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ADRENERGIC CONTROL OF RED CELL pH IN SALMONID FISH: ROLES OF THE SODIUM/PROTON EXCHANGE, JACOBS–STEWART CYCLE AND MEMBRANE POTENTIAL

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Summary

We investigated the mechanisms by which adrenergic activation of sodium/proton exchange reduces the pH gradient across the membrane of rainbow trout red cells. In untreated cells, adrenergic stimulation caused a significant increase in the proton distribution ratio ($[H^+]_e/[H^+]_i$) across the red cell membrane. The increase in the proton distribution ratio caused by adrenergic stimulation was inhibited by the protonophore 2,4-dinitrophenol (2,4-DNP). Thus, sodium/proton exchange displaces protons from electrochemical equilibrium. Active regulation of intracellular pH by sodium/proton exchange is possible, because the extracellular dehydration of carbonic acid to carbon dioxide is uncatalyzed. The increase in proton distribution ratio caused by adrenergic stimulation was inhibited in red cell suspensions to which extracellular carbonic anhydrase had been added before stimulation. In contrast, inhibition of intracellular carbonic anhydrase markedly increased the pH changes induced by adrenergic stimulation, suggesting that the net direction of the intracellular hydration/dehydration reaction may markedly affect the intracellular pH changes. Membrane potential changes are not a necessary component of the adrenergic response. The increases in red cell volume and sodium and chloride concentrations induced by adrenergic stimulation were unaffected in cells 'voltage-clamped' by valinomycin.

Introduction

Despite the presence of a functional, rapid anion exchange (Romano and Passow, 1984), adrenergic stimulation of rainbow trout red cells decreases the pH gradient across the red cell membrane both in nominally bicarbonate-free medium (Nikinmaa, 1983; Cossins and Richardson, 1985) and in a medium buffered with a carbon dioxide–bicarbonate system (Nikinmaa, 1982; Heming *et al.* 1987; Nikinmaa *et al.* 1987; Motais *et al.* 1989).

However, there is a much larger drop in the extracellular pH of adrenergically stimulated cells suspended in their own plasma (i.e. buffered with a carbon

Key words: intracellular pH, sodium/proton exchange, adrenergic stimulation, teleost red cell, Jacobs–Stewart cycle.

dioxide–bicarbonate system) than in the extracellular pH of adrenergically stimulated cells suspended in Hepes- or Tris-buffered saline (see Motais *et al.* 1989). This pronounced drop in the external pH can be prevented by adding carbonic anhydrase to the external medium (Motais *et al.* 1989), indicating that it is mainly due to the slow dehydration of carbonic acid to carbon dioxide in the plasma.

However, because the adrenergically induced increase in intracellular pH was similar in saline-incubated cells and plasma-incubated cells in the absence and presence of carbonic anhydrase (Motais *et al.* 1989), the mechanism by which the increase in intracellular pH can be achieved was not fully clarified.

There are two explanations for the adrenergic pH changes. The first is an extension of the findings of Motais *et al.* (1989). Protons are displaced from electrochemical equilibrium by the sodium/proton exchanger, because the rate constant for proton extrusion *via* the sodium/proton exchanger approaches the rate constant of the slowest step of passive proton equilibration, the uncatalysed extracellular reaction:

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$$

(Hladky and Rink, 1977). If this were the case, the reduction of pH gradient across the red cell membrane should be prevented by using higher carbonic anhydrase activities in the extracellular medium than those used by Motais *et al.* (1989).

Adrenergically induced pH changes would also occur if protons remained passively distributed across the red cell membrane during adrenergic stimulation, but the stimulation caused membrane depolarization. As a result of the membrane depolarization, the pH gradient across the membrane would decrease.

We have investigated which of the two alternatives explains the adrenergic pH changes. In addition, we have investigated how the adrenergic response is influenced by inhibition of the intracellular formation of bicarbonate from carbon dioxide. This information is important because, in the blood going through the gills, the net direction of the intracellular reaction is from bicarbonate to carbon dioxide. This must inhibit the net rate of intracellular hydration.

Materials and methods

Rainbow trout *Salmo gairdneri* Richardson (80–170 g, N=30) were obtained from commercial fish farms. The fish were acclimated to laboratory conditions (dechlorinated Helsinki tap water, at 8–10°C) for a minimum of 2 weeks before they were used in experiments.

Blood samples were taken from anaesthetized fish (MS-222, 0.1 g l^{-1}, 4 min) by caudal puncture. Red cells and plasma were separated by centrifugation, and the red cells were washed twice for at least 30 min with the saline used in the experiments. The composition of the saline was: (in mmol l^{-1}) NaCl 128; KCl 3; $CaCl_2$ 1.5; $MgCl_2$ 1.5; $NaHCO_3$ 1; D-glucose 2.8; pyruvate 1; and imidazole buffer 10, pH 7.35–7.45, osmolality 290 mosmol kg^{-1}. All washes and experiments were carried out at room temperature (22°C).

Before each experiment the washed red cells were diluted to a haematocrit value of 20 in the experimental saline. The experiments were carried out with a carbon dioxide tension of 0.2 kPa (1.5 mmHg) and oxygen tension of 0.4 kPa (3 mmHg), the remainder being nitrogen. The low oxygen tension was used because the sodium/proton exchanger is activated to a greater degree in hypoxic than in normoxic conditions (see Motais *et al.* 1987). The gas mixture was obtained using Wösthoff gas-mixing pumps.

