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ADRENERGIC INHIBITION OF CARBON DIOXIDE EXCRETION BY TROUT RED BLOOD CELLS *IN VITRO* IS MEDIATED BY ACTIVATION OF Na^+/H^+ EXCHANGE

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Summary

We have used a sensitive new technique to assess the mechanism(s) of adrenergic inhibition of rainbow trout (*Oncorhynchus mykiss)* red blood cell (RBC) carbon dioxide excretion *in vitro*. The effect was only apparent using blood acidified to simulate metabolic acidosis. Red blood cell CO_2 excretion was inhibited in a dose-dependent manner by physiologically relevant concentrations of noradrenaline ($10–1000\,nmol\,l^{-1}$) or adrenaline ($100–1000\,nmol\,l^{-1}$). The β-adrenoceptor antagonist propranolol abolished the inhibitory effect of noradrenaline, whereas the α-adrenoceptor antagonist phentolamine was without effect. The action of noradrenaline on RBC CO_2 excretion was mimicked by the β-adrenoceptor agonist isoproterenol, but not by the α-adrenoceptor agonist phenylephrine. Therefore, adrenergic inhibition of CO_2 excretion is mediated by RBC β-adrenoceptors, presumably of the β_1 subtype. The Na^+/H^+ exchange inhibitor amiloride effectively blocked adrenergic stimulation of Na^+/H^+ exchange (as indicated from measurements of pHe and RBC pHi) and entirely prevented the inhibition of CO_2 excretion. Noradrenaline significantly reduced the rate of CO_2 excretion even in the presence of the Cl^-/HCO_3^- exchange inhibitor SITS. Therefore, adrenergic inhibition of CO_2 excretion is accomplished *via* activation of RBC Na^+/H^+ exchange rather than by a direct inhibition of Cl^-/HCO_3^- exchange. The observed relationship between CO_2 excretion rates and the RBC transmembrane pH difference (pHe−pHi) and the occurrence of the inhibition only at low pHe provide further evidence of the linkage with RBC Na^+/H^+ exchange. We suggest that adrenergic activation of RBC

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Na^+/H^+ exchange impedes CO_2 excretion by causing a rise in intracellular HCO_3^- levels concurrent with a reduction of intracellular P_{CO_2}. The net result is a reduced gradient for HCO_3^- entry into the RBC in conjunction with a diminution of the outwardly directed P_{CO_2} gradient. Thus, the rate of formation of CO_2 from the dehydration of plasma HCO_3^- is reduced and, in turn, a portion of this CO_2 is not excreted but recycled through the red blood cell.

Introduction

In the previous paper, we have developed a sensitive new *in vitro* assay to measure the rate of CO_2 excretion by trout blood under conditions comparable to those *in vivo* (Wood and Perry, 1991). Using this assay, we confirmed that, under the typical post-exercise condition of metabolic acidosis, catecholamines cause a transient inhibition of CO_2 excretion. This observation supports the 'CO_2 retention theory' (Wood and Perry, 1985; Perry, 1986; Perry and Wood, 1989) to explain the increase in Pa_{CO_2} commonly observed after strenuous exercise or catecholamine infusion in salmonids. However, the mechanism of this inhibition remains unclear.

Our original idea was that catecholamines directly inhibited the HCO_3^- entry step, perhaps *via* a direct action on Cl^-/HCO_3^- exchange *via* band 3 (Wood and Perry, 1985; Perry, 1986). An alternative possibility, however, is that the inhibition is a consequence of β-adrenergic activation of the RBC Na^+/H^+ antiporter, the well-known RBC pHi regulatory response (see reviews by Nikinmaa and Tufts, 1989; Thomas and Motais, 1990). Indeed, in the previous paper, the magnitude of the inhibition was correlated with the magnitude of this RBC adrenergic response, though the time courses did not appear to be identical (Wood and Perry, 1991). In theory, adrenergic stimulation of Na^+/H^+ exchange and the attendant alkalization of the RBC could impede CO_2 excretion by reducing the gradient for HCO_3^- entry. In consequence, the net rate of dehydration of plasma-derived HCO_3^- by erythrocytic carbonic anhydrase would be reduced. Furthermore, the abrupt alkalization of the RBC could reduce (or temporarily abolish) the normally outwardly directed P_{CO_2} gradient between RBC and plasma, as suggested previously by Thomas and Motais (1990).

The aim of the present study was to employ the new *in vitro* assay to elucidate the underlying mechanisms for adrenergic inhibition of RBC CO_2 excretion. In particular, we were interested in the dose-dependency of the response with the two naturally occurring catecholamines (adrenaline and noradrenaline), the pharmacological identification of the adrenoceptors involved, and the relationship of the inhibition (if any) to activation of Na^+/H^+ exchange and/or to a direct action on Cl^-/HCO_3^- exchange.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of either sex weighing

between 175 and 225 g were obtained from Thistle Springs Trout Farm (Ashton, Ontario). Fish were maintained indoors in flowing dechlorinated and vigorously aerated City of Ottawa tapwater at 10°C, as described by Wood and Perry (1991). Prior to experiments, the trout were fitted with indwelling dorsal aortic catheters (Soivio *et al.* 1975) and allowed to recover for 48 h in opaque Perspex boxes.

Assessment of RBC CO_2 excretion in vitro

The new technique for assessing CO_2 excretion within trout RBCs has been described in detail in the accompanying paper (Wood and Perry, 1991) and is therefore only briefly reiterated here. Approximately 20 ml of whole blood was required for a typical single experimental run (i.e. $N=1$). Thus, it was necessary to use pooled blood obtained by slow withdrawal from the dorsal aortic cannulae of 4–5 fish (3–4 ml per fish). The pooled blood (on ice) was assayed for haematocrit (17.5±1.5%), whole-blood pH (pHe=7.84±0.03) and total carbon dioxide content (C_{CO_2}=5.3±0.3 mmol l^{-1}). Appropriate volumes of HCl (140 mmol l^{-1}) were added to simulate typical whole blood acidosis in trout immediately after exhaustive exercise (i.e. pHe=7.40±0.02; C_{CO_2}=2.02±0.07 mmol l^{-1}; Wood and Perry, 1985). In one experimental series the blood was not acidified and in this case an equivalent volume of 140 mmol l^{-1} NaCl was added. Endogenous catecholamine levels (adrenaline plus noradrenaline) were always less than 7 nmol l^{-1}. Samples (1 ml) of acidified blood (see below) or true plasma were added to glass scintillation vials (20 ml), stoppered and gassed with a humidified gas mixture to yield P_{CO_2}=0.25 kPa (1.91 mmHg), P_{O_2}=20.7 kPa (155 mmHg), remainder N_2, for 2 h at ambient water temperature in a shaking water bath. The gas mixture was provided by a gas-mixing pump (Wösthoff model M 301a/f).

2 μCi (10 μl of 200 μCi ml^{-1}) of sodium [^{14}C]bicarbonate (in teleost Ringer) was added to each 1 ml of blood or plasma. The vial was then immediately sealed with a rubber septum, from which was suspended a plastic well containing a filter paper trap (150 μl of hyamine hydroxide) for CO_2, and shaking was started. After exactly 3 min of shaking, the filter was removed and assayed for ^{14}C activity. Whole-blood or true plasma pH was determined and the remaining blood centrifuged (12 000 *g* for 2 min). The pellet was utilized to determine RBC pHi according to the freeze–thaw method (Zeidler and Kim, 1977). Samples of true plasma were assayed for ^{14}C activity (50 μl) and C_{CO_2} (100 μl) to determine plasma HCO_3^- specific activity (disints min^{-1} μmol $^{-1}$). Analytical techniques and calculations were identical to those described by Wood and Perry (1991). RBC CO_2 excretion rate was calculated by subtracting the plasma CO_2 excretion rate from the whole-blood rate. This procedure, therefore, allows an assessment of the rate of CO_2 excretion arising only from the dehydration of plasma HCO_3^-.

Assays were performed 5 min after addition of 10 μl of freshly prepared catecholamine (L-adrenaline bitartrate, L-noradrenaline bitartrate; Sigma) or adrenoceptor agonist (L-isoproterenol bitartrate, phenylephrine HCl; Sigma) in 140 mmol l^{-1} NaCl to yield nominal final concentrations of 10–1000 nmol l^{-1} (adrenaline or noradrenaline) or 1000 nmol l^{-1} (isoproterenol, phenylephrine). In

controls, 10 μl of 140 mmol l^{-1} NaCl was added to each vial. In one experimental series, 50 μl of the adrenoceptor antagonists (2×10^{-4} mol l^{-1}) propranolol (β-adrenoceptor antagonist; Sigma) or phentolamine (α-adrenoceptor antagonist; Ciba-Geigy) was added to samples of blood 30 min prior to addition of 1000 nmol l^{-1} noradrenaline to achieve final nominal antagonist levels of 1×10^{-5} mol l^{-1}; 50 μl of 140 mmol l^{-1} NaCl was added to control vials. In another experimental series, utilising 1000 nmol l^{-1} noradrenaline, blood was pre-incubated for 30 min with 50 μl of the antiporter inhibitors amiloride HCl (Na^+/H^+ exchange blocker; Sigma) or SITS (4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid; Cl^-/HCO_3^- exchange blocker; Sigma) dissolved in 2 % dimethyl sulphoxide (DMSO) to yield final nominal concentrations of 1×10^{-4} mol l^{-1} in 0.1 % DMSO; 50 μl of 2 % DMSO was added to control vials.

An additional series of experiments was performed using non-acidified blood. In this instance a simple comparison was made between the effects of 1000 nmol l^{-1} noradrenaline on HCO_3^- dehydration in acidified and non-acidified blood.

Statistical analysis

All values shown are means±1 standard error of the mean (S.E.M.). The results have been statistically analyzed using factorial analysis of variance followed by Fisher's LSD multiple-comparison test; 5 % was taken as the fiducial limit of significance.

Results

The acute addition of catecholamines to acidified whole blood caused a dose-dependent inhibition of CO_2 excretion within RBCs. The effect of noradrenaline was more pronounced than that of adrenaline (Fig. 1). A concentration of 10 nmol l^{-1} noradrenaline caused a significant 23 % inhibition of CO_2 excretion, whereas a 10-fold higher concentration of adrenaline was required to elicit a

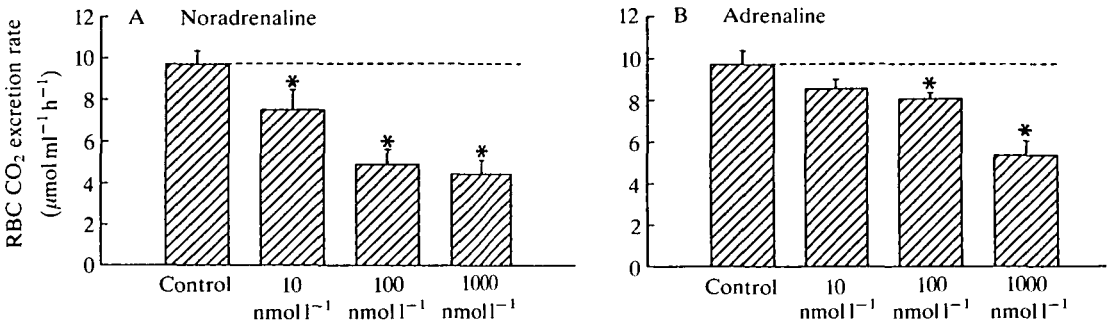

Fig. 1. The dose-dependent effects of (A) noradrenaline or (B) adrenaline on red blood cell (RBC) CO_2 excretion rate in trout blood *in vitro*. Values shown are means±1 standard error of the mean (S.E.M.); N=6 in all cases. * indicates a significant difference (P<0.05) from the control value.

Table 1. *The dose-dependent effects of the catecholamines, noradrenaline (NA) or adrenaline (AD) on whole-blood pH (pHe), RBC intracellular pH (pHi) and the RBC transmembrane pH gradient (pHe−pHi)*

Treatment	pHe	pHi	pHe−pHi
Control	7.421±0.04 (5)	7.117±0.02 (5)	0.304±0.02 (5)
10 nmol l^{-1} NA	7.308±0.06 (5)	7.146±0.02 (5)	0.162±0.05 (5)*
100 nmol l^{-1} NA	7.084±0.04 (5)*	7.156±0.02 (5)*	−0.074±0.02 (5)*
1000 nmol l^{-1} NA	7.007±0.03 (5)*	7.197±0.02 (5)*	−0.190±0.02 (5)*
10 nmol l^{-1} AD	7.287±0.03 (5)*	7.150±0.03 (5)	0.137±0.03 (5)*
100 nmol l^{-1} AD	7.282±0.03 (5)*	7.151±0.02 (5)*	0.131±0.04 (5)*
1000 nmol l^{-1} AD	7.123±0.02 (5)*	7.185±0.02 (5)*	−0.064±0.01 (5)*

All measurements were made at the end of the assay, 8 min after the addition of catecholamine.

Values shown are means±1 S.E.M. (*N*); * indicates significantly different ($P<0.05$) from control value.

similar degree of inhibition. At the highest concentrations (1000 nmol l^{-1}) utilised, RBC CO_2 excretion was reduced by 54% and 45% by noradrenaline and adrenaline, respectively. The catecholamines also induced pronounced and dose-dependent reductions in the RBC transmembrane pH gradient (pHe−pHi), owing to simultaneous changes in both pHe and pHi (Table 1).

Fig. 2 illustrates the effects of a variety of adrenoceptor antagonists and agonists on RBC CO_2 excretion. Pre-incubation of whole blood for 30 min with the β-adrenoceptor antagonist propranolol (10^{-5} mol l^{-1}) entirely abolished the inhibitory effect of noradrenaline (1000 nmol l^{-1}) on CO_2 excretion as well as preventing the usual changes in pHe, pHi and pHe−pHi (Table 2). In contrast, pre-incubation with an equivalent concentration of the α-adrenoceptor antagonist phentolamine did not significantly alter the effects of noradrenaline on either CO_2 excretion or pHe, pHi or pHe−pHi (Fig. 2; Table 2). Addition of these antagonists alone was without effect on any measured or calculated variable (Fig. 2; Table 2). The β-adrenoceptor agonist isoproterenol (1000 nmol l^{-1}) mimicked the effects of an equivalent dose of noradrenaline, whereas the α-adrenoceptor agonist phenylephrine (1000 nmol l^{-1}) had no effect on CO_2 excretion (Fig. 2), although it did significantly affect blood acid–base status (Table 2).

In an attempt to elucidate the post-receptor mechanism by which catecholamines inhibit CO_2 excretion, the effects of noradrenaline (1000 nmol l^{-1}) were assessed in the presence or absence of the Na^+/H^+ exchange blocker amiloride (10^{-4} mol l^{-1}) or the Cl^-/HCO_3^- exchange blocker SITS (10^{-4} mol l^{-1}). Incubation of whole blood with amiloride prevented the usual adrenergic inhibition of CO_2 excretion (Fig. 3). In addition, the changes in pHe and pHe−pHi were significantly reduced by amiloride (Table 3), indicating that this treatment was indeed effective in blocking (at least partially) the Na^+/H^+ exchanger. Amiloride alone did not affect the rate of RBC CO_2 excretion (Fig. 3). The addition of SITS

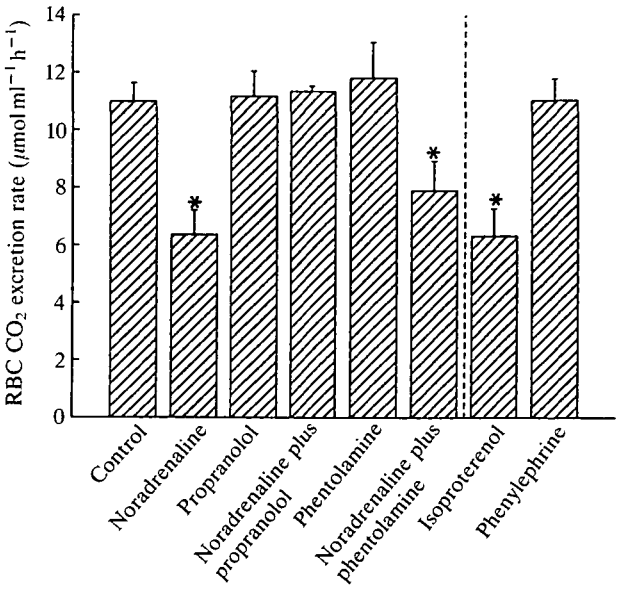

Fig. 2. The effects of 1000 nmol l^{-1} noradrenaline, β-adrenoceptor blockade with propranolol (10^{-5} mol l^{-1}), α-adrenoceptor blockade with phentolamine (10^{-5} mol l^{-1}), the β-adrenoceptor agonist isoproterenol (1000 nmol l^{-1}) and the α-adrenoceptor agonist phenylephrine (1000 nmol l^{-1}) on red blood cell (RBC) CO_2 excretion rate *in vitro*. Values shown are means±1 S.E.M.; N=6 in all cases. * indicates a significant difference (P<0.05) from the control value.

Table 2. *The effects of noradrenaline, β-adrenoceptor blockade (propranolol, 10^{-5} mol l^{-1}) α-adrenoceptor blockade (phentolamine, 10^{-5} mol l^{-1}), the β-adrenoceptor agonist isoproterenol (1000 nmol l^{-1}) and the α-adrenoceptor agonist phenylephrine (1000 nmol l^{-1}) on whole-blood pH (pHe), RBC intracellular pH (pHi) and the RBC transmembrane pH gradient (pHe−pHi)*

Treatment	pHe	pHi	pHe−pHi
Control	7.471±0.03 (6)	7.132±0.01 (6)	0.339±0.02 (6)
1000 nmol l^{-1} NA	7.058±0.04 (6)*	7.219±0.03 (6)*	−0.161±0.04 (6)*
Propranolol	7.437±0.03 (6)	7.140±0.01 (6)	0.298±0.03 (6)
NA+propranolol	7.463±0.02 (6)	7.135±0.02 (6)	0.327±0.03 (6)
Phentolamine	7.454±0.03 (6)	7.161±0.02 (6)	0.293±0.03 (6)
NA+phentolamine	7.128±0.04 (6)*	7.228±0.01 (6)*	−0.100±0.04 (6)*
Isoproterenol	7.003±0.04 (6)*	7.244±0.01 (6)*	−0.241±0.03 (6)*
Phenylephrine	7.323±0.02 (6)*	7.176±0.02 (6)	0.147±0.03 (6)*

All measurements were made at the end of the assay, 8 min after the addition of 1000 nmol l^{-1} noradrenaline (NA) or isoproterenol.

Values shown are means±1 S.E.M. (N); * indicates significantly different (P<0.05) from control value.

caused a marked 70% inhibition of CO_2 excretion. However, this reduced rate was further substantially inhibited by the presence of noradrenaline. Indeed, on a relative basis, the percentage inhibition (53%) by noradrenaline in SITS-treated blood was identical to that (54%) in control blood (Fig. 3).

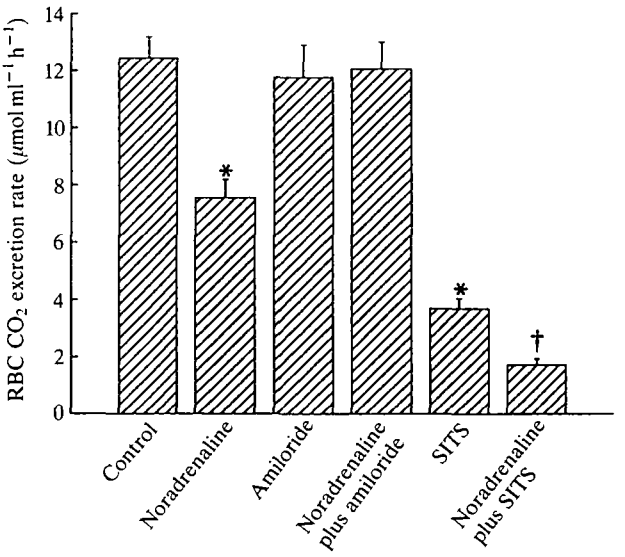

Fig. 3. The effects of 1000 nmol l^{-1} noradrenaline on red blood cell (RBC) CO_2 excretion rates *in vitro* in the presence or absence of the Na^+/H^+ and Cl^-/HCO_3^- exchange blockers amiloride (10^{-4} mol l^{-1}) and SITS (10^{-4} mol l^{-1}), respectively. Values shown are means±1 S.E.M.; N=6 in all cases. * indicates a significant difference (P<0.05) from the control value; † from the value using SITS alone.

Table 3. *The effects of noradrenaline (NA) on whole-blood pH (pHe), RBC intracellular pH (pHi) and the RBC transmembrane pH gradient (pHe−pHi) in the presence or absence of the Na^+/H^+ exchange and Cl^-/HCO_3^- exchange blockers amiloride and SITS*

Treatment	pHe	pHi	pHe−pHi
Control	7.461±0.01 (6)	7.171±0.02 (6)	0.293±0.04 (6)
1000 nmol l^{-1} NA	7.069±0.02 (6)*	7.202±0.04 (6)	−0.133±0.05 (6)*
Amiloride	7.450±0.02 (6)	7.172±0.02 (6)	0.278±0.03 (6)
NA+amiloride	7.354±0.01 (6)*†	7.188±0.03 (6)	0.166±0.03 (6)*†
SITS	7.364±0.02 (6)*	7.171±0.02 (6)	0.199±0.04 (6)
SITS+NA	6.983±0.01 (6)*	7.293±0.03 (6)*	−0.313±0.03 (6)*

All measurements were made at the end of the assay, 8 min after the addition of NA (1000 nmol l^{-1}).

Values shown are means±1 S.E.M. (N); * indicates significantly different (P<0.05) from control group; † significantly different from value in the NA group.

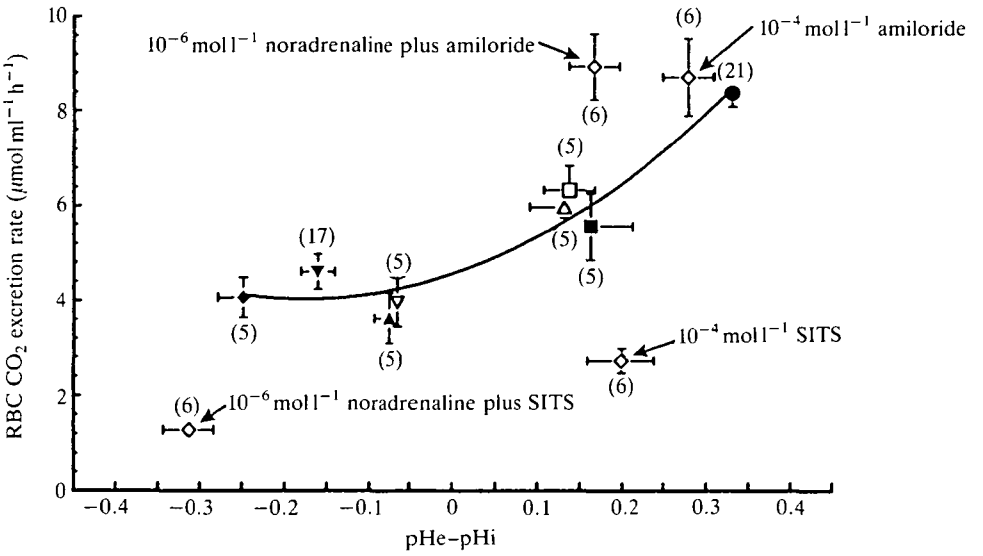

Fig. 4. The relationship (curve drawn by eye) between red blood cell (RBC) CO_2 excretion rate *in vitro* and the transmembrane pH gradient (pHe−pHi). This relationship was generated by monitoring dehydration rates in control blood (●; endogenous [noradrenaline]=0.84±0.2 nmol l^{-1}, endogenous [adrenaline]=4.71±1.7 nmol l^{-1}) and after addition of exogenous noradrenaline (10 nmol l^{-1}=■, 100 nmol l^{-1}=▲, 1000 nmol l^{-1}=▼), adrenaline (10 nmol l^{-1}=□, 100 nmol l^{-1}=△, 1000 nmol l^{-1}=▽) or 1000 nmol l^{-1} isoproterenol (◆). Superimposed about this relationship are the rates of CO_2 excretion after addition of the Na^+/H^+ and Cl^-/HCO_3^- exchange blockers amiloride (10^{-4} mol l^{-1}) and SITS (10^{-4} mol l^{-1}), respectively, in the presence or absence of 1000 nmol l^{-1} noradrenaline. Values shown are means±1 S.E.M.; sample sizes are shown in parentheses.

Fig. 4 illustrates the curvilinear relationship between RBC CO_2 excretion and pHe−pHi which was generated using a range of concentrations (10–1000 nmol l^{-1}) of noradrenaline and adrenaline and 1000 nmol l^{-1} isoproterenol. These data support the hypothesis that activation of the Na^+/H^+ exchanger (as indicated by the changes in pHe−pHi) is the mechanism underlying the adrenergic inhibition of CO_2 excretion. It is noteworthy that, in the presence of amiloride, noradrenaline did not elicit a reduction of CO_2 excretion, despite a small reduction of pHe−pHi (Fig. 4). Indeed, the rate of CO_2 excretion obtained in the presence of noradrenaline plus amiloride was significantly greater than that predicted from the curvilinear relationship. Possible reasons for this deviation are discussed below. Predictably, SITS (in the presence or absence of noradrenaline) caused a more pronounced inhibition of CO_2 excretion than predicted (Fig. 4), owing to direct inhibition of the Cl^-/HCO_3^- exchanger.

All the experiments described above were performed using acidified blood, 5–8 min after catecholamine addition, based on the protocol of Wood and Perry (1991), because the adrenergic inhibition of RBC CO_2 excretion was well-defined under these conditions. Furthermore, the situation was appropriate because w

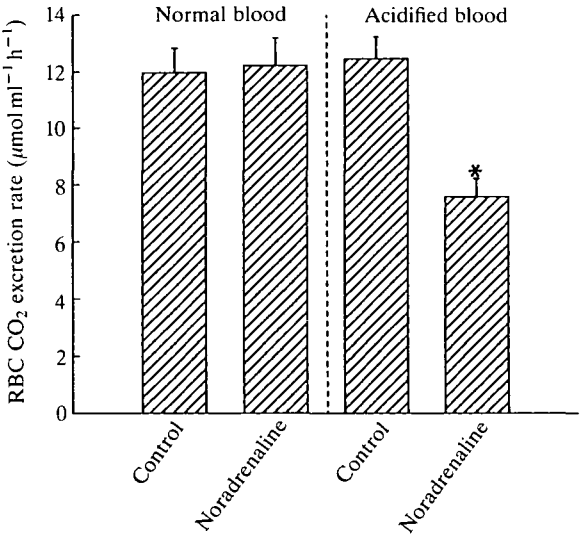

Fig. 5. The effect of 1000 nmol l^{-1} noradrenaline on red blood cell (RBC) CO_2 excretion rate *in vitro* in normal (non-acidified; pH=7.88; $[HCO_3^-]$=5.5 mmol l^{-1}) or acidified (pH=7.46; $[HCO_3^-]$=2.1 mmol l^{-1}) blood. Values shown are means ±1 S.E.M.; N=6 in each case. * indicates a significant difference (P<0.05) from the control value.

were testing the hypothesis that the effect is linked to activation of the Na^+/H^+ exchanger, and acidification is known to enhance the responsiveness of this antiporter to catecholamines. Preliminary experiments by Wood and Perry (1991) suggested that the adrenergic inhibition of CO_2 excretion was absent if the blood was kept at normal pHe. However, those trials were performed at inappropriate times (30–120 min after catecholamine addition).

Therefore, in a separate series of experiments, we tested whether acidification of the blood was indeed required to evoke adrenergic inhibition of RBC CO_2 excretion. The trials were run 5–8 min after addition of noradrenaline (1000 nmol l^{-1}). The same original blood (N=6), half pre-equilibrated for 2 h at normal acid–base status (pHe=7.88±0.02, plasma $[HCO_3^-]$=5.5±0.1 mmol l^{-1}) the other half pre-equilibrated for the same period under metabolic acidosis (pHe=7.46±0.01, plasma $[HCO_3^-]$=2.1±0.1 mmol l^{-1}), was used in these experiments. The results (Fig. 5) clearly demonstrated that RBC CO_2 excretion is totally insensitive to noradrenaline when the blood is non-acidified, thereby supporting our contention of linkage to Na^+/H^+ exchange. However, the absence of acidosis only reduced and did not abolish the Na^+/H^+ exchange response. Whole-blood pH (pHe) still fell (to 7.58±0.03) and pHi still increased slightly (from 7.37±0.03 to 7.42±0.02) in response to noradrenaline in non-acidified blood. Interestingly, on Fig. 4, this point lies above the predicted relationship in an identical position to the other anomalous point (noradrenaline after amiloride).

Discussion

The results of the present study unequivocally demonstrate adrenergic inhibition of RBC CO_2 excretion *in vitro* and suggest that the underlying mechanism is linked to β-adrenoceptor-mediated activation of the Na^+/H^+ exchanger rather than *via* a direct action on the Cl^-/HCO_3^- exchanger. The involvement of RBC β-adrenoceptors is indicated by the experiments utilizing β-adrenoceptor agonists or antagonists. Although there is conflicting experimental evidence (Bennett and Rankin, 1985; Tetens *et al.* 1988), the current consensus is that the RBC β-adrenoceptors are of the β_1 subtype (see review by Nikinmaa and Tufts, 1989). Our observation that noradrenaline is a more potent inhibitor than adrenaline of CO_2 excretion further supports this consensus.

RBC CO_2 excretion can be modified not only by direct antagonism of the Cl^-/HCO_3^- exchange on the RBC membrane (e.g. the inhibition caused by SITS; see also Perry *et al.* 1982), but also by a linkage between the Cl^-/HCO_3^- and Na^+/H^+ exchange pathways. It is the latter mechanism that is the basis of the adrenergic inhibition of RBC HCO_3^- dehydration. Catecholamines cause a β-adrenergic, cyclic-AMP-dependent stimulation of the Na^+/H^+ antiporter in the RBCs of trout and many other teleost species (Nikinmaa and Tufts, 1989). The consequent extrusion of H^+ from the RBC causes a pronounced reduction of pHe, a smaller rise in RBC pHi and therefore a marked alteration of the transmembrane pH gradient (pHe−pHi). The change in pHe−pHi after adrenergic stimulation is directly related to the activity of the Na^+/H^+ exchanger and is largely blocked by amiloride. Thus, the inhibitory effects of amiloride on adrenergic impairment of CO_2 excretion and the observed relationship between CO_2 excretion rates and pHe−pHi (Fig. 4) both support the idea that adrenergic activation of Na^+/H^+ exchange is responsible for the inhibition of RBC CO_2 excretion.

Although Wood and Perry (1991) portrayed a simple correlation between RBC CO_2 excretion and pHe−pHi, the more extensive data of the present study suggest that the true relationship is curvilinear (Fig. 4). The inhibition by SITS was far greater than that predicted by this curvilinear relationship, and reveals the additional effect of direct inhibition of the Cl^-/HCO_3^- exchanger. The finding that noradrenaline caused an additional 53% reduction in CO_2 excretion after SITS treatment (equal, on a relative basis, to its effect before SITS; Fig. 3), correlated with a large decrease in pHe−pHi (Table 3; Fig. 4), provides further evidence that the adrenergic effect is not directly on the Cl^-/HCO_3^- exchanger, but rather *via* Na^+/H^+ exchange.

It is noteworthy that there was significant deviation from the predicted relationship between CO_2 excretion and pHe−pHi in adrenergically stimulated blood pre-treated with amiloride (Fig. 4), and also in non-acidified blood treated with noradrenaline (identical position on Fig. 4). Specifically, the rate of CO_2 excretion was greater than predicted if activation of Na^+/H^+ exchange was the solitary determinant of the change in CO_2 excretion rates after catecholamine addition. The most likely explanation is that these data may reflect a minor adrenergic stimulation of CO_2 excretion which was unmasked by these treatments

This apparent stimulation of CO_2 excretion may simply reflect the pHe-dependence of the Cl^-/HCO_3^- exchanger, the activity of which (as measured using SO_4^{2-}) increases as extracellular pH is reduced (Romano and Passow, 1984; Fievet *et al.* 1988). The observation of stable rates of CO_2 excretion in blood treated with the α-adrenoceptor agonist phenylephrine (Fig. 2), despite a significant reduction of pHe−pHi (Table 2), also supports this hypothesis. The net effect of catecholamines on CO_2 excretion may be the result of two opposing phenomena; an inhibitory effect linked to activation of the Na^+/H^+ exchanger as well as a stimulatory component linked to the pHe-dependence of the Cl^-/HCO_3^- exchanger. Clearly, under most conditions, the inhibitory response is dominant.

The proposed mechanisms underlying the linkage between adrenergic activation of Na^+/H^+ exchange and inhibition of RBC CO_2 excretion are as follows. Activation of the Na^+/H^+ exchanger and concurrent alkalization of the RBC must cause an immediate rise in intracellular HCO_3^- levels, owing to physicochemical buffering and the presence of intra-erythrocytic carbonic anhydrase (Thomas and Motais, 1990). This results in a diminution of the electrochemical gradient for the inward movement of HCO_3^- and hence reduces the rate of HCO_3^- dehydration within the RBC. Unfortunately, the extracellular acid–base disequilibrium after adrenergic stimulation prevents us from calculating the changes in the transmembrane HCO_3^- gradient. It is obvious, nonetheless, that the pronounced changes in pHe−pHi must be associated with significant reductions in this gradient. Owing to the pronounced sensitivity of the RBC CO_2 excretion rate to acute changes in the transmembrane HCO_3^- gradient (see Fig. 3 in Wood and Perry, 1991), it is possible to achieve significant inhibition with relatively minor changes in the HCO_3^- gradient. The other proposed mechanism causing the adrenergic reduction of CO_2 excretion is CO_2 recycling between the plasma and the interior of the RBC immediately after activation of the Na^+/H^+ exchanger (Motais *et al.* 1989; Thomas and Motais, 1990). Under normal conditions, P_{CO_2} decreases from RBC to plasma to water, which permits effective excretion of CO_2 according to existing partial pressure gradients. In our experimental apparatus, the P_{CO_2} decreases from RBC to plasma to gas phase (see Wood and Perry, 1991), which is essentially analogous to the *in vivo* situation. The massive stimulation of proton extrusion from the RBC after addition of catecholamine causes rapid formation of intracellular HCO_3^- *via* the catalysed hydration of CO_2. Since a significant portion of this HCO_3^- must leave the RBC in exchange for Cl^-, and CO_2 cannot be reintroduced into the cell as rapidly as it is being hydrated to HCO_3^-, a major reduction of intracellular P_{CO_2} must ensue. Therefore, a portion of the CO_2 formed at the uncatalysed rate from HCO_3^- and H^+ in the plasma is recycled through the RBC rather than diffusing into the water (or the gas phase in the *in vitro* assay). Both the reduction of the transmembrane HCO_3^- gradient and CO_2 recycling will cause a transient inhibition of net CO_2 excretion *in vivo*. The CO_2 recycling is associated with the phase of extracellular acid–base disequilibrium. Red blood cell HCO_3^- levels presumably remain elevated (since pHi remains elevated; see Fig. 6 of Wood and Perry, 1991) after the Na^+/H^+

exchanger is once again inactivated and can therefore contribute to a more prolonged inhibition of RBC HCO_3^- dehydration. Despite this, however, we suggest that normal CO_2 excretion rates are re-established *in vivo* owing to a rise in P_{CO_2} as CO_2 backs up in the system, thereby restoring the transbranchial flux.

Adrenergic inhibition of RBC CO_2 excretion, mediated by activation of Na^+/H^+ exchange, is sufficient to account for the transitory retention of CO_2 in fish blood after exhaustive exercise (Wood and Perry, 1985; Perry and Wood, 1989) and during intra-arterial catecholamine infusion (Perry and Vermette, 1987; Vermette and Perry, 1988). In the present study, this effect was demonstrated at realistic levels of catecholamines (10–1000 nmol l^{-1}) using physiological levels of HCO_3^- in blood appropriately acidified to mimic post-exercise acidosis. Previous studies *in vivo* (Steffensen *et al.* 1987; Playle *et al.* 1990) or *in vitro* (Tufts *et al.* 1988) were unable to demonstrate adrenergic inhibition of whole-animal CO_2 excretion and RBC CO_2 excretion, respectively. There are several possible reasons for the discrepancy. First, we now know that the inhibitory effect of catecholamines occurs only under acidotic conditions, and not when blood pH is normal (Fig. 5). Presumably, this reflects the potentiating effects of extracellular acidosis on adrenergic stimulation of the RBC Na^+/H^+ antiporter (see review by Nikinmaa and Tufts, 1989) in addition to possible allosteric modification of the Na^+/H^+ antiporter itself (e.g. Aronson *et al.* 1982). Second, the response is transient, reaching a maximum after approximately 5 min and disappearing after about 30 min (Wood and Perry, 1991). Third, inhibition of HCO_3^- dehydration *in vivo* would cause a rise in P_{CO_2} of the blood and quickly re-establish CO_2 excretion at a new steady state (see above). Thus, inappropriate methodology and/or sampling times may have prevented detection of adrenergic responses in other studies. Fourth, the protocol of acutely raising the blood HCO_3^- concentration to 100 mmol l^{-1} in the 'boat' technique (Tufts *et al.* 1988) may have obscured the proposed linkage between RBC CO_2 excretion and activation of Na^+/H^+ exchange in that study.

The functional significance of adrenergic inhibition of CO_2 excretion is unclear but may be related to the control of ventilation. For example, after exhausting exercise fish are motionless and blood oxygen status is unchanged so stimulation of neither proprioceptors nor oxygen chemoreceptors can account for the observed hyperventilation at this time. Recent studies (Heisler *et al.* 1988; R. Kinkead and S. F. Perry, unpublished results; Wood *et al.* 1990) have shown that relatively small changes in blood pH and/or P_{CO_2} have marked effects on ventilation in fish. Moreover, injection of bovine carbonic anhydrase into the circulation of rainbow trout not only reduced the rise in Pa_{CO_2} but also significantly depressed the post-exercise hyperventilation (Perry and Wood, 1989). Thus, we suggest that adrenergic inhibition of RBC CO_2 excretion and the associated respiratory acidosis after vigorous exercise serve to increase gill ventilation in the absence of other appropriate ventilatory stimulants.

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