J. exp. Biol. ***192****, 239–252 (1994)*
Printed in Great Britain © The Company of Biologists Limited 1994

ACTIVE Na^+-, Cl^-- AND HCO_3^--DEPENDENT ACID EXTRUSION IN ATLANTIC COD RED BLOOD CELLS IN WINTER ACTIVATED BY HYPERCAPNIA

M. BERENBRINK AND C. R. BRIDGES*

Institut für Zoologie, Lehrstuhl für Tierphysiologie, Heinrich-Heine-Universität, Universitätsstraße 1, 40225 Düsseldorf, Germany and *Biologische Anstalt Helgoland, Meeresstation Helgoland, Postfach 180, 27498 Helgoland, Germany*

Accepted 29 March 1994

Summary

The relationship between intracellular pH (pHi) and extracellular pH (pHe) was investigated in red blood cells from the Atlantic cod (*Gadus morhua*) in carbon dioxide/bicarbonate-buffered salines. In summer animals (August/September), similar transmembrane distribution ratios of chloride ($[Cl^-]_i/[Cl^-]_e$=$r Cl^-$) and protons ($[H^+]_e/[H^+]_i$=rH^+) suggested a passive Donnan distribution of these ions across the red blood cell membrane at pHe 6.7–8.4. In winter animals (February/March), a marked discrepancy occurred between rH^+ and rCl^- at low pHe values. The pronounced increase in rH^+ resulted in significantly higher pHi values compared with those of red blood cells from summer animals and at pHe 6.7 pHi exceeded pHe by 0.3 units. The increases in rH^+ values were completely abolished by cyanide and 2,4-dinitrophenol. The high disequilibrium rH^+ values were sodium-, chloride- and bicarbonate-dependent. During hypercapnic acidosis, proton equivalents were extruded from the red blood cell. The resulting high rH^+ values were accompanied by a reduced chloride shift into the red blood cell and a ouabain-insensitive net sodium influx. The net sodium influx into red blood cells from winter animals was significantly reduced in the presence of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid). The results suggest the activation of a Na^+-dependent Cl^-/HCO_3^-exchanger at low pHe in the red blood cells of the Atlantic cod in winter.

Introduction

Protons are passively distributed across the membrane of mammalian red blood cells according to a Donnan equilibrium and any change in the extracellular pH (pHe) affects the intracellular compartment (Hladky and Rink, 1977). The intracellular pH (pHi) is dependent on the properties of the intracellular buffers (mainly haemoglobin), the enzyme carbonic anhydrase within the cells and on three passive transport processes

*To whom reprint requests should be addressed at: Institut für Zoologie, Lehrstuhl für Tierphysiologie, Heinrich-Heine-Universität, Universitätsstraße 1, 40225 Düsseldorf, Germany.

Key words: intracellular pH, ion regulation, red blood cells, cod, *Gadus morhua*, season.

across the red blood cell membrane. These are (1) the diffusion of carbon dioxide, (2) osmotically induced transfer of water and (3) the electroneutral exchange of chloride for bicarbonate mediated by the anion exchanger (Hladky and Rink, 1977). The red blood cell anion exchanger has been described in the nucleated red blood cells of teleosts (Romano and Passow, 1984) and it is generally accepted that chloride and protons are in Donnan equilibrium across the red blood cell membranes of teleosts. In a study on carp red blood cells, Albers and Goetz (1985) showed that between pHe 7.0 and 8.2 the transmembrane distribution ratio of protons ($rH^+=[H^+]_e/[H^+]_i$) closely resembled the transmembrane distribution ratio of chloride ($rCl^-=[Cl^-]_i/[Cl^-]_e$). rH^+ and rCl^- both varied with pHe as expected for a passive Donnan distribution. Similar results, over the same range of pHe values, were obtained with rainbow trout red blood cells (Heming *et al.* 1986). In contrast to these findings, Nikinmaa *et al.* (1987) observed a significant difference between the measured pHi and the pHi calculated from rCl^- in rainbow trout red blood cells below pHe 7.3.

The aim of the present study was to investigate the distribution of protons across the red blood cell membrane in the Atlantic cod, *Gadus morhua*. The lower value of the pHe range investigated was extended to 6.7 since such acidotic pH values may be reached in blood passing through the teleost gas gland in the swimbladder (Steen, 1963). The study was conducted on Atlantic cod obtained both in summer and winter, since changes in the plasma ionic composition with ambient water temperature have been reported in this species (Woodhead and Woodhead, 1959).

Materials and methods

Animals and preparation of red blood cells

Atlantic cod, *Gadus morhua* (200–1000 g), were caught in the German Bight near Helgoland in August 1991 and September 1993 (referred to as summer animals) and in February 1992 and March 1993 (winter animals). They were kept in running, aerated sea water (at 14–16 °C in summer and 8–10 °C in winter) at the Marine Biological Station, Helgoland, for between 1 and 3 weeks before they were used in the experiments. Winter animals would not take any food, summer animals were fed five times a week on small pieces of fish.

Animals were killed using a sharp blow to the head and blood was sampled immediately from the caudal vein. Red blood cells and plasma were separated by centrifugation in a table centrifuge (5 min at 1000 ***g***, RT; Heraeus Christ, Osterode, Germany). To remove residual catecholamines, red blood cells were washed three times in 2–4 volumes of ice-cold experimental saline. The saline consisted of (in $mmol\,l^{-1}$): NaCl, 144; $NaHCO_3$, 10; KCl, 6; $CaCl_2$, 5; $MgSO_4$, 1; D-glucose, 5. The red blood cells were then incubated overnight at 4 °C to ensure that they were no longer in a catecholamine-stimulated condition (Bourne and Cossins, 1982). At the onset of the experiments, the red blood cells were washed again in the experimental saline, resuspended at a haematocrit value of 20 % and equilibrated for 40 min with humidified air in an intermittently rotating glass tonometer at 15 °C (Zentralwerkstatt für Biologie, Universität Düsseldorf). All subsequent experiments were carried out at 15 °C.

Experimental protocol

The pHe of red blood cells from summer and winter animals was changed by equilibrating 800 μl samples of red blood cell suspensions for 20 min with humidified CO_2/air mixtures supplied by a gas-mixing pump (model 2M303/a-F, Wösthoff KG, Bochum, Germany). CO_2/air mixtures in the range from 0 % CO_2+100 % air to 10 % CO_2+90 % air (P_{CO_2} 0.03–10.16 kPa) yielded mean pHe values between 8.4 and 6.7. Following the equilibration period, pHe, pHi and red blood cell ion and water content were determined. The same experiment was carried out on red blood cells from summer animals which had received 10 μl of DIDS (10^{-4} mol l^{-1} final concentration, dissolved in dimethylsulphoxide) prior to the equilibration.

In a second series, red blood cells from winter animals were incubated under various conditions and pHe, pHi and red blood cell ion and water content were measured at the end of each incubation. The effects of KCN (10^{-3} mol l^{-1} final concentration) and 2,4-dinitrophenol (10^{-4} mol l^{-1} final concentration, dissolved in ethanol) were tested by equilibrating red blood cells for 40 min with 10 % CO_2 and 90 % air in the presence of these inhibitors. CO_2/HCO_3^--free conditions were achieved by incubating red blood cells under a nitrogen atmosphere in Hepes buffer (pH 6.7) with the same composition as the saline given above, except for $NaHCO_3$, which was replaced by 15 mmol l^{-1} Hepes. Additionally, red blood cells were washed (three times) in media containing low sodium (10 mmol l^{-1}) or low chloride (10 mmol l^{-1}) concentrations and equilibrated for 40 min with 10 % CO_2 and 90 % air. In this case, choline or nitrate was substituted for sodium or chloride to maintain the osmolality of the salines. Finally, the effect of DIDS (10^{-4} mol l^{-1} final concentration, dissolved in dimethylsulphoxide) was tested by equilibrating red blood cell suspensions for 1 h with 1 % CO_2 and 99 % air and then adding the drug. Samples for red blood cell water and ion content were taken immediately before and 10 min after DIDS had been added.

In a third series, the effect of acute hypercapnia was tested in winter animals by pre-equilibrating red blood cells for 40 min with 1 % CO_2 and 99 % air and then, at time zero, switching to 10 % CO_2 and 90 % air. Samples for the determination of pHe, pHi and red blood cell ion and water content were taken at intervals from −20 to 40 min. The experiment was repeated in the presence of ouabain (dissolved in dimethylsulphoxide, 10^{-4} mol l^{-1} final concentration, added 1 min before the change in P_{CO_2}).

Analytical procedures

Haematocrit was measured using a Micro-Compur haematocrit centrifuge (M1100, Compur Elektronik, München, Germany). Extracellular pH was determined using a Radiometer BMS3 Mk2 apparatus with a G 299A micro pH glass electrode, thermostatted at 15 °C, and a PHM73 monitor (Radiometer, Copenhagen, Denmark). Red blood cells were separated from the incubation medium by centrifugation in two 400 μl Eppendorf tubes (2 min 10 000 revs min^{-1}, RT, Centrifuge 5415 C, Eppendorf GmbH, Hamburg, Germany) and quickly frozen in liquid nitrogen.

One tube was cut with a razor blade 2 mm below and above the boundary between supernatant and pellet. The pellet was thawed, frozen and thawed again within 10 min and

pHi was measured directly in the red blood cell haemolysate. Control experiments showed that storage in liquid nitrogen for up to 24 h did not affect the measured pHi values, even in samples equilibrated with 10 % CO_2. To avoid errors resulting from liquid junction potentials at the pH electrode, 20–30 μl of the experimental saline was introduced between the haemolysate or red blood cell suspension and the saturated KCl solution of the reference electrode (Boutilier *et al.* 1985).

The second 400 μl tube was kept frozen at −80 °C until water and ion content were measured. The red blood cell fraction and the supernatant were separated as described above. Red blood cell water content was determined after drying the preweighed pellet for 16 h at 75 °C. The proportion of trapped extracellular fluid in the red blood cell pellet was determined by adding 5 μl of an [^{3}H]inulin solution (3700 MBq l^{-1}, specific activity 47 GBq $mmol^{-1}$, Amersham, England) to red blood cell suspensions (300 μl, haematocrit 20 %) that had been equilibrated for 20 min with different CO_2/air mixtures). The red blood cell suspensions were then centrifuged in 400 μl Eppendorf tubes and the red blood cell fraction was separated from the supernatant as described above. 50 μl of the supernatant and about 20 mg of the red blood cell pellet were precipitated with 100 μl of 0.6 mol l^{-1} perchloric acid. The solutions were centrifuged again to remove the debris and the relative amount of [^{3}H]inulin in the resulting supernatants was determined by liquid scintillation counting (scintillation fluid, Ready Protein; counter, model LS-1801; both Beckman, München, Germany). The proportion of trapped extracellular fluid was independent of the CO_2 tension in the equilibration gas and amounted to 60.1±10.8 μl per milligram of the wet red blood cell pellet (N=8 equilibrations). This trapping correction was routinely applied to all final calculations.

Dried red blood cells were homogenized in 200 μl of distilled water using a dentist's drill (1 min at 1000 revs min^{-1}, then 45 s at 10 000 revs min^{-1}; K9 type 950, KaVo GmbH, Leutkirch, Germany). The homogenate was vigorously mixed (1 min) and then centrifuged (5 min at 10 000 ***g***, RT). The chloride content of the resulting supernatant was determined coulometrically using a chloride titrator (CMT 10, Radiometer, Copenhagen, Denmark). Sodium and potassium in the supernatant were either measured directly by flame spectroscopy (Jenway PFP7, Felsted, England) or diluted 1:2 (v/v) with acetonitrile (Merck, Darmstadt, Germany), centrifuged at 4 °C (5 min, 10 000 ***g***) and determined with high performance ion chromatography on a DX-100 system (DIONEX GmbH, Idstein, Germany). Extracellular chloride was measured directly in the thawed supernatants of the 400 μl tubes using the method described above.

Calculations and statistics

The transmembrane chloride and proton distribution ratios ($r\mathrm{Cl}^-$ and $r\mathrm{H}^+$) were calculated from the intracellular and extracellular concentrations assuming similar activity coefficients inside and outside the red blood cells: $r\mathrm{Cl}^-=[\mathrm{Cl}^-]_i/[\mathrm{Cl}^-]_e$ and $r\mathrm{H}^+=[\mathrm{H}^+]_e/[\mathrm{H}^+]_i=10^{(\mathrm{pHi}-\mathrm{pHe})}$. The lines fitting a data set were constructed with a curve-fitting programme (SigmaPlot 4.1, Jandel Scientific, Corte Madera, USA) using appropriate polynomial, hyperbolic and exponential functions. All values are given as means ± S.D. Differences between means were statistically evaluated with Student's t-test for independent samples with $P<0.05$ being taken as the level of significance.

Results

In Atlantic cod red blood cells obtained in summer, any change in pHe between 6.7 and 8.4 was transferred to the intracellular compartment and affected pHi (Fig. 1). The changes in pHi per unit change in pHe were most pronounced below pHe 7.2 and above pHe 7.9. Within this interval, in the flat part of the curve, the relationship between pHi and pHe followed the equation: pHi=0.41pHe+4.07 (r=0.96, N=12). In winter animals, equilibration of red blood cells with 0 % and 1 % CO_2 + air produced virtually the same intracellular and extracellular pH values as those in summer animals (Fig. 1). At 10 % CO_2, however, the pHi was significantly elevated (about 0.3 pH units) above the corresponding value in summer animals.

To test whether protons were passively distributed across the red blood cell membrane of both summer and winter animals, $r\mathrm{H}^+$ was calculated and compared with $r\mathrm{Cl}^-$ (Fig. 2). In summer animals, there was a small but significant difference between $r\mathrm{Cl}^-$ and $r\mathrm{H}^+$ but both $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ showed characteristic slightly S-shaped and strikingly similar pHe-dependencies over the whole range studied. Red blood cells from winter animals at 0 % and 1 % CO_2 showed the same $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ values as their counterparts in summer. However, at 10 % CO_2, $r\mathrm{H}^+$ was significantly higher and $r\mathrm{Cl}^-$ significantly lower than in summer animals. The marked discrepancy developing between $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ at high CO_2 tensions in winter animals suggested that either protons or chloride ions or both were no longer passively distributed across the red blood cell membrane.

The possibility of an actively maintained proton gradient across the red blood cell

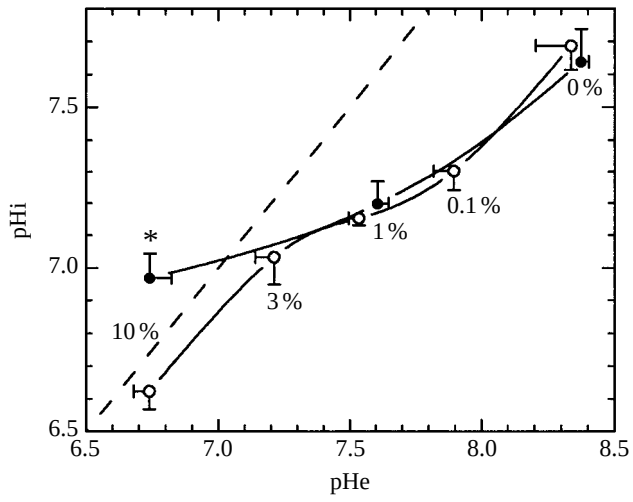

Fig. 1. The *in vitro* relationship between pHe and pHi in Atlantic cod red blood cells in CO_2/HCO_3^--buffered media (open circles, summer animals; filled circles, winter animals). Each point represents the mean ± S.D. of 3–8 equilibrations of blood from 3–5 individual animals. Numbers below the symbols give the percentage of CO_2 in the equilibration gas (balance air; nominal 10 mmol l^{-1} HCO_3^- in the saline). The dashed line represents the line of identity; an asterisk indicates a significant difference between the pHi of red blood cells from winter and summer animals under similar experimental conditions.

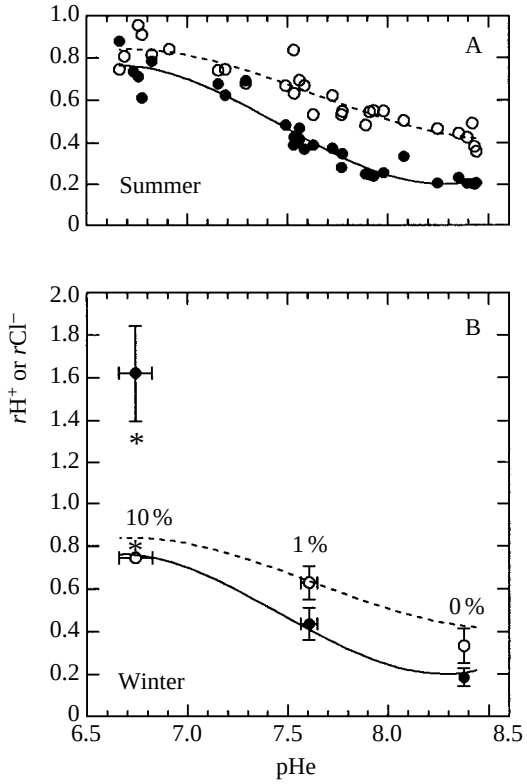

Fig. 2. Proton (filled circles, rH^+) and chloride (open circles, rCl^-) distribution ratios across the Atlantic cod red blood cell membrane in the experiment from Fig. 1. (A) Summer animals. The two data sets were fitted to polynomial functions following the equation: rH^+ or $rCl^-=a+b\text{pHe}+c\text{pHe}^2+d\text{pHe}^3$ (solid line, rH^+; dashed line, rCl^-; 29 equilibrations, blood from five individuals). (B) Winter animals. Each point represents the mean ± S.D. of four individuals. For comparison, the regression lines for the same variables in summer animals (Fig. 2A) are given. An asterisk indicates a significant difference between the respective mean rH^+ and rCl^- values in summer animals. Percentages of CO_2 in the equilibration gas (balance air) are given in B.

membrane in winter animals at low pHe was tested using metabolic inhibitors (Fig. 3). Incubation with the protonophore 2,4-dinitrophenol or with cyanide completely abolished the highly elevated rH^+ values in red blood cells from winter animals compared with those of summer animals. In contrast, incubation of red blood cells from summer animals in the presence of DIDS produced even higher rH^+ values than those observed in untreated red blood cells from winter animals. The apparent proton disequilibrium in Atlantic cod red blood cells in winter at high CO_2 tension was significantly diminished when the cells were incubated in media containing low sodium or low chloride concentrations and it was not seen under CO_2/HCO_3^--free conditions at similar low pHe values.

Fig. 4 shows the effect of acute hypercapnia on red blood cell ion and water content in

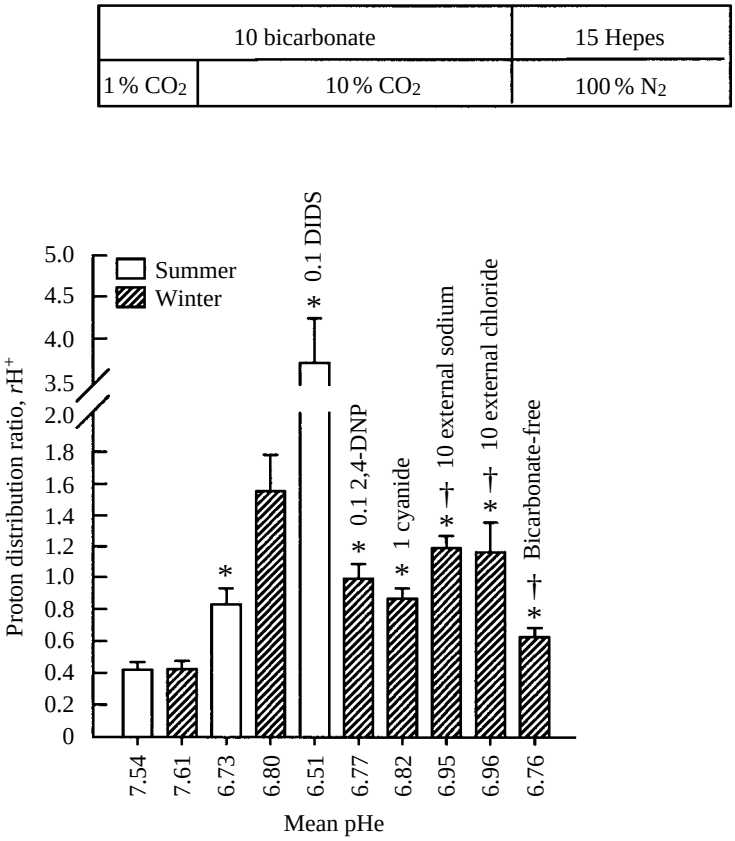

Fig. 3. The proton distribution ratio across the Atlantic cod red blood cell membrane under various experimental conditions (means ± S.D., N=4). Open and shaded bars refer to summer and winter animals, respectively. An asterisk denotes a significant difference from the value at 10 % CO_2/10 mmol l^{-1} HCO_3^- in untreated cells from winter animals, whereas a dagger denotes a significant difference from the value at 10 % CO_2/10 mmol l^{-1} HCO_3^- in untreated cells from summer animals. Concentrations are given in mmol l^{-1}.

Atlantic cod in winter. When the CO_2 tension was changed from 1 % to 10 %, pHe and pHi decreased rapidly during the first 10 min and then reached a new steady-state level with pHi exceeding pHe (Fig. 4A). The high rH^+ values characteristic of a proton disequilibrium across the red blood cell membrane are reached as early as 5 min after the onset of hypercapnia and remain elevated for at least 40 min (Fig. 4B). The drop in pH caused a shift of chloride into the red blood cells (Fig. 4D), but the new steady-state red blood cell chloride content was significantly lower than the respective value obtained in summer (not shown). The increase in cellular ion content was followed by cell swelling, as revealed by the increase in red blood cell water content (Fig. 4C). While the red blood cell potassium content did not change significantly during the experiment, hypercapnia resulted in a significant elevation of the red blood cell sodium content (Fig. 4D). A net gain of cellular sodium was also observed in ouabain-treated red blood cells (not shown),

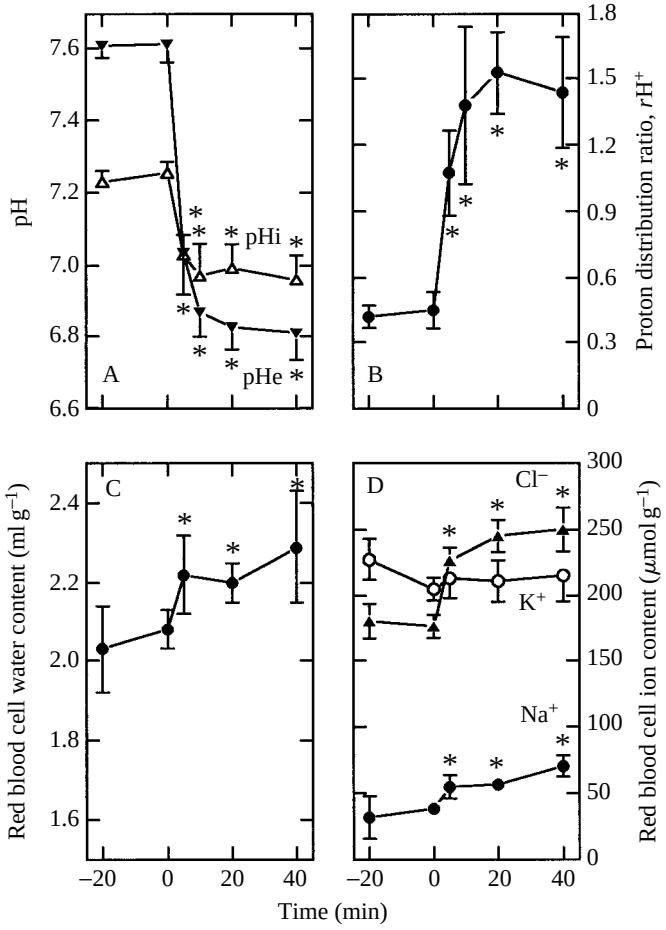

Fig. 4. Changes in pHe and pHi (A), rH^+ (B), cellular water content (C) and ion content (D) in red blood cells of winter Atlantic cod during an increase in the equilibrating CO_2 tension. At time zero, the equilibration gas was rapidly changed from 1 % to 10 % CO_2 (balance air, means ± S.D., N=4). An asterisk indicates values significantly different from the value at time zero. Ion and water contents are given per gram dry cell solids.

which tended to reach higher intracellular sodium contents than untreated red blood cells (after 40 min of hypercapnia 70.5±8.0 and 61.8±5.2 μmol g^{-1} dry cell solids, respectively, N=4), indicating that the sodium/potassium pump was extruding part of the sodium influx in untreated red blood cells. During the first 5 min, the hypercapnia-induced net sodium influx in ouabain-treated red blood cells amounted to 3.6 μmol g^{-1} dry cell solids min^{-1}.

Atlantic cod red blood cells equilibrated for 20 min generally showed a significantly lower sodium content at 0 % and a higher sodium content at 10 % CO_2 than at 1 % CO_2 (Fig. 5A). This phenomenon was more pronounced in winter animals, where the red blood cell sodium content was significantly higher than the respective value for summer animals at 1 % CO_2. The highest cellular sodium content was found in winter in red blood

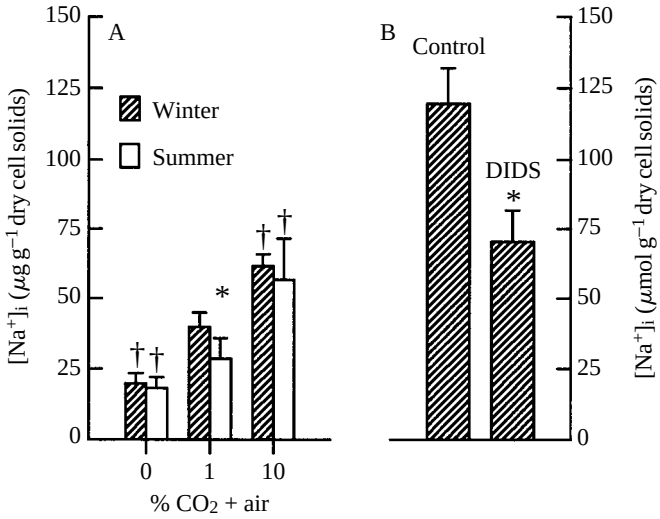

Fig. 5. (A) Sodium content of red blood cells from summer (open bars) and winter (shaded bars) animals incubated for 20 min at different CO_2 tensions (means ± S.D., N=4). A dagger indicates a significant difference from the value at 1 % CO_2 within the same season; an asterisk indicates a significant difference between summer and winter animals at the same CO_2 tension. (B) The effects of DIDS on the red blood cell sodium content of winter Atlantic cod (means ± S.D., N=4). Red blood cells were incubated in 1 % CO_2 for 1 h prior to the addition of DIDS. An asterisk denotes a significant difference in the sodium content of red blood cells immediately before (control) and 10 min after the addition of 0.1 mmol l^{-1} DIDS.

cells that had been equilibrated for 1 h at 1 % CO_2 (Fig. 5B). Within 10 min after addition of DIDS (10^{-4} mol l^{-1} final concentration), the red blood cell sodium content of these cells was significantly reduced by 47.6±11.1 μmol g^{-1} dry cell solids (N=4).

Discussion

Determinants of the pHi of Atlantic cod red blood cells in summer

The strikingly similar pH-dependencies of rH^+ and rCl^- over the whole pHe range studied (8.4–6.7) are in good agreement with a Donnan distribution of protons and chloride across the red blood cell membrane (Fig. 2). A small but significant difference between rH^+ and rCl^- is seen in anucleated and nucleated red blood cells of various species with chloride and protons in Donnan equilibrium across their red blood cell membrane (human, Funder and Wieth, 1966; carp, Albers and Goetz, 1985; *Bufo marinus*, Tufts and Randall, 1988). Possible causes for this slight difference are (1) different activity coefficients of chloride in red blood cells and the suspension medium, (2) protein binding of chloride, (3) the method used for pHi and intracellular chloride measurements and (4) intracellular heterogeneity (Funder and Wieth, 1966; Roos and Boron, 1981; Albers and Goetz, 1985).

With protons and chloride in Donnan equilibrium across the red blood cell membrane, any perturbation affecting the extracellular acid–base status will also affect the

intracellular pH. In this case, during extracellular acidosis, proton equivalents are transferred to the intracellular compartment, leading to the titration of fixed negative charges within the red blood cell (mainly haemoglobin). To maintain electroneutrality, this provokes the entry of permeable anions (mainly chloride) into the cell. The result is an increase in $r\mathrm{Cl}^-$ and $r\mathrm{H}^+$ together with cell swelling, since the gain of osmolytes increases the cellular water content *via* osmosis. The same sequence of events takes place in the opposite direction when the pH of the extracellular medium is increased (Hladky and Rink, 1977).

A similar S-shaped relationship between $r\mathrm{Cl}^-$ and pHe to that seen in Fig. 2 has been described in human erythrocytes, where it was explained by the titration characteristics of the haemoglobin inside the red blood cells (Dalmark, 1975). Within the pH range 6–9, all teleostean haemoglobins studied have far fewer titratable groups than mammalian haemoglobins (Jensen, 1989). However, the pronounced influence of pH on teleost haemoglobin conformation (Root effect) will promote additional proton binding below pH 7.4, where these haemoglobins readily adopt the deoxy-conformation with more proton binding sites than in the oxy-conformation (for a review, see Pelster and Weber, 1991). Consequently, the changes in $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ are small between pHe 8.4 and 7.9 (pHi 7.7–7.3) because the haemoglobin is almost completely in the oxy-conformation. Further pH reductions promote the Root effect and accentuate the changes in $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ since the net charge inside the red blood cell will be decreased not only by simple titration of haemoglobin but also by its enhanced transition to the deoxy-conformation. Finally, when the Root effect is maximally displayed, further acidification will again result in less accentuated changes in $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$. Therefore the S-shaped relationship between pHe and pHi (Fig. 1) can be accounted for by the passive distribution of protons across the red blood cell membrane together with changes in the net charge on haemoglobin provoked by titration and oxy–deoxy transitions. In the flat part of the curve in Fig. 1 (pHe range 7.2–7.9), the relationship between pHi and pHe is linear and similar to the relationships obtained for red blood cells from various freshwater and marine teleosts, such as rainbow trout (Tetens and Lykkeboe, 1981), carp (Albers and Goetz, 1985), plaice (Wood *et al.* 1975) and starry flounder (Milligan and Wood, 1987).

Determinants of the pHi of Atlantic cod red blood cells in winter

Between pHe 8.4 and 7.5, Atlantic cod red blood cells obtained in winter showed no significant difference in pHi, $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ from the respective values in red blood cells from summer animals. (Figs 1, 2). At 10 % CO_2, however, the red blood cell pHi of winter animals is about 0.3 pH units higher than the red blood cell pHi in summer. Simultaneously, $r\mathrm{H}^+$ is significantly elevated and $r\mathrm{Cl}^-$ significantly reduced. The marked difference between $r\mathrm{H}^+$ and $r\mathrm{Cl}^-$ is no longer in accordance with a passive distribution of both ions across the red blood cell membrane.

There are two general mechanisms that could account for this phenomenon in Atlantic cod red blood cells in winter: (1) reduced chloride and proton permeabilities and (2) active transport of chloride and protons across the red blood cell membrane at low pHe. Although each of the two mechanisms is not necessarily exclusive and both may be required, they will be discussed separately in the following paragraphs.

First, the deviation of rH^+ and rCl^- from their equilibrium distribution and the marked difference between them does not automatically imply that the ion gradients for protons and chloride are actively maintained. In Atlantic cod red blood cells in summer, inhibition of the anion exchanger with DIDS and thus inhibition of the passive Jacobs–Stewart cycle, which usually equilibrates protons and chloride across the red blood cell membrane, produces similar disequilibrium rH^+ and rCl^- values to those observed in untreated red blood cells from winter animals (Fig. 3). Therefore, a possible explanation for the situation in winter red blood cells is a reduced anion exchange activity of the red blood cell membrane at low pHe. The pH-dependence of the anion exchanger has been described in human and rainbow trout red blood cells (Gunn *et al.* 1973; Romano and Passow, 1984). When measured as chloride equilibrium self-exchange, anion exchange activity in rainbow trout red blood cells was highly pH-dependent and decreased by about 97 % when the pHe was reduced from 7.4 to 6.7 (Romano and Passow, 1984). Thus, the effects of a generally reduced red blood cell anion exchange activity in winter animals (e.g. by a reduced number of anion exchangers in the red blood cell membrane) will be more pronounced at low pHe (10 % CO_2), whereas it may not be detectable at a higher pHe (1 % CO_2). A markedly reduced anion exchange activity has important consequences for the equilibration of protons across the red blood cell membrane. During hypercapnia, CO_2 will still diffuse across the red blood cell membrane, but the bicarbonate formed by its hydration inside the red blood cell will no longer be readily able to leave the cell. The protons formed by the hydration reaction will be largely buffered by haemoglobin and the accumulation of bicarbonate inside the red blood cell would hinder further CO_2 hydration, resulting in a high pHi despite a low pHe (that is a high rH^+ value; Fig. 4A,B). When these red blood cells are incubated with the protonophore 2,4-DNP, a second pathway is opened for proton equilibration across the red blood cell membrane and the high rH^+ value is reduced to the equilibrium value. Similarly, no disequilibrium rH^+ values are observed in bicarbonate-free media at low pHe in red blood cells of winter animals since bicarbonate cannot accumulate inside the red blood cells.

Second, the assumption of a reduced red blood cell anion exchange activity at low pH in winter Atlantic cod offers an explanation for the high pHi, for the marked difference between rH^+ and rCl^- and for the effects of 2,4-DNP and bicarbonate-free media. However, it is not sufficient to explain all experimental observations. The marked discrepancy seen between rH^+ and rCl^- in Atlantic cod red blood cells at 10 % CO_2 in winter is at least in part the result of active pHi regulation. This is supported by the significant reduction of rH^+ in the presence of cyanide, a known inhibitor of aerobic energy metabolism (Fig. 3). The effect of 2,4-DNP on rH^+ also matches the concept of active pHi regulation, since the protonophore short-circuits any actively maintained proton gradient and is also a potent inhibitor of aerobic energy production. More evidence for active pHi regulation comes from the study of the cellular ion contents during a stepwise decrease in pHe (Fig. 4). In parallel with the marked increase in rH^+, hypercapnic acidosis activates a ouabain-insensitive net sodium influx. The development of high rH^+ values is significantly reduced when the net sodium influx is inhibited by reducing the extracellular sodium concentration (Fig. 3). The initial (0–5 min) net sodium influx of $3.6\,\mu mol\,g^{-1}$ dry cell solids min^{-1} under hypercapnia is relatively small

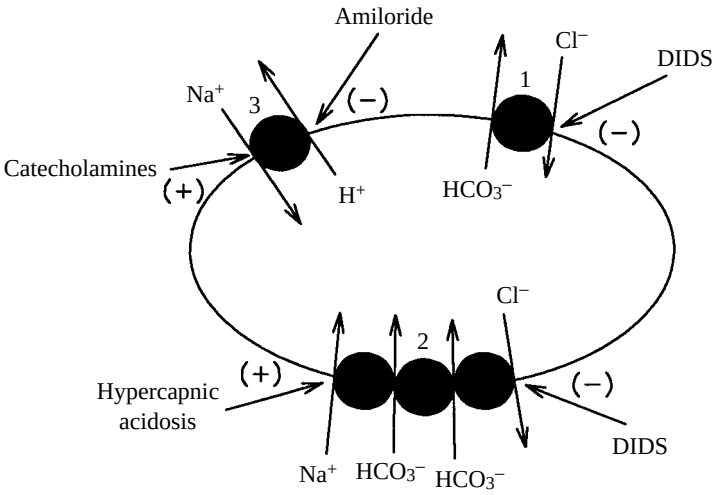

Fig. 6. Possible mechanisms affecting the pHi of Atlantic cod red blood cells in winter: (1) chloride/bicarbonate exchange, (2) hypercapnia-induced sodium-dependent chloride/bicarbonate exchange and (3) catecholamine-induced sodium/proton exchange (Berenbrink and Bridges, 1994). Arrows and (+) or (−) indicate a stimulatory or inhibitory effect of a given treatment.

compared with the catecholamine-induced initial net sodium influx of 12 μmol g^{-1} min^{-1} dry cell solids under similar conditions (Berenbrink and Bridges, 1994). Assuming the extrusion of one proton equivalent for each sodium ion entering the red blood cell (as expected for Na^+/H^+ exchange), it seems paradoxical that the much smaller hypercapnia-induced sodium influx should have the same strong impact on rH^+ as the catecholamine-induced sodium influx. In addition, if the elevated red blood cell rH^+ values under hypercapnia in winter were due to activation of Na^+/H^+ exchange, rCl^- should also increase (Berenbrink and Bridges, 1994). However, the sodium-dependent elevation of rH^+ is associated with significantly lower rCl^- values compared with the equilibrium value in summer (Fig. 2). Apparently, some of the chloride entering the red blood cells at 10 % CO_2 by the Jacobs–Stewart cycle is extruded again, leading to significantly lower intracellular chloride concentrations in red blood cells from winter animals compared with those of summer animals. This movement of chloride seems to form an important part of the sodium-dependent acid extrusion mechanism, since the high disequilibrium rH^+ values are significantly lowered when the external (and also the internal) chloride concentration is reduced (Fig. 3). The absolute requirement for bicarbonate (Fig. 3) and the inhibitory effect of DIDS on the net sodium influx (Fig. 5B) are further evidence against a Na^+/H^+ exchange mechanism. In contrast, the results presented above suggest the activation of a Na^+-dependent Cl^-/HCO_3^--exchange mechanism like that described previously for various invertebrate and vertebrate preparations (for a review, see Boron, 1985). This electroneutral exchanger promotes the extrusion of two proton equivalents for each sodium ion entering and chloride ion leaving the cell (Fig. 6). It has an absolute requirement for external sodium, internal chloride and external bicarbonate (or an

equivalent ion species) and it is inhibited by cyanide and DIDS. The energy for the transport is provided by the steep sodium gradient across the cell membrane, which is established by the sodium/potassium pump. The existence of a certain threshold pHi for the activation of Na^+-dependent Cl^-/HCO_3^- exchange (Thomas, 1977; Boron *et al.* 1979) conforms with the high disequilibrium rH^+ values at 10 % CO_2 (pHi 6.97) and the rH^+ values closer to a passive proton distribution at 1 % CO_2 (pHi 7.20).

To our knowledge this is the first report that provides evidence for Na^+-dependent Cl^-/HCO_3^- exchange in red blood cells. Evidence for this transporter might have been missed in previous studies on red blood cell pHi regulation since it is common to use CO_2/HCO_3^--free incubation media to avoid the problems associated with volatile buffers. In addition, the transport is most effective at low pHe values that might be thought to lie below the physiological range. However, at least in fish, these low pHe values may be achieved regularly in blood passing the gas glands (Steen, 1963). The relatively low pHi threshold of the transporter would allow for maximal exploitation of the Root effect but hinder further acidification of the red blood cell interior. The need for this kind of pHi regulation seems to vary with season, since it appears to be absent in red blood cells from Atlantic cod in the summer.

We would like to thank Frau Andrea Köster for excellent technical assistance. Thanks are also due to the Biologische Anstalt Helgoland for providing experimental facilities. Financial support for this work was provided by the Studienstiftung des deutschen Volkes (M.B.) and the Deutsche Forschungsgemeinschaft (Gr 456/12–2).

References

ALBERS, C. AND GOETZ, K. H. (1985). H^+ and Cl^- ion equilibrium across the red cell membrane in the carp. *Respir. Physiol.* **61**, 209–219.

BERENBRINK, M. AND BRIDGES, C. R. (1994). Catecholamine-activated sodium/proton exchange in red blood cells of the marine teleost *Gadus morhua*. *J. exp. Biol.* **192**, 253–267.

BORON, W. F. (1985). Control of intracellular pH. In *The Kidney: Physiology and Pathophysiology*, chapter 61 (ed. D. W. Seldin and G. Giebisch), pp. 1417–1439. New York: Raven Press.

BORON, W. F., MCCORMICK, W. C. AND ROOS, A. (1979). pH regulation in barnacle muscle fibers: Dependence on intracellular and extracellular pH. *Am. J. Physiol.* **237**, C185–C193.

BOURNE, P. K. AND COSSINS, A. R. (1982). On the instability of K^+ influx in erythrocytes of the rainbow trout, *Salmo gairdneri* and the role of catecholamine hormones in maintaining *in vivo* influx activity. *J. exp. Biol.* **101**, 93–104.

BOUTILIER, R. G., IWAMA, G. K., HEMING, T. A. AND RANDALL, D. J. (1985). The apparent pK of carbonic acid in rainbow trout plasma between 5 and 15 °C. *Respir. Physiol.* **61**, 237–254.

DALMARK, M. (1975). Chloride and water distribution in human red cells. *J. Physiol., Lond.* **250**, 65–84.

FUNDER, J. AND WIETH, J. O. (1966). Chloride and hydrogen ion distribution between human red cells and plasma. *Acta physiol. scand.* **68**, 234–245.

GUNN, R. B., DALMARK, M., TOSTESON, D. C. AND WIETH, J. O. (1973). Characteristics of chloride transport in human red blood cells. *J. gen. Physiol.* **61**, 185–206.

HEMING, T. A., RANDALL, D. J., BOUTILIER, R. G., IWAMA, G. K. AND PRIMMET, D. (1986). Ionic equilibria in red blood cells of rainbow trout (*Salmo gairdneri*): Cl^-, HCO_3^- and H^+. *Respir. Physiol.* **65**, 223–234.

HLADKY, S. B. AND RINK, T. J. (1977). pH equilibrium across the red cell membrane. In *Membrane Transport in Red Cells* (ed. J. C. Ellory and V. L. Lew), pp. 115–135. London: Academic Press.

JENSEN, F. B. (1989). Hydrogen ion equilibria in fish haemoglobins. *J. exp. Biol.* **143**, 225–234.

MILLIGAN, C. L. AND WOOD, C. M. (1987). Regulation of blood oxygen transport and red cell pHi after exhaustive activity in rainbow trout (*Salmo gairdneri*) and starry flounder (*Platichthys stellatus)*. *J. exp. Biol*. **133**, 263–282.

NIKINMAA, M., STEFFENSEN, J. F., TUFTS, B. L. AND RANDALL, D. J. (1987). Control of red cell volume and pH in trout: effects of isoproterenol, transport inhibitors and extracellular pH in bicarbonate/carbon dioxide-buffered media. *J. exp. Zool*. **242**, 273–281.

PELSTER, B. AND WEBER, R. E. (1991). The physiology of the Root effect. *Adv. comp. env. Physiol*. **8**, 51–77.

ROMANO, L. AND PASSOW, H. (1984). Characterization of anion transport system in trout red blood cell. *Am. J. Physiol.* **246**, C330–C338.

ROOS, A. AND BORON, W. F. (1981). Intracellular pH. *Physiol. Rev*. **61**, 296–434.

STEEN, J. B. (1963). The physiology of the swimbladder of the eel *Anguilla vulgaris.* III. The mechanism of gas secretion. *Acta physiol. scand*. **59**, 221–241.

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

THOMAS, R. C. (1977). The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurons. *J. Physiol., Lond.* **273**, 317–338.

TUFTS, B. L. AND RANDALL, D. J. (1988). The distribution of protons and chloride ions across the erythrocyte membrane of the toad *Bufo marinus. Can. J. Zool*. **66**, 2503–2506.

WOOD, S. C., JOHANSEN, K. AND WEBER, R. E. (1975). Effects of ambient P_{O_2} on hemoglobin–oxygen affinity and red cell ATP concentrations in a benthic fish, *Pleuronectes platessa*. *Respir. Physiol*. **25**, 259–267.

WOODHEAD, P. M. J. AND WOODHEAD, A. D. (1959). The effects of low temperatures on the physiology and distribution of the cod, *Gadus morhua* L., in the Barents sea. *Proc. zool. Soc. Lond.* **133**, 181–199.