The effects of isoproterenol on the ion and water content of the red cell and on proton distribution across the cell membrane were studied in the following manner. A cell suspension (7–10 ml) was incubated in saline in a shaking tonometer for 30 min. 10 μl of ^{14}C-labelled DMO (5,5-dimethyl-2,4-oxazolidine-dione; 10 μCi ml^{-1}; Amersham) was added to the suspension at the onset of equilibration. After the initial equilibration, two successive control level samples were taken at 30 min intervals before adding 10^{-5} mol l^{-1} (−)isoproterenol hydrochloride (final concentration; Sigma) to the incubation medium. Samples were taken 2, 5, 10, 20 and 40 min after the addition of isoproterenol.

The role of membrane potential in the adrenergic response was examined using valinomycin- and 2,4-dinitrophenol-treated rainbow trout red cells. The experiment with valinomycin-treated cells was done in the following manner: The cells were incubated for 2 h in the presence of 10 μg l^{-1} valinomycin. They were then adrenergically stimulated using isoproterenol (final concentration 10^{-5} mol l^{-1}). The membrane potential, chloride distribution ratio across the red cell membrane, intracellular sodium concentration and cellular water content were measured both before and 20 min after stimulation. Qualitative changes of membrane potential were followed using the lipophilic, radioactively labelled cation [^{14}C]tetraphenyl-phosphonium (TPP^+, Heinz *et al.* 1975). We estimated changes in membrane potential from changes in the extracellular concentration of TPP^+ at a constant number of red cells per unit volume.

2,4-dinitrophenol was used to prevent the build-up of actively maintained proton gradients across the red cell membrane. 10^{-4} mol l^{-1} (final concentration) 2,4-dinitrophenol was added to the suspension at the onset of the incubation. Thereafter the samples were treated in the same manner as those in the control experiments.

The role of the Jacobs–Stewart cycle in the adrenergic response was studied in two ways. First, carbonic anhydrase from bovine erythrocytes [3 g l^{-1}, 2500 Wilbur–Anderson units mg^{-1} protein; 1 unit will cause the pH of a 0.012 mol l^{-1} veronal buffer to drop from 8.3 to 6.3 in 1 min at 0°C; Sigma] was added to the external medium at the onset of incubation to speed up extracellular interconversion between carbon dioxide, bicarbonate and protons. The samples were then treated as in the control experiments. Second, acetazolamide (final concentration 10^{-4} mol l^{-1}; Sigma) was added to the suspension at the onset of the incubation to inhibit carbonic anhydrase activity in the cells, and the samples were then treated in the same manner as in the control experiments.

Determinations of intra- and extracellular pH, and the water and ion contents of

the red cells were carried out in the same way in all experiments. Immediately after sampling, extracellular pH was measured using a Radiometer BMS3 Mk2 and PHM 73 apparatus thermostatted at the experimental temperature. The incubation medium and red cells were then separated by centrifugation in two Eppendorf tubes (2 min, 10 000 *g*). The supernatants were used for extracellular ion and DMO determinations. The red cell pellets were used for the determinations of red cell ion contents, DMO concentration and water content. The red cell water content was determined by weighing, drying and reweighing the cell pellet. With the centrifugation used, the proportion of trapped extracellular water, estimated as polyethylene glycol space, was 6.5±0.2 % (N=16) of the volume of packed cells, and independent of treatment. The samples for ion and DMO determinations were deproteinized in 0.6 mol l^{-1} perchloric acid, then stored in liquid nitrogen until measurements were taken. Sodium contents were measured using a flame photometer (FLM3, Radiometer, Copenhagen) and chloride contents using a Radiometer CMT 10 chloride titrator. In the Results, the ion contents are given in mmol kg^{-1} dry mass of cells. In addition, the sodium ratio across the red cell membrane ($[Na^+]_e/[Na^+]_i$) was calculated from the measured extracellular and intracellular concentrations. The intracellular pH was determined from the extracellular pH and from the distribution of the radioactively labelled weak acid DMO across the cell membrane, as described by Nikinmaa and Huestis (1984), using the formula:

$$pH_i = pK_{DMO} + \log\{[DMO]_i/[DMO]_e(10^{pHe-pK_{DMO}} + 1) - 1\} .$$

The samples were analysed for [^{14}C]DMO using a liquid scintillation counter (LKB Wallac 1211 Minibeta). We had earlier checked that DMO reaches equilibrium in our red cell suspensions within 2 min. In the Results, the extracellular pH and the proton distribution ratio ($[H^+]_e/[H^+]_i$) are given. The proton distribution ratio is used instead of intracellular pH for two reasons: first, it (together with the sodium distribution ratio) allows comparison of the driving forces for the sodium/proton exchanger in the different experiments and, second, in the experiments with 2,4-DNP and carbonic anhydrase, the extracellular and intracellular pH decreased for unknown reasons, even in the absence of adrenergic stimulation during the incubation.

Results

The effects of adrenergic stimulation on the water, sodium and chloride content of the red cell and on proton distribution across the cell membrane

As expected, adrenergic stimulation caused a marked accumulation of sodium and chloride in the cells (Fig. 1A), an increase in the red cell water content (Fig. 2A), a rapid decrease in the extracellular pH (Fig. 2B) and an increase in the proton distribution ratio (Fig. 3A). From the data on sodium and chloride contents, the net fluxes of sodium and chloride into the cells can be estimated (Fig. 1B). The net sodium flux decreased markedly after the initial peak

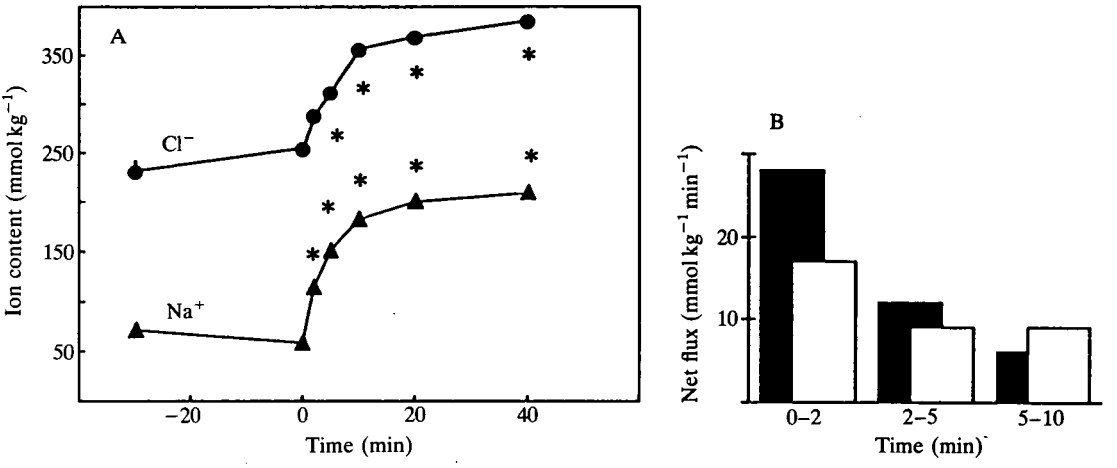

Fig. 1. (A) Effects of adrenergic stimulation (10^{-5} mol l^{-1} isoproterenol) on the sodium and chloride content of untreated rainbow trout red cells (in mmol kg^{-1} dry cell mass; $N=6$) as a function of time. Means are given, bars indicate S.E.M. (whenever the bar is not visible, the S.E.M. is smaller than the diameter of the symbol). An asterisk by the symbol indicates that the mean value at that time point after adrenergic stimulation differed significantly from the mean value of the second control sample. The probability level was set at $P<0.05$. Paired t-tests were used for comparisons. (B) The mean net sodium and chloride influx (in mmol kg^{-1} dry cell mass min^{-1}) at the times indicated (0–2, 2–5 and 5–10 min). Filled columns, sodium flux; empty columns, chloride flux.

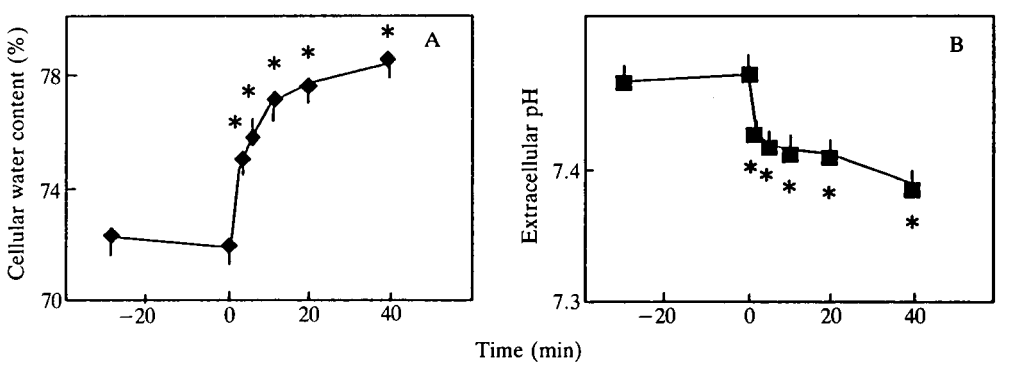

Fig. 2. Effects of adrenergic stimulation on (A) cellular water content (%) and (B) extracellular pH of untreated rainbow trout red cells ($N=6$) as a function of time. For further details, see legend to Fig. 1.

(29 mmol kg^{-1} dry cell mass min^{-1} during the first 2 min), possibly reflecting the reduction in the driving force for inward sodium transport (the sodium distribution ratio decreased from about 5 to about 3 within the first 2 min, see Fig. 3B). The net sodium flux exceeded the net chloride flux by 10 mmol kg^{-1} dry cell mass min^{-1} in the first 2 min of adrenergic stimulation, and by 3 mmol kg^{-1} dry cell mass min^{-1}

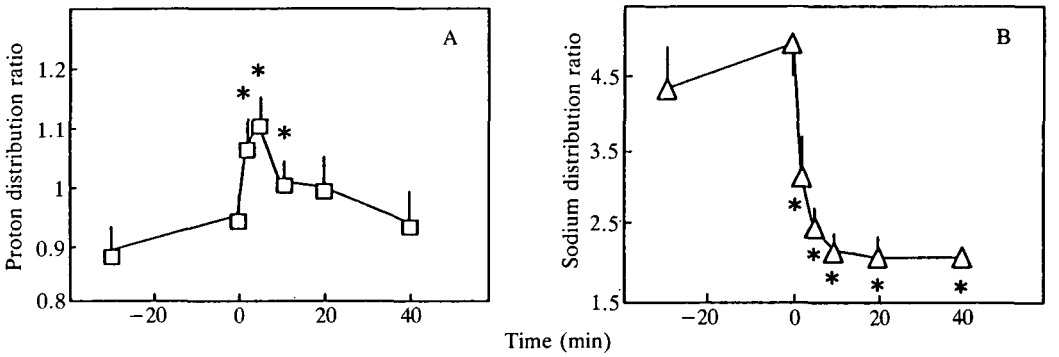

Fig. 3. Effects of adrenergic stimulation on (A) the proton ($[H^+]_e/[H^+]_i$) and (B) the sodium ($[Na^+]_e/[Na^+]_i$) distribution ratios across the red cell membrane in untreated rainbow trout red cells (N=6) as a function of time. For further details, see legend to Fig. 1.

between 2 and 5 min; after this the net flux of chloride exceeded that of sodium. Since the net sodium and chloride fluxes probably occur *via* loosely coupled sodium/proton and chloride/bicarbonate exchangers, the greater influx of sodium than chloride indicates a net extrusion of acid equivalents. Such an extrusion is also seen as an increase in the proton distribution ratio (Fig. 3A) at the onset of adrenergic stimulation. Towards the end of the incubation, in contrast, the proton distribution ratio decreased towards the initial value.

The role of membrane potential in the adrenergic response

Changes in membrane potential were followed by measuring changes in the extracellular concentration of tetraphenylphosphonium (TPP^+). Although it was not possible to measure quantitative values of membrane potential with this method (see Heinz *et al.* 1975), several pieces of evidence suggest that qualitative changes in membrane potential can be reliably estimated. First, since valinomycin markedly increases the permeability of red cells to potassium, the membrane potential hyperpolarized, and the external TPP^+ concentration decreased. Second, after the initial hyperpolarization, potassium ions moved towards an electrochemical equilibrium, which was seen as a membrane depolarization, i.e. an increase in extracellular TPP^+ concentration. Third, an increase in external potassium concentration in valinomycin incubations was always associated with an increase in the external TPP^+ concentration, as expected from the dependence of membrane hyperpolarization on the external K^+ concentration. Fourth, treatment of the cells with gramicidin led to membrane depolarization, and was seen as an increase in external TPP^+ concentration. Thus, the external TPP^+ concentration appears to respond to any changes in membrane potential.

After a 2-h equilibration, no changes were observed in the external TPP^+ concentration of valinomycin-treated cells. At this time, the cells were adrenergically stimulated. As shown in Table 1, no changes in the external TPP^+

Table 1. *Effects of 20 min of stimulation with 10^{-5} mol l^{-1} isoproterenol on the extracellular TPP^+ concentration, the $[Cl^-]_i/[Cl^-]_e$ ratio, intracellular sodium concentration and cellular water content in valinomycin-treated cells*

	Control	Isoproterenol-treated
TPP^+ (disints min^{-1})	47100±880(9)	47600±830(9)
$[Cl^-]_i/[Cl^-]_e$	0.601±0.008(6)	0.675±0.019(6)*
Na (mmol l^{-1})	63.0±14.7(6)	84.7±11.8(6)*
Cell water (%)	66.7±0.8(7)	70.6±0.6(7)*

Values are mean±S.E.M. (*N*).

Asterisks indicate a statistically significant difference between the means of the control and isoproterenol-treated cells ($P<0.01$).

concentration were observed after stimulation. This result suggests that the membrane potential was not affected by adrenergic stimulation in this case. Despite this, however, the chloride distribution ratio, the intracellular sodium concentration and the cell water content all increased, suggesting that the adrenergic response takes place in the cells without a change in membrane potential.

If protons and chloride ions were passively distributed during the course of the adrenergic response, and the change in the pH gradient was caused by membrane depolarization, increasing the proton permeability of the red cell membrane should not affect the response. This is clearly not the case. In the presence of the protonophore 2,4-DNP, adrenergic stimulation did not significantly affect the proton distribution ratio across the red cell membrane (Fig. 4A), although the extracellular pH was somewhat reduced (Fig. 5B). Furthermore, the adrenergically induced red cell swelling (Fig. 5A), the decrease in sodium distribution ratio

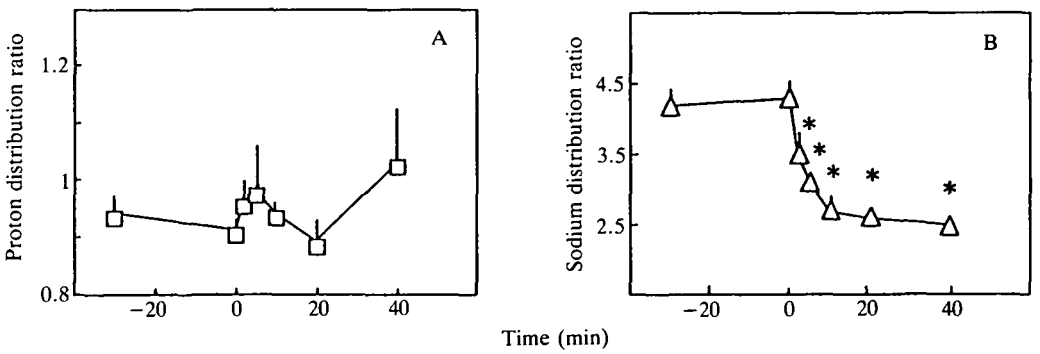

Fig. 4. Effects of adrenergic stimulation on (A) the proton ($[H^+]_e/[H^+]_i$) and (B) the sodium ($[Na^+]_e/[Na^+]_i$) distribution ratios across the red cell membrane in 2,4-dinitrophenol-treated rainbow trout red cells ($N=6$) as a function of time. For further details, see legend to Fig. 1.

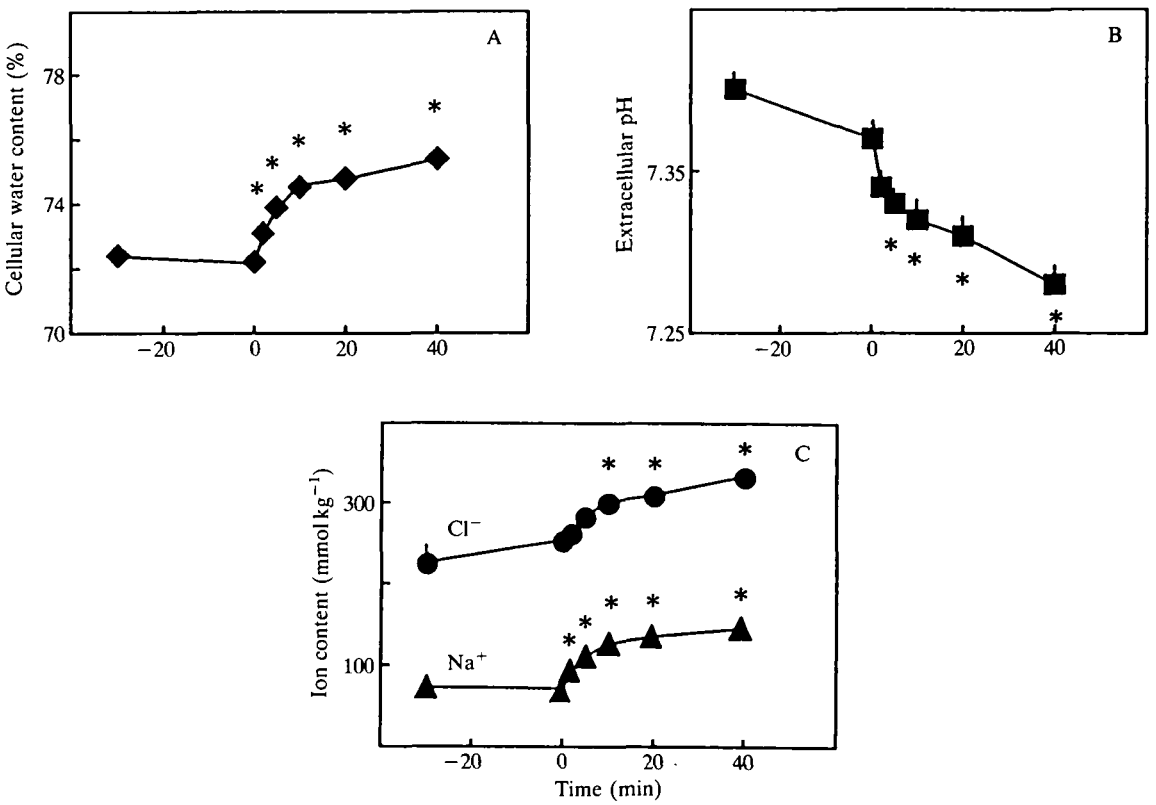

Fig. 5. Effects of adrenergic stimulation on (A) the water content, (B) the extracellular pH and (C) the sodium and chloride contents of 2,4-dinitrophenol-treated rainbow trout red cells (N=6) as a function of time. For further details, see legend to Fig. 1.

(Fig. 4B) and the accumulation of sodium and chloride in the cell were markedly reduced (Fig. 5C) compared with untreated, adrenergically stimulated cells.

The role of the Jacobs–Stewart cycle in the adrenergic response

The effects of 3 g l^{-1} (specific activity 2500 W–A units mg^{-1} protein) extracellular carbonic anhydrase on the adrenergic response are shown in Figs 6–8. There was a marked accumulation of sodium and chloride in the cells (Fig. 6A) and an increase in cellular water content (Fig. 7A), but the extracellular acidification was reduced (Fig. 7B) and there was no change in the proton distribution ratio across the red cell membrane (Fig. 8A). The initial sodium flux of 28 mmol kg^{-1} dry cell-mass min^{-1} was similar to the 29 mmol kg^{-1} dry cell mass min^{-1} found in untreated cells (cf. Figs 1B, 6B). However, since the extracellular uncatalyzed reaction:

$$H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$$

was speeded up by carbonic anhydrase, thereby coupling the proton and bicarbonate movements, the net sodium and chloride fluxes were similar through-

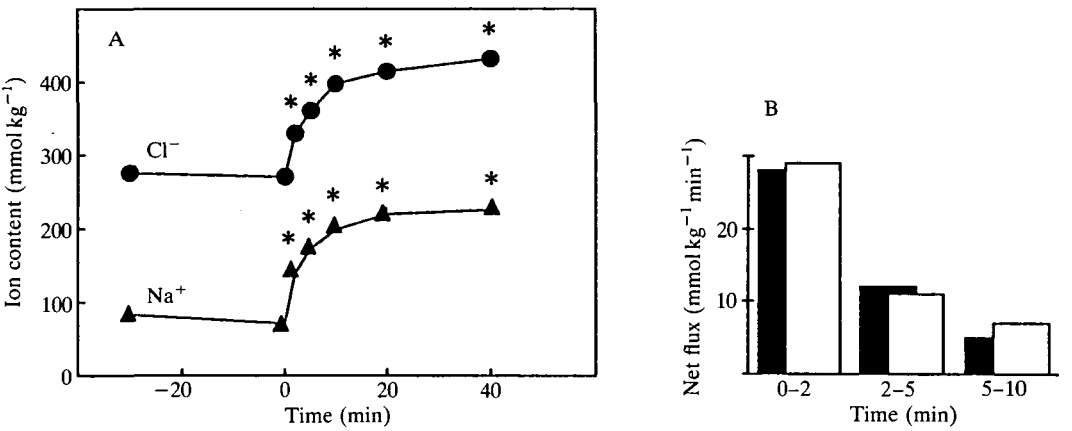

Fig. 6. (A) Effects of adrenergic stimulation on the sodium and chloride content of rainbow trout red cells (N=6) incubated in the presence of 3 g l^{-1} carbonic anhydrase (specific activity 2500 Wilbur–Anderson units mg^{-1}). (B) The mean net sodium and chloride influx at the times indicated. For further details, see legend to Fig. 1.

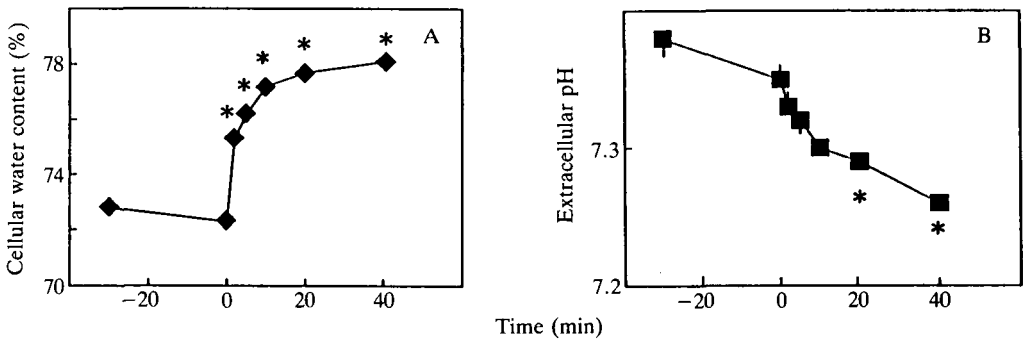

Fig. 7. Effects of adrenergic stimulation on (A) the cellular water content and (B) extracellular pH of rainbow trout red cells (N=6) incubated in the presence of 3 g l^{-1} carbonic anhydrase (specific activity 2500 Wilbur–Anderson units mg^{-1}). For further details, see legend to Fig. 1.

out the incubation (Fig. 6B). Consequently, no net proton efflux occurred, and the proton distribution ratio was not affected by adrenergic stimulation in carbonic-anhydrase-treated cells. Similar results were obtained with cells incubated in carbonic-anhydrase-containing, air-equilibrated, Tris-buffered (pH 7.3) medium in which the bicarbonate concentration was about 0.2 mmol l^{-1}. Despite the marked accumulation of sodium, chloride and water in the cell, no changes were observed in the proton distribution ratio across the red cell membrane (not shown).

In acetazolamide-treated cells, adrenergic stimulation was followed by a marked drop in the extracellular pH (Fig. 11B) and a marked increase in the

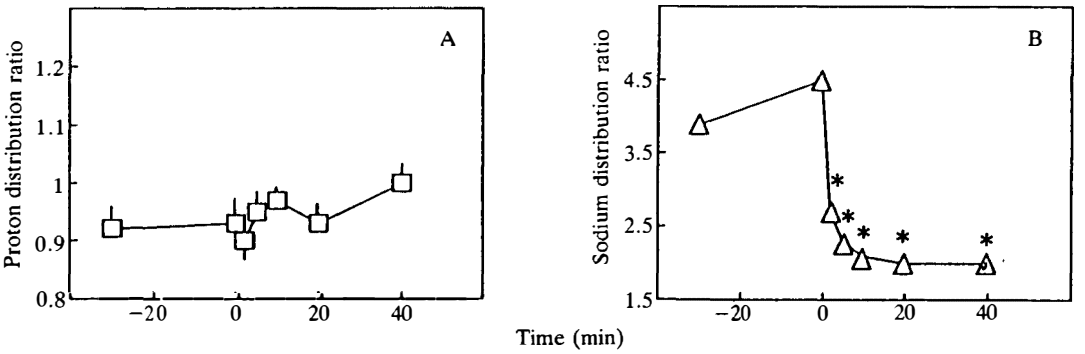

Fig. 8. Effects of adrenergic stimulation on (A) the proton ($[H^+]_e/[H^+]_i$) and (B) the sodium ($[Na^+]_e/[Na^+]_i$) distribution ratios across the red cell membrane in rainbow trout red cells ($N=6$) incubated in the presence of $3\,g\,l^{-1}$ carbonic anhydrase (specific activity 2500 Wilbur–Anderson units mg^{-1}). For further details, see legend to Fig. 1.

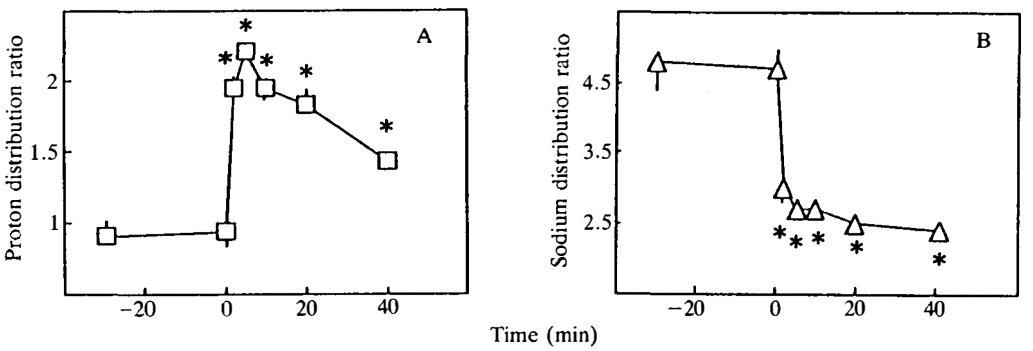

Fig. 9. Effects of adrenergic stimulation on (A) the proton ($[H^+]_e/[H^+]_i$) and (B) the sodium ($[Na^+]_e/[Na^+]_i$) distribution ratios across the red cell membrane in acetazolamide-treated rainbow trout red cells ($N=6$). For further details, see legend to Fig. 1.

proton distribution ratio across the red cell membrane (Fig. 9A). Also, the red cell sodium content (Fig. 10A) and the red cell water content (Fig. 11A) increased, whereas the red cell chloride content was initially not affected (Fig. 10A). The initial sodium influx exceeded the initial chloride influx by approximately $20\,mmol\,kg^{-1}$ dry cell mass min^{-1} (Fig. 10B). The initial sodium influx was reduced compared with that of adrenergically stimulated, untreated cells. This is probably because, as the sodium (Fig. 9B) and proton distribution ratios approach each other, the driving force for sodium/proton exchange is markedly reduced.

Discussion

Motais *et al.* (1989) showed that the large extracellular acidification which is observed after adrenergic stimulation of rainbow trout red cells in a carbon

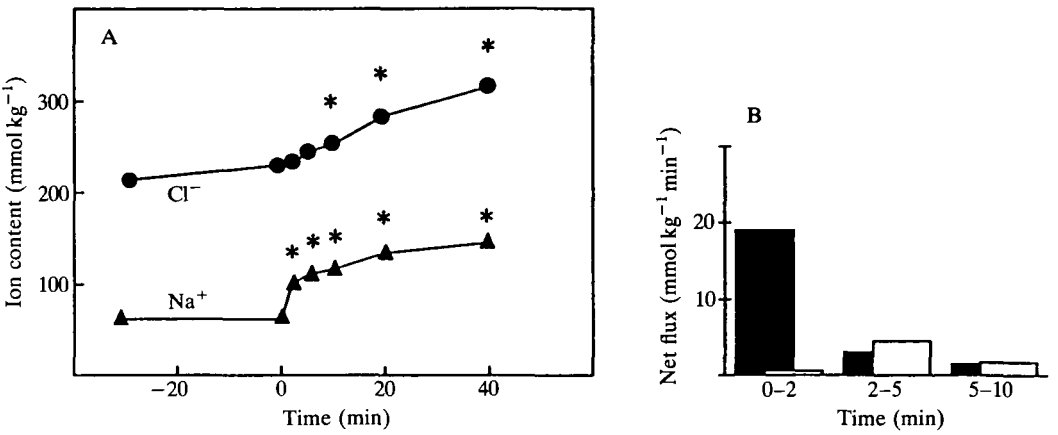

Fig. 10. (A) Effects of adrenergic stimulation on the sodium and chloride content of acetazolamide-treated rainbow trout red cells (N=6). (B) The mean net sodium and chloride influx at the times indicated. For further details, see legend to Fig. 1.

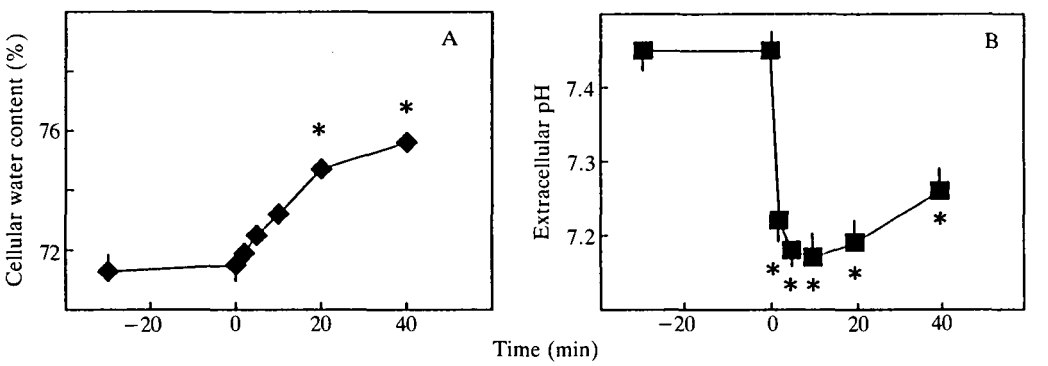

Fig. 11. Effects of adrenergic stimulation on (A) the cellular water content and (B) the extracellular pH of acetazolamide-treated rainbow trout red cells (N=6). For further details, see legend to Fig. 1.

dioxide–bicarbonate-buffered medium occurs because the extracellular dehydration of carbonic acid to carbon dioxide is slow. The protons extruded from the cells by the adrenergically stimulated sodium/proton exchange are accumulated in the incubation medium and cause a strong acid disequilibrium pH before the carbonic acid formed from the accumulated protons and bicarbonate is dehydrated to carbon dioxide to a significant extent.

Our results further show the importance of the intra- and extracellular hydration/dehydration reactions between carbonic acid and carbon dioxide in the adrenergically induced changes in intra- and extracellular pH. It is important to remember that, in the absence of hydration/dehydration reactions between carbonic acid and carbon dioxide, bicarbonate ions would not act as basic

equivalents within the physiological pH range (7–8), because these pH values are far higher than the pK value for carbonic acid–bicarbonate equilibrium (approximately 3.5 at 25°C). In this case, protons would not be buffered by bicarbonate ions. As a result, there would be no coupling of proton and bicarbonate activities. The hydration/dehydration reactions between carbonic acid and carbon dioxide shift the apparent pK value for the carbon dioxide–bicarbonate equilibrium to values above pH 6, and thus bicarbonate ions can act as basic equivalents.

The following paragraphs describe how the rates of the intracellular and extracellular hydration/dehydration reactions between carbonic acid and carbon dioxide affect the adrenergically induced pH changes in suspensions of rainbow trout red blood cells. Initially, adrenergic stimulation activates the sodium/proton exchange across the red cell membrane (Nikinmaa and Huestis, 1984; Baroin *et al.* 1984; Cossins and Richardson, 1985). The proton extrusion generates disequilibrium for the reaction sequence:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

in the extracellular compartment. The rate-limiting step of this sequence, and the whole passive equilibration of protons across the red cell membrane *via* the Jacobs–Stewart cycle, is the uncatalyzed dehydration of carbonic acid.

The carbonic acid concentration can be given as a function of proton and bicarbonate concentrations:

$$[H_2CO_3] = [H^+][HCO_3^-]/K_A ,$$

in which K_A is the acid constant of carbonic acid. Thus, if the external carbon dioxide tension can be held constant by tonometry (Motais *et al.* 1989), the net rate of carbonic acid dehydration to carbon dioxide will depend on the increase in external proton (and/or bicarbonate) concentration, and the rate constants for carbonic acid dehydration and carbon dioxide hydration.

If both the intra- and extracellular hydration/dehydration reactions can be considered instantaneous, the protons excreted *via* the sodium/proton exchanger will immediately react with external bicarbonate to form carbon dioxide. Consequently, bicarbonate ions will leave the cell *via* the anion exchanger, to be replaced by chloride, and will replenish the external bicarbonate pool. In this situation, the proton and bicarbonate activities, and proton and bicarbonate movements *via* the two exchangers, will be fully coupled and no changes in the proton gradient will occur after adrenergic stimulation. This is the case when cells are incubated with carbonic anhydrase. The difference between our study, in which carbonic anhydrase inhibited the changes in proton distribution ratio, and the study of Motais *et al.* (1989), in which the proton distribution ratio decreased even in the presence of carbonic anhydrase, is possibly due to the sixfold higher concentration of carbonic anhydrase used in the present study.

If only the intracellular hydration/dehydration reactions are catalyzed (and can be considered instantaneous), and the external reaction occurs at the uncatalyzed reaction rate, intracellular proton and bicarbonate activities will be coupled. The

onset of proton extrusion, and consequent increase in intracellular pH, will cause an increase in intracellular bicarbonate concentration. This will generate a driving force for net bicarbonate efflux and chloride influx *via* the anion exchanger. However, because the extracellular dehydration reaction is uncatalyzed, there is no immediate coupling of proton and bicarbonate activities in the extracellular compartment. Instead, the formation of carbon dioxide from protons and bicarbonate occurs at the uncatalyzed rate. In this situation, the fluxes of sodium and protons *via* the sodium/proton exchanger exceed the fluxes of chloride and bicarbonate *via* the anion exchanger until the external pH has decreased so much that the rate of carbon dioxide formation in the uncatalyzed reaction catches up with the activity of the sodium/proton exchanger. In the present experiments this was observed after about 5 min of net proton extrusion. Consequently, measurable changes in both the intra- and the extracellular pH occurred, despite a functional anion exchange.

If both the intracellular and extracellular hydration/dehydration reactions between carbonic acid and carbon dioxide occur at the uncatalyzed rate, the initial proton efflux will not lead to changes in either the intracellular or extracellular bicarbonate concentration. Consequently, as shown by the experiment with acetazolamide-treated cells, the changes in proton gradient will be accentuated and, initially, there will be no net movement of chloride into the cell. Thus, the accumulation of intracellular chloride is secondary to the accumulation of bicarbonate in the cell.

The acetazolamide experiment also shows that the rapid hydration of intracellular carbon dioxide to bicarbonate and protons can significantly limit the increase in intracellular pH. As a consequence, if the conditions favour a constant formation of carbon dioxide, as in the tissues, most of the protons excreted will be replenished by the rapid hydration reaction, and the effect of adrenergic stimulation on the intracellular pH will be small. If, however, the conditions favour a net loss of carbon dioxide to the environment, as in the gills, protons and bicarbonate cannot readily be formed from carbon dioxide, and the increase in intracellular pH may be greater. In accordance with this suggestion, Milligan and Wood (1986) observed that the pH gradient across the red cell membrane decreased much more during physical disturbance in dorsal aortic blood than in ventral aortic blood.

The net flux of protons stops as soon as the inward flux of protons *via* the Jacobs–Stewart cycle equals the outward flux of protons *via* the sodium/proton exchanger. The direction of net proton movements may then be reversed, because of the apparent self-inhibition of the sodium/proton exchanger (Garcia-Romeu *et al.* 1988), and the continuous proton equilibration *via* the Jacobs–Stewart cycle. However, even during this period, the red cell volume will increase because net fluxes of sodium and chloride are occurring *via* the exchangers.

According to the present results, changes in the membrane potential are not required for the adrenergic response. This was shown by two experiments. First, adrenergic stimