of the various Na^+/H^+ antiporter activators. Erythrocyte intracellular cyclic AMP contents were also determined after addition of noradrenaline or forskolin. To complete the investigation, radioreceptor binding assays were conducted on separate blood samples to characterize the numbers and affinities of the surface population of β-adrenoceptors of erythrocytes maintained under normoxic or hypoxic conditions.

Exposure of erythrocytes to noradrenaline, forskolin or 8-bromo-cyclic AMP resulted in dose-dependent reductions in pHe as a result of Na^+/H^+ antiporter activation. In all cases, the effects were significantly more pronounced under hypoxic than normoxic conditions. Hypoxia significantly increased the production of cyclic AMP in the presence of noradrenaline but did not affect the forskolin-induced production of cyclic AMP. Blood oxygen status also affected the number of β-adrenoceptors expressed at the erythrocyte surface; hypoxic erythrocytes possessed 880.7±28.6 (N=6) receptors per cell whereas normoxic erythrocytes possessed 532.6±43.2 (N=6) receptors per cell.

These results suggest that *in vitro* exposure of trout erythrocytes to hypoxic conditions results in at least two significant alterations in the catecholamine signal transduction system: (1) an enhancement in erythrocyte cyclic AMP production, in part by virtue of an increase in the number of surface β-adrenoceptors, and (2) a hypoxia-induced increase in

*Present address: Fish Physiology Laboratory, Department of Biology, McMaster University, 1280 Main St West, Hamilton, Ontario, Canada L8S 5K1.

†Present address: Laboratoire de Physiologie Animale, Faculté des Sciences et Techniques, Université de Bretagne Occidentale, 6 Avenue Victor Le Gorgeu, F-29287 Brest, France.

Key words: erythrocyte, trout, *Oncorhynchus mykiss*, hypoxia, β-adrenoceptors, adenylate cyclase, cyclic AMP, catecholamines, proton extrusion, Na^+/H^+ exchange.

the cyclic AMP sensitivity of one or more of the steps culminating in Na^+/H^+ antiporter activation. These events ultimately increase the responsiveness of the erythrocyte Na^+/H^+ antiporter to catecholamines during hypoxia.

Introduction

Exposure of trout erythrocytes to exogenous catecholamines *in vitro* (Baroin *et al.* 1984; Cossins and Richardson, 1985; Borgese *et al.* 1986; Reid and Perry, 1991; see also review by Nikinmaa and Tufts, 1989) or endogenous catecholamines *in vivo* (Fievet *et al.* 1987) activates a membrane-associated Na^+/H^+ antiporter, thereby causing H^+ extrusion, Na^+ (and Cl^-) accumulation (Baroin *et al.* 1984; Cossins and Richardson, 1985; for recent reviews, see Nikinmaa and Tufts, 1989; Fievet and Motais, 1991) and consequent cell swelling (for a recent review, see Motais and Garcia-Romeu, 1987) and alkalization (Nikinmaa, 1983; Tetens *et al.* 1988; Claireaux *et al.* 1988; for a recent review, see Perry and Wood, 1989). These responses are thought to enhance blood oxygen transport during periods of increased O_2 demand (for recent reviews, see Thomas and Perry, 1992; Randall and Perry, 1992). Both the cell swelling-associated and metabolically mediated reductions of intracellular nucleoside triphosphate (NTP) concentrations (Tetens and Lykkeboe, 1981; Ferguson and Boutilier, 1989) and the intracellular alkalization can increase haemoglobin oxygen-binding affinity (Nikinmaa, 1983; Tetens and Christensen, 1987).

Hypoxia is a strong stimulus for catecholamine mobilization in teleost fish (Fievet *et al.* 1987, 1990; Tetens and Christensen, 1987; Boutilier *et al.* 1988; Ristori and Laurent, 1989; Perry *et al.* 1991). Additionally, it has been demonstrated that the responsiveness of erythrocytes to catecholamines is affected by blood oxygen partial pressure (Motais *et al.* 1987; Nikinmaa *et al.* 1987; Salama and Nikinmaa, 1988, 1990; Reid and Perry, 1991). Both catecholamine-stimulated erythrocyte swelling (carp: Salama and Nikinmaa, 1990) and Na^+/H^+ antiporter proton extrusion activity (trout: Motais *et al.* 1987; Reid and Perry, 1991) are significantly enhanced following *in vitro* reductions in blood oxygen tension (P_{O_2}).

The catecholamine signal transduction pathway in trout erythrocytes appears to utilize a typical β-adrenoceptor–adenylate cyclase system, which, when activated, results in increased intracellular concentrations of cyclic AMP. The ultimate result of cyclic AMP accumulation is the activation of the Na^+/H^+ antiporter. Clearly, such a system provides multiple sites for the expression of the oxygen sensitivity of erythrocyte catecholamine responsiveness. Marttila and Nikinmaa (1988) showed that acute hypoxia results in the appearance of high-affinity surface β-adrenoceptors in carp (*Cyprinus carpio* Linnaeus) erythrocytes, while in trout, hypoxia stimulates the redistribution of erythrocyte β-adrenoceptors (Reid and Perry, 1991), which results in a significant increase in surface receptor numbers. Erythrocyte cyclic AMP content and production also appear to be influenced by blood oxygen tension in these fish (carp: Salama and Nikinmaa, 1990; trout: Reid and Perry, 1991), although the generality of this response is in question (Motais *et al.* 1987). However, the mechanism(s) underlying the enhancement of erythrocyte cyclic AMP production and Na^+/H^+ antiporter activity during hypoxia have

not yet been elucidated. The goal of this study was to identify modifications in the catecholamine signal transduction system of trout erythrocytes resulting from exposure of cells to acute hypoxia *in vitro*. Specifically, experiments were designed to: (1) confirm hypoxia-sensitive alterations in trout erythrocyte surface β-adrenoceptor numbers, (2) determine the basis for previously observed increases in intracellular cyclic AMP content, and (3) identify possible oxygen-sensitive alterations in Na^+/H^+ antiporter responsiveness to endogenous and exogenous cyclic AMP.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex weighing between 189 and 293g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the University of Ottawa. Fish were held in large fibreglass tanks supplied with flowing, aerated and dechlorinated City of Ottawa tap water [see Perry *et al.* (1988) for water ionic composition]. Fish were acclimated to these conditions for at least 4 weeks prior to experimentation at water temperatures which varied between 11 and 13 °C. Photoperiod was kept constant at 12h light:12h dark. Fish were fed daily with commercial trout pellets (Martins Feed Mill Ltd) and tanks were syphoned after feeding to prevent the build-up of organic material. All experiments reported in this study were undertaken in May.

Blood collection and preparation

Fish were anaesthetized in a 1:10000 (w/v) solution of ethyl *m*-aminobenzoate (MS-222, Sigma) adjusted to pH7.5 with sodium bicarbonate. Fish were then placed onto an operating table that permitted continuous retrograde irrigation of the gills with either the anaesthetic solution or fresh water. Dorsal aortic cannulae were implanted following standard techniques (Soivio *et al.* 1975) using flexible polyethylene tubing (Clay-Adams PE-50, i.d. 0.580mm, o.d. 0.965mm).

After surgery, fish were placed in black Perspex boxes (volume 3l) supplied with continuously flowing, dechlorinated and aerated water. Fish were allowed at least 48h to recover from surgery before experiments commenced. Dorsal aortic cannulae were flushed at least once daily with 0.2–0.3ml of heparinized (10i.u.ml^{-1} ammonium heparin) Cortland saline (Wolf, 1963).

Blood was withdrawn from the dorsal aortic cannula and placed within heparinized (100i.u.ml^{-1} blood, as ammonium heparin) round-bottomed tonometer flasks. Typically, 2 ml of blood was removed per fish and the blood of several animals (2–3) was pooled to provide an adequate volume for a single (i.e. N=1) experiment. Flasks of blood were collected, gassed briefly with O_2, placed on ice 12h prior to experimentation, and slowly agitated until required. Preliminary experiments were conducted to ensure that this procedure did not result in any modification of the responsiveness of the blood to noradrenaline as trout erythrocytes are typically unresponsive to catecholamines immediately following sampling by either dorsal aortic cannula or caudal puncture, with full catecholamine responsiveness becoming established 2–4h after sampling. The pools

of blood were handled, maintained and gassed in an identical manner, irrespective of the particular component of the study being completed.

Experimental protocol

Responsiveness to noradrenaline

The standard protocol consisted of measuring the change in whole-blood pH (pHe) in round-bottomed tonometer flasks containing 400 μl of blood after the addition (20 μl) of noradrenaline (L-noradrenaline, bitartrate salt dissolved in saline) to yield final nominal concentrations ranging from 10 to 1000nmol l^{-1}. The rationale for measuring catecholamine-induced alterations in pHe is that they directly reflect changes in trout erythrocyte Na^+/H^+ antiporter activity (e.g. Thomas and Perry, 1992). The blood was maintained at 12 °C in a shaking water bath and gassed continuously for 30min with a humidified gas mixture (normoxia: 20% O_2, 79.8% N_2, 0.2% CO_2; hypoxia: 2% O_2, 97.8% N_2, 0.2% CO_2) supplied by a gas-mixing pump (Wösthoff, model M301-A/F). pHe was measured on five consecutively drawn 10 μl samples immediately before additions of noradrenaline and 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20 and 30min thereafter. Additional blood samples (200 μl) were removed prior to and 5min after addition of noradrenaline to determine erythrocyte cyclic AMP concentration and P_{O_2} (pre-noradrenaline sample only).

Characterization of erythrocyte β*-adrenoceptors*

The characterization of the erythrocyte β-adrenoceptors was accomplished on intact erythrocyte suspensions using the β-antagonist (±)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one ([^{3}H]-CGP 12177; CGP, specific activity 1.26–1.70TBq mmol $^{-1}$; New England Nuclear) as described by Reid *et al.* (1991). Briefly, samples of the erythrocyte suspension were contained in round-bottomed tonometer flasks placed in a 12 °C shaking water bath and gassed under the appropriate conditions for 30min prior to initiating β-adrenoceptor characterization. Radioligand binding was initiated by the addition of 40 μl of this erythrocyte suspension to 100 μl of Cortland saline into which the radioligand CGP at a range of concentrations (5–40nmol l^{-1}) had been added with either 200 μmol l^{-1} of the β-agonist (−)-isoproterenol (Sigma) or alone. The number of erythrocytes added to each incubation was determined by cell counting using a haemocytometer (American Optical). All binding assays were completed at room temperature (19 °C).

Incubations were terminated following a 60min radioligand incubation by transferring the erythrocytes to borosilicate filters (no. 32, Mandel Scientific) using a cell membrane harvester (Brandel 24R) with subsequent repeated washings (four times) with 5ml of ice-cold 0.9% NaCl. The filters were placed into glass liquid scintillation vials containing 8 ml of fluor (ACS II, Amersham) and allowed to settle for at least 24h. Sample radioactivity was then determined using a Canberra Packard (2500 TR) liquid scintillation counter, with all counts corrected for quenching using an external standard technique.

The maximal number of isoproterenol-displaceable binding sites (B_{max}, in

disintsmin^{-1} cell^{-1}) and the apparent dissociation constants (K_D) were determined using Scatchard plot analysis (Scatchard, 1949). Receptor density (B_{max}) was converted to, and expressed on, a receptor per erythrocyte basis by multiplying the maximal number of specific receptor sites (disintsmin^{-1} cell^{-1}) by the radioligand specific activity and Avogadro's number. The specific erythrocyte β-adrenoceptors detected using [^{3}H]-CGP in combination with isoproterenol are referred to as high-affinity erythrocyte surface β-adrenoceptors in accordance with our previously reported findings (Reid *et al.* 1991).

Responsiveness to forskolin

Blood was taken from a 400 μl sample of pooled normoxic or hypoxic blood, before and following (5–15, 20, 30min) the addition (20 μl) of forskolin [Sigma, St Louis; dissolved in 2% dimethylsulphoxide (DMSO) saline] to yield final nominal concentrations ranging from 10^{-7} to 10^{-5} mol l^{-1} in the presence of 10^{-4} mol l^{-1} theophylline (1,3-dimethylxanthine, Sigma, St Louis; dissolved in 2% DMSO saline). An additional sample was removed at 10min to determine erythrocyte cyclic AMP concentration. Theophylline was used to minimize possible hypoxia-associated alterations in phosphodiesterase activity and was added to samples of blood 15min prior to the addition of forskolin.

Responsiveness to 8-bromo-cyclic AMP

Hypoxia-associated modifications in Na^+/H^+ antiporter activity were assessed by monitoring pHe in 200 μl blood samples following the addition of 40 μl of 8-bromo-cyclic AMP (8-bromoadenosine 3′,5′-cyclic monophosphate, Sigma, St Louis) using a protocol similar to those describe above. 8-Bromo-cyclic AMP (dissolved in 2% DMSO saline) was prepared to yield final nominal concentrations ranging from 10^{-4} to 10^{-2} mol l^{-1}. Erythrocytes were exposed to 8-bromo-cyclic AMP in the presence of 10^{-4} mol l^{-1} theophylline as detailed previously. Erythrocyte cyclic AMP concentrations were not determined during these experiments.

Analytical techniques

Erythrocyte cyclic AMP content was determined on 40 μl of packed erythrocytes, obtained by centrifugation (12000 ***g***, 2min), according to the protocol of a commercially available radioimmunoassay (Amersham Canada Inc.). Plasma protein concentrations were determined according to the procedure of Lowry *et al.* (1951). Haemoglobin levels were measured in duplicate on 20 μl blood samples using a commercial spectrophotometric assay kit (Sigma, St Louis). pHe and P_{O_2} were measured using Radiometer microcapillary pH (G299A) and P_{O_2} (E5046) electrodes adjusted to 12 °C, in conjunction with a Radiometer PHM 71 acid–base analyzer and BMS3 MK2 blood microsystem. The change in pHe (ΔpHe) elicited by additions of noradrenaline, forskolin or 8-bromo-cyclic AMP was determined as the difference between pHe prior to and the maximal reduction in pHe following Na^+/H^+ activation. The measured values of ΔpHe were corrected for differences in blood haemoglobin content (Hb) in accordance with Thomas *et al.* (1991) by standardizing and expressing ΔpHe on a per 10g Hb basis. The apparent activation constant (K_a), calculated from an Eadie–Hofstee plot of erythrocyte

intracellular cyclic AMP concentration *vs* maximal reduction in whole-blood pH (ΔpHe), was used to estimate the second messenger sensitivity of the steps culminating in Na^+/H^+ activation.

Statistical analysis

All experiments were performed six times with differences between mean values assessed by analysis of variance (ANOVA) followed by Fisher's LSD for multiple comparison, at a 95% level of confidence (Statview 512$^+$). The difference between cyclic AMP apparent activation constants (K_a) was assessed by comparison of the slopes of the Eadie–Hofstee plots using Student's *t* at a 95% level of confidence (Zar, 1984). All values have been reported as means ± 1 S.E.

Results

Erythrocytes maintained under normoxic conditions (P_{O_2}=17.9±0.2kPa, N=12) were found to possess 532.6±43.2 (B_{max}, N=6) β-adrenoceptors per cell (Fig. 1A), with an apparent dissociation constant of 4.3±1.5nmoll^{-1} (K_D, N=6; Fig. 1B). These findings are in agreement with previously reported receptor characteristics for trout erythrocytes from our laboratory. A reduction in *in vitro* blood oxygen tension (P_{O_2}=2.8±0.1kPa, N=12) did not significantly alter the apparent dissociation constant of the erythrocyte surface β-adrenoceptors (Fig. 1B) but did cause a significant 1.5-fold enhancement in the number of β-adrenoceptors (880.7±28.6; Fig. 1A) expressed at the erythrocyte surface.

Under normoxic conditions, additions of noradrenaline resulted in a dose-dependent depression of pHe (Fig. 2A) and a dose-dependent increase in intracellular cyclic AMP concentration (Fig. 2B). When erythrocytes were maintained under hypoxic conditions, identical concentrations of noradrenaline resulted in an average 1.5-fold greater increase in erythrocyte cyclic AMP content and a 2.3-fold greater reduction in pHe. During hypoxia, ΔpHe reached a maximum which was both greater, and obtained at a considerably lower noradrenaline concentration, than that obtained during normoxic

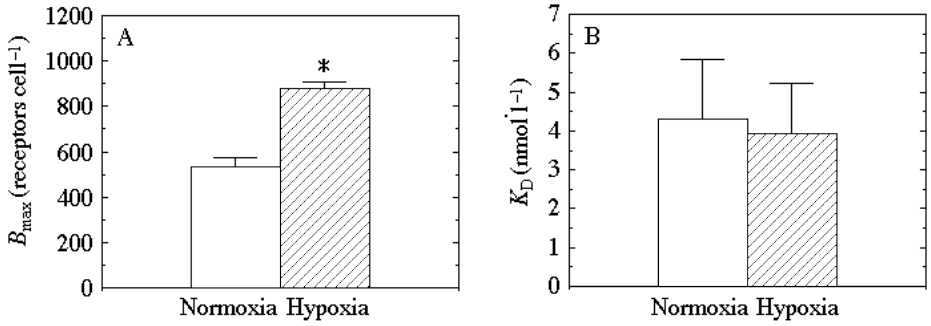

Fig. 1. The effects of acute hypoxia on (A) erythrocyte surface β-adrenoceptor numbers (B_{max}) and (B) apparent dissociation constant (K_D). All values are means ±1 standard error of the mean. N=6 for erythrocytes maintained under both normoxic (open histograms) and hypoxic (hatched histograms) conditions. An asterisk indicates that the mean value for hypoxic erythrocytes differs significantly (P<0.05) from the corresponding normoxic mean.

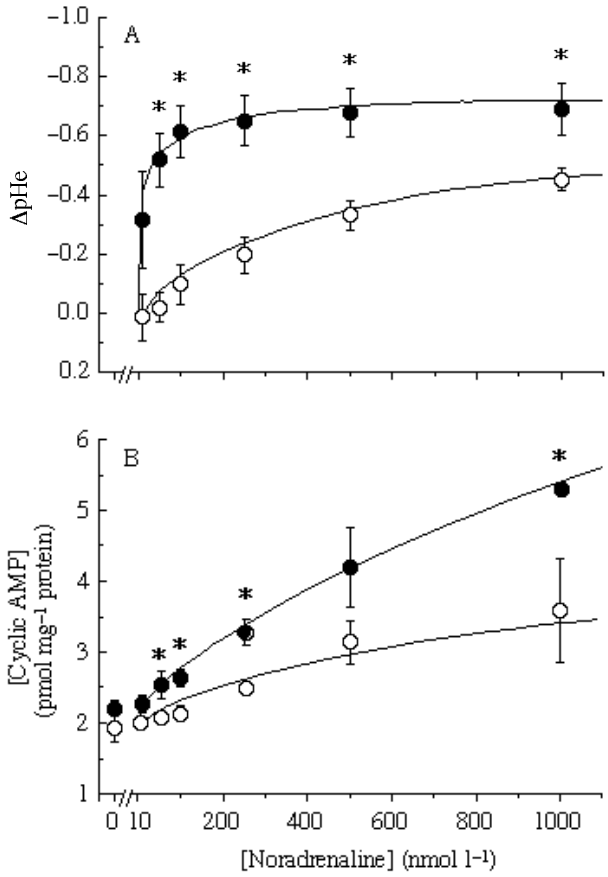

Fig. 2. The effect of *in vitro* blood oxygen status on noradrenaline-stimulated changes in (A) whole-blood pH (ΔpHe) and (B) erythrocyte cyclic AMP concentration ([cyclic AMP]). All values are means ±1 standard error of the mean (where not indicated, the S.E. lies within the symbol). N=6 for erythrocytes stimulated under both normoxic (P_{O_2}=17.9±0.2kPa; open circles) and hypoxic (30min hypoxia, P_{O_2}=2.8±0.1kPa; filled circles) conditions. An asterisk indicates that the mean value for hypoxic erythrocytes differs significantly (P<0.05) from the corresponding normoxic mean. Lines are fitted to the data by eye.

noradrenaline exposures. No such maximal noradrenaline-induced erythrocyte cyclic AMP content was achieved (Fig. 2B), although significantly greater cyclic AMP concentrations were obtained after noradrenaline exposure during hypoxia than during normoxia at nearly all noradrenaline concentrations.

The incubation of erythrocytes in the presence of forskolin, a direct activator of adenylate cyclase, caused dose-dependent reductions in pHe and increases in erythrocyte cyclic AMP content (Fig. 3). The potency of forskolin as an activator of erythrocyte Na^+/H^+ antiporter activity was markedly enhanced during acute hypoxia (Fig. 3A). Hypoxia increased the responsiveness of the Na^+/H^+ antiporter to forskolin at all concentrations. No maximal hypoxia-induced reduction of pHe was observed within the concentration range used. In contrast to the effect of hypoxia on noradrenaline-stimulated

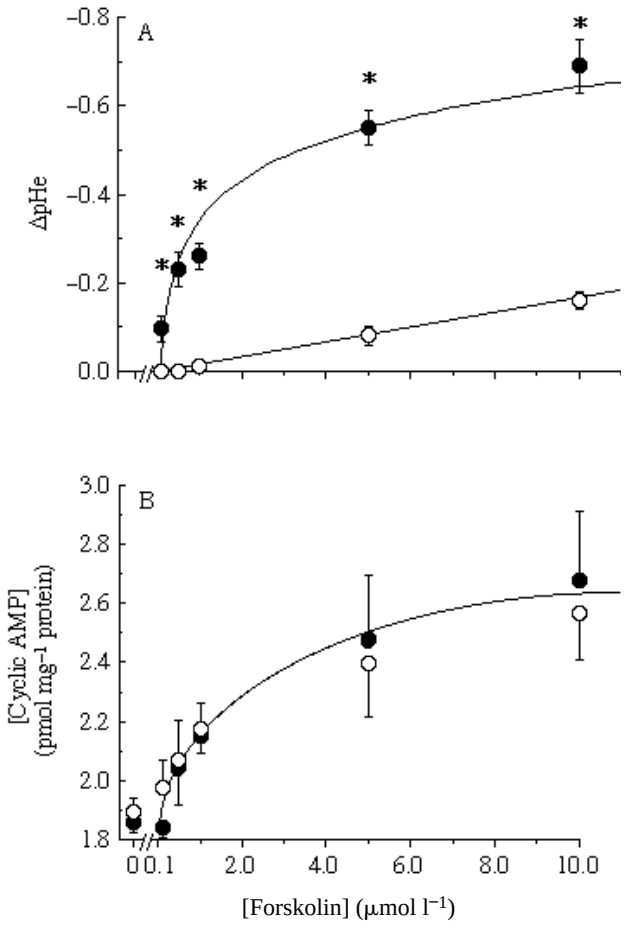

Fig. 3. The effect of *in vitro* blood oxygen status on forskolin-stimulated changes in (A) whole-blood pH (ΔpHe) and (B) erythrocyte cyclic AMP concentration ([cyclic AMP]). All values are means ±1 standard error of the mean (where not indicated, the S.E. lies within the symbol). *N*=6 for erythrocytes stimulated under both normoxic (open circles) and hypoxic (filled circles) conditions. An asterisk indicates that the mean value for hypoxic erythrocytes differs significantly ($P<0.05$) from the corresponding normoxic mean. Lines are fitted to the data by eye.

erythrocyte cyclic AMP content, hypoxia did not affect the amount of cyclic AMP produced following forskolin-activated adenylate cyclase activity (Fig. 3B).

Mean values, taken from Figs 1 and 2, have been replotted to illustrate the relationship between erythrocyte cyclic AMP content and the depression of pHe and the impact of hypoxia on this relationship (Fig. 4). Under normoxic conditions, there was a positive correlation between erythrocyte cyclic AMP content and the resultant depression of pHe. However, during acute hypoxia, there was a significant increase in the responsiveness (normoxia K_a=3.51±0.35 *vs* hypoxia K_a=2.68±0.28pmolmg^{-1} protein) of the erythrocyte Na^+/H^+ antiporter to elevations of intracellular cyclic AMP concentration.

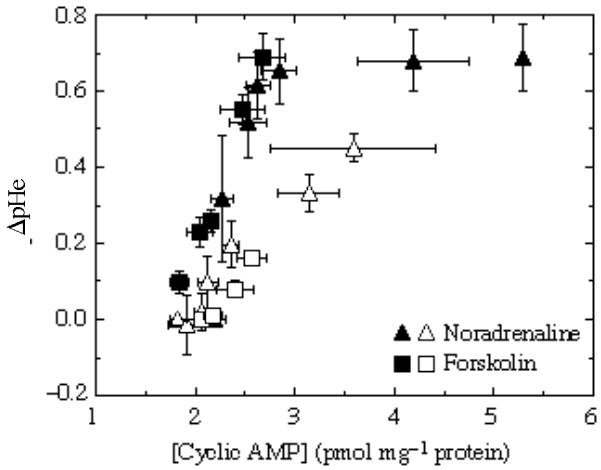

Fig. 4. Maximal reductions in whole-blood pH (ΔpHe) as a function of intracellular cyclic AMP concentration in normoxic (open symbols) and acutely (30min) hypoxic (filled symbols) erythrocytes. Data represent means ±1 standard error of the mean (*N*=6) for both noradrenaline- (triangles) and forskolin-stimulated (squares) erythrocytes.

The activation of the erythrocyte Na^+/H^+ antiporter by 8-bromo-cyclic AMP and the interactive effects of hypoxia, are illustrated in Fig. 5. Under normoxic conditions, incubation of trout erythrocytes with the membrane-permeable cyclic AMP analogue resulted in a dose-dependent reduction of pHe, which was significantly enhanced an average of 7.5 times under hypoxic conditions.

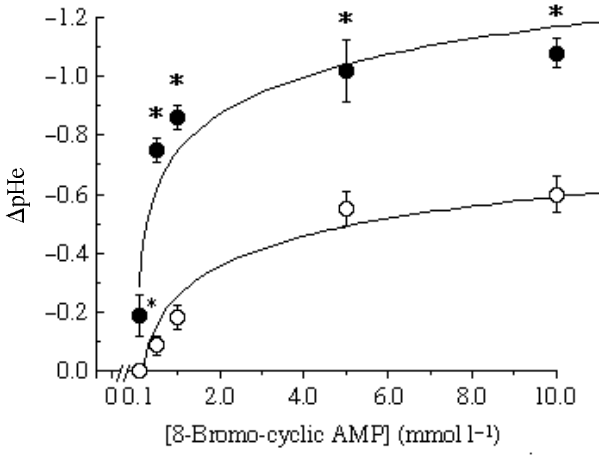

Fig. 5. The effect of *in vitro* blood oxygen status on 8-bromo-cyclic-AMP-stimulated changes in whole-blood pH (ΔpHe). All values are means ±1 standard error of the mean (where not indicated, the S.E. lies within the symbol). *N*=6 for erythrocytes stimulated under both normoxic (open circles) and hypoxic (filled circles) conditions. An asterisk indicates that the mean value for hypoxic erythrocytes differs significantly ($P<0.05$) from the corresponding normoxic mean. Lines are fitted to the data by eye.

Discussion

It has been previously documented (Nikinmaa *et al.* 1987; Motais *et al.* 1987; Salama and Nikinmaa, 1988, 1990; Reid and Perry, 1991) that the catecholamine-responsiveness of teleost erythrocytes is significantly increased by acute hypoxia. Reductions in blood oxygen tension, *in vitro*, lead to an increase in the number of β-adrenoceptors at the erythrocyte surface (trout: Reid and Perry, 1991; carp: Marttila and Nikinmaa, 1988), increased cyclic AMP content of catecholamine-stimulated cells (trout: Reid and Perry, 1991; eel: Perry and Reid, 1992; carp: Salama and Nikinmaa, 1990) and enhanced β-adrenergic swelling (trout: Motais *et al.* 1987; carp: Salama and Nikinmaa, 1990). Thus, previous studies have established that the increased responsiveness of teleost erythrocytes to catecholamines during hypoxia is related, at least in part, to enhanced production of cyclic AMP, in turn a consequence of increased numbers of β-adrenoceptors. In the present study, we have confirmed these findings while identifying an additional stimulatory factor (increased responsiveness of the Na^+/H^+ antiporter, either direct or indirect, to cyclic AMP) and eliminated another (increased responsiveness of adenylate cyclase).

The alkalization of the erythrocyte cytosol is initiated by the occupancy of the erythrocyte surface β-adrenoceptors to catecholamines. In addition to these physiologically active receptors, lower-affinity internalized receptors have been identified, which may act as a reservoir for normal receptor cycling (Reid *et al.* 1991), agonist-dependent down-regulation (Thomas *et al.* 1991) and surface receptor population augmentation (Reid and Perry, 1991). In trout, the increase in cell surface β-adrenoceptors was shown to occur in response to acute (*in vitro*) and chronic (*in vivo*) exposure to elevated plasma cortisol levels (Reid and Perry, 1991) and *in vitro* reductions in blood P_{O_2} (Fig. 1, this study; Reid and Perry, 1991). Marttila and Nikinmaa (1988) demonstrated an oxygen sensitivity in the quantification of the carp erythrocyte β-adrenoceptor. Under normoxic conditions (P_{O_2}=155mmHg; 20.7kPa), β-adrenoceptors were absent or non-detectable on the carp erythrocyte surface, while significant numbers of these receptors were apparent following 1h of hypoxia *in vitro* (P_{O_2}=8mmHg; 1.1kPa).

These increases in surface β-adrenoceptor numbers were postulated to be the explanation for increased agonist-stimulated erythrocyte cyclic AMP concentrations observed under hypoxic conditions (Salama and Nikinmaa, 1990; Reid and Perry, 1991). Motais *et al.* (1987) reported a reduction in isoproterenol-stimulated trout erythrocyte cyclic AMP concentration under *in vitro* anoxia (100% N_2) compared with that measured under 100% O_2. However, several more recent studies have shown significant increases in cyclic AMP concentrations under less extreme *in vitro* reductions in blood P_{O_2}. Using carp erythrocytes, Salama and Nikinmaa (1990) reported that, under hypoxic conditions (P_{O_2}=8mmHg; 1.1kPa), the cyclic AMP concentrations obtained after stimulation with noradrenaline or adrenaline were greater than in normoxia (P_{O_2}=150mmHg; 20kPa). Under comparable *in vitro* conditions, we have observed similar hypoxia-associated changes in cyclic AMP concentration in both trout and eel erythrocytes (Fig. 2, this study; Reid and Perry, 1991; Perry and Reid, 1992).

The oxygen-sensitivity of the β-adrenoceptor population suggests that post-agonist-receptor binding events, namely steps culminating in Na^+/H^+ activation and/or activation of the Na^+/H^+ antiporter itself, might also be modulated by changes in blood P_{O_2}. These possibilities were tested in the present study. The data suggest, however, that the increased concentration of cyclic AMP following adrenergic stimulation in hypoxic erythrocytes was not caused by any enhancement in adenylate cyclase activity but was simply the result of the increased number of physiologically active β-adrenoceptors (Fig. 1). Evidence for this was the lack of an effect of hypoxia on forskolin-activated cyclic AMP production (Fig. 3). In the absence of receptor-based activation of adenylate cyclase, no significant difference in cyclic AMP production was observed between normoxic and hypoxic erythrocytes in the presence of forskolin. These data indicate that the erythrocyte complement or activity of adenylate cyclase is unaffected by hypoxia unlike the erythrocyte receptor population, which is dynamic in its ability to change its numbers in response to changes in blood oxygen status (Fig. 1, this study; Reid and Perry, 1991).

This study has established that the distribution or the expression of β-adrenoceptors at the erythrocyte surface is not the only step in the catecholamine signal transduction system that is significantly altered by variations in blood oxygen status. In addition, the endogenous (Fig. 4) or exogenous (Fig. 5) cyclic AMP responsiveness of one or more steps culminating in the activation of the Na^+/H^+ antiporter was significantly enhanced by hypoxia. For example, incubation of the erythrocytes in the presence of 8-bromo-cyclic AMP under hypoxia resulted in an average 3.9-fold increase in the reduction of pHe (an indicator of proton extrusion activity) compared to normoxia (Fig. 5). It is possible that the change in cyclic AMP sensitivity under hypoxic conditions was related to alterations in erythrocyte membrane permeability to 8-bromo-cyclic AMP, thereby increasing the effective intracellular 8-bromo-cyclic AMP concentrations in comparison to normoxia. However, our data suggest that this explanation is unlikely. First, when cyclic AMP production was stimulated using forskolin under both normoxic and hypoxic conditions, significantly greater Na^+/H^+ antiporter activity was achieved under hypoxia (Fig. 3A) despite identical erythrocyte intracellular cyclic AMP concentrations in both conditions (Fig. 3B). Second, Fig. 4 clearly illustrates that there is a significant hypoxia-associated increase in the sensitivity of the erythrocyte Na^+/H^+ antiporter system to endogenous cyclic AMP that appears to be independent of the mode of stimulation; findings contrary to the oxygen-independent cyclic AMP sensitivity of carp erythrocyte swelling observed by Salama and Nikinmaa (1990). These data, therefore, indicate that hypoxia results in modifications in the cyclic AMP affinity of one or more of the steps culminating in the activation of the exchanger.

The nature and number of the steps following the production of cyclic AMP that are eventually expressed as the extrusion of intracellular H^+ and accumulation of intracellular Na^+ have yet to be elucidated. However, in the majority of systems in which the Na^+/H^+ antiporter has been characterized, cyclic AMP is an inhibitor of antiporter activity (for a recent review, see Clark and Limbird, 1991). In these cell systems, inhibition of antiporter activity results from the phosphorylation of the antiporter or a closely associated protein. To date, only erythrocytes (rabbit: Escobales and Rivera, 1987; trout: Fig. 4, this study;

Mahé *et al.* 1985; Motais *et al.* 1987; Reid and Perry, 1991; eel: Perry and Reid, 1992; carp: Salama and Nikinmaa, 1990) have been shown to possess a Na^+/H^+ antiporter stimulated by cyclic AMP. For cyclic AMP to activate antiporters directly in these erythrocytes, one would have to speculate that, unlike the majority of Na^+/H^+ exchangers, these antiporters are in an active state when phosphorylated. This significance of the uniqueness of erythrocyte Na^+/H^+ exchange is unknown.

The findings of this study suggest that there are at least two possible cyclic-AMP-independent mechanisms required to achieve enhanced Na^+/H^+ antiporter activity under hypoxia: changes in the cyclic AMP sensitivity of one or more of the steps culminating in antiporter activation and/or an increase in the Na^+/H^+ antiporter numbers. Both possibilities are based on the results depicted in Fig. 4. The left-ward shift in the cyclic AMP–ΔpHe curve suggests that some, as yet unknown, step(s) becomes more responsive to cyclic AMP and would result in the observed increase in Na^+/H^+ exchange in the absence of any change in antiporter numbers. However, as we were unable to achieve the same maximal intracellular cyclic AMP concentration under normoxia as under hypoxia, it is conceivable that hypoxia could trigger the recruitment of Na^+/H^+ antiporters in a manner similar to that observed for trout erythrocyte surface β-adrenoceptors (Fig. 1, this study; Reid and Perry, 1991). Little is known concerning the regulation of cell Na^+/H^+ numbers, but Watson *et al.* (1992) have recently demonstrated in a human cell line that regulation of Na^+/H^+ exchange does occur and involves the endocytotic removal or addition of the antiporter.

The exact mechanism by which reductions in blood oxygen partial pressure are translated into cyclic-AMP-independent enhanced Na^+/H^+ antiporter activity in trout erythrocytes is unknown. However, Motais *et al.* (1987) suggested that haemoglobin can act as a signal transducer in these cells according to its degree of oxygenation and thereby modulate Na^+/H^+ exchange through conformational alterations. It is well established that haemoglobin reversibly binds to band 3 protein in human erythrocytes (Shaklai *et al.* 1977; Salhany and Shaklai, 1979; Salhany *et al.* 1980; Cassoly, 1983) and that deoxygenated haemoglobin has a higher binding affinity for this anion exchanger than does oxygenated haemoglobin (Walder *et al.* 1984; Chetrite and Cassoly, 1985). Our findings do not challenge this role for haemoglobin oxygen-saturation in modifying erythrocyte catecholamine-responsiveness since it is plausible that the observed enhancement in the cyclic AMP sensitivity of Na^+/H^+ exchange, through increased cyclic AMP affinity, Na^+/H^+ antiporter numbers or Na^+/H^+ turnover rate, is induced by hypoxia-associated alterations in haemoglobin conformation.

In summary, this study has identified that *in vitro* exposure of trout erythrocytes to hypoxic conditions results in at least two significant alterations in the catecholamine signal transduction system. First, an enhancement in erythrocyte cyclic AMP production, in part by virtue of an increase in the number of surface β-adrenoceptors, and second, a hypoxia-induced increase in the cyclic AMP sensitivity of the steps culminating in Na^+/H^+ antiporter activation. These events ultimately increase the responsiveness of the erythrocyte Na^+/H^+ antiporter to catecholamines during hypoxia.

This work was supported by an NSERC operating grant (S.F.P.). The authors wish to

thank the 'Pernod Twins' (The Disco Kids) for their positive influence and keen interest in this study.

References

BAROIN, A., GARCIA-ROMEU, F., LAMARRE, T. AND MOTAIS, R. (1984). A transient sodium/hydrogen exchange system induced by catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol., Lond.* **356**, 21–32.

BORGESE, F., GARCIA-ROMEU, F. AND MOTAIS, R. (1986). Catecholamine-induced transport system in trout erythrocyte. Na^+/H^+ countertransport or NaCl cotransport? *J. gen. Physiol.* **87**, 551–556.

BOUTILIER, R. G., DOBSON, G., HOEGER, U. AND RANDALL, D. J.(1988). Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): metabolic and respiratory adaptations. *Respir. Physiol.* **71**, 69–82.

CASSOLY, R.(1983). Quantitative analysis of the association of human hemoglobin with the cytoplasmic fragment of band 3 protein. *J. biol. Chem* **258**, 3859–3864.

CHETRITE, G. AND CASSOLY, R. (1985). Affinity of hemoglobin for the cytoplasmic fragment of human erythrocyte membrane band 3. Equilibrium measurements at physiological pH using matrix-bound proteins: the effects of ionic strength, deoxygenation and of 2-3 diphosphoglycerate. *J. molec. Biol.* **185**, 639–644.

CLAIREAUX, G., THOMAS, S., FIEVET, B. AND MOTAIS, R. (1988). Adaptative respiratory responses of trout to acute hypoxia. II. Blood oxygen carrying properties during hypoxia. *Respir. Physiol*. **74**, 91–98.

CLARK, J. D. AND LIMBIRD, L. E. (1991). Na^+–H^+ exchanger subtypes: a predictive review. *Am. J. Physiol.* **261**, C945–C953.

COSSINS, A. R. AND RICHARDSON, P. A. (1985). Adrenaline-induced Na^+/H^+ exchange in trout erythrocytes and its effects upon oxygen carrying capacity. *J. exp. Biol.* **118**, 229–246.

ESCOBALES, N. AND RIVERA, A.(1987). Na^+ for H^+ exchange in rabbit erythrocytes. *J. cell. Physiol.* **132**, 73–80.

FERGUSON, R. A. AND BOUTILIER, R. G.(1989). Metabolic membrane coupling in red blood cells of trout: the effects of anoxia and adrenergic stimulation. *J. exp. Biol.* **143**, 149–164.

FIEVET, B., CAROFF, J. AND MOTAIS, R. (1990). Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effect on red blood cell Na/H exchanger activity. *Respir. Physiol*. **79**, 81–90.

FIEVET, B. AND MOTAIS, R. (1991). Na^+/H^+ exchange and red cell function in fish. In *Advances in Comparative and Environmental Physiology*, vol. 8, pp. 79–104. Berlin, Germany: Springer-Verlag.

FIEVET, B., MOTAIS, R. AND THOMAS, S. (1987). Role of adrenergic-dependent H^+ release from red cells in acidosis induced by hypoxia in trout. *Am. J. Physiol.* **252**, R269–R275.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *J. biol. Chem* **193**, 265–275.

MAHÉ, Y., GARCIA-ROMEU, F. AND MOTAIS, R. (1985). Inhibition by amiloride of both the adenylate cyclase activity and Na^+/H^+ antiporter in fish erythrocytes. *Eur. J. Pharmac.* **116**, 199–206.

MARTTILA, O. N. T. AND NIKINMAA, M. (1988). Binding of β-adrenergic antagonists ^{3}H-DHA and ^{3}H-CGP 12177 to intact rainbow trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) red blood cells. *Gen. comp. Endocr*. **70**, 429–435.

MOTAIS, R. AND GARCIA-ROMEU, F. (1987). Cell volume control by catecholamines in erythrocytes. Mechanisms and physiological role. In *Comparative Physiology and Environmental Adaptations*, vol. 1, (ed. R. Kirsch and B. Lahlou), pp. 13–25. Basel: Karger.

MOTAIS, R., GARCIA-ROMEU, F. AND BORGESE, F. (1987). The control of Na^+/H^+ exchange by molecular oxygen in trout erythrocytes. A possible role of hemoglobin as a transducer. *J. gen. Physiol.* **90**, 197–207.

NIKINMAA, M. (1983). Adrenergic regulation of hemoglobin oxygen affinity in rainbow trout red cells. *J. comp. Physiol.* **152**B, 67–72.

NIKINMAA, M., CECH, J. J., JR, RYHÄNEN, E.-L. AND SALAMA, A. (1987). Red cell functions of carp (*Cyprinus carpio*) in acute hypoxia. *Expl Biol.* **47**, 53–58.

NIKINMAA, M. AND TUFTS, B. (1989). Regulation of acid and ion transfer across the membrane of nucleated erythrocytes. *Can. J. Zool.* **67**, 3039–3045.

PERRY, S. F., FRITSCHE, R., KINKEAD, R. AND NILSSON, S. (1991). Control of catecholamine release *in vivo* and *in situ* in the Atlantic cod (*Gadus morhua*) during hypoxia. *J. exp. Biol.* **155**, 549–566.

PERRY, S. F. AND REID, S. D. (1992). The relationship between β-adrenoceptors and adrenergic responsiveness in trout (*Oncorhynchus mykiss*) and eel (*Anguilla rostrata*) erythrocytes. *J. exp. Biol.* **167**, 235–250.

PERRY, S. F., WALSH, P. J., MOMMSEN, T. P. AND MOON, T. W. (1988). Metabolic consequences of hypercapnia in the rainbow trout, *Salmo gairdneri*: β-Adrenergic effects. *Gen. comp. Endocr.* **69**, 439–447.

PERRY, S. F. AND WOOD, C. M. (1989). Control and coordination of gas transfer in fishes. *Can. J. Zool.* **67**, 2961–2970.

RANDALL, D. J. AND PERRY, S. F. (1992). Catecholamines. In *Fish Physiology*. vol. 12, *The Cardiovascular System* (ed. D. J. Randall and W. S. Hoar). New York: Academic Press (in press).

REID, S. D., MOON, T. W. AND PERRY, S. F. (1991). Characterization of β-adrenoreceptors of rainbow trout (*Oncorhynchus mykiss*) erythrocytes. *J. exp. Biol.* **158**, 199–216.

REID, S. D. AND PERRY, S. F. (1991). The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Oncorhynchus mykiss*) erythrocyte β-adrenoceptors. *J. exp. Biol.* **158**, 217–240.

RISTORI, M. T. AND LAURENT, P. (1989). Plasma catecholamines in rainbow trout (*Salmo gairdneri*) during hypoxia. *Expl Biol.* **48**, 285–290.

SALAMA, A. AND NIKINMAA, M. (1988). The adrenergic responses of carp (*Cyprinus carpio*) red cells: effects of P_{O_2} and pH. *J. exp. Biol.* **136**, 405–416.

SALAMA, A. AND NIKINMAA, M.(1990). Effect of oxygen tension on catecholamine-induced formation of cyclic AMP and on swelling of carp red blood cells. *Am. J. Physiol.* **259**, C723–C726.

SALHANY, J. M., CORDES, K. A. AND GAINES, E. D. (1980). Light-scattering measurements of hemoglobin binding to the erythrocyte membrane. Evidence for transmembrane effects related to a disulfonic stilbene binding to band 3. *Biochemistry, N.Y.* **19**, 1147–1154.

SALHANY, J. M. AND SHAKLAI, N. (1979). Functional properties of human hemoglobin bound to the erythrocyte membrane. *Biochemistry, N.Y.***18**, 893–899.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**, 660–672.

SHAKLAI, N., YGUERABIDE, J. AND RANNEY, H. M.(1977). Classification and localization of hemoglobin binding sites on the red blood cell membrane. *Biochemistry, N.Y.***16**, 5593–5597.

SOIVIO, A., WESTMAN, K. AND NYHOLM, K.(1975). A technique for repeated blood sampling of the blood of individual resting fish. *J. exp. Biol.* **62**, 207–217.

TETENS, V. AND CHRISTENSEN, N. J. (1987). Beta-adrenergic control of blood oxygen affinity in acutely hypoxia exposed rainbow trout. *J. comp. Physiol.* **157**B, 667–675.

TETENS, V. AND LYKKEBOE, G. (1981). Blood respiratory properties of rainbow trout, *Salmo gairdneri*: responses to hypoxia acclimation and anoxic incubation of blood *in vitro*. *J. comp. Physiol.* **145**, 117–125.

TETENS, V., LYKKEBOE, G. AND CHRISTENSEN, N. J. (1988). Potency of epinephrine and norepinephrine for β-adrenergic proton extrusion frm red blood cells of rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **134**, 267–280.

THOMAS, S., KINKEAD, R., WALSH, P. J., WOOD, C. M. AND PERRY, S. F. (1991). Desensitization of adrenaline-induced red cell H^+ extrusion *in vitro* after chronic exposure of rainbow trout to moderate environmental hypoxia. *J. exp. Biol.* **156**, 233–248.

THOMAS, S. AND PERRY, S. F. (1992). Control and consequences of adrenergic activation of red blood cell Na^+/H^+ exchange on blood oxygen and carbon dioxide transport. *J. exp. Zool.* **263**, 160–175.

WALDER, J. A., CHATTERJEE, R., STECK, T. L., LOW, P. S., MUSSO, G. F., KAISER, E. T., ROGERS, P. H. AND ARNOVE, A.(1984). The interaction of hemoglobin with the cytoplasmic domain of band 3 of the human erythrocyte membrane. *J. biol. Chem* **259**, 10238–10246.

WATSON, A. J. M., LEVINE, S., DONOWITZ, M. AND MONTROSE, M. H. (1992). Serum regulates Na^+/H^+ exchange in Caco-2 cells by a mechanism which is dependent on F-actin. *J. biol. Chem* **267**, 956–962.

WOLF, K.(1963). Physiological salines for freshwater teleosts. *Progve Fish Cult.* **25**, 135–140.

ZAR, J. H.(1984). *Biostatistical Analysis.* London: Prentice-Hall International Limited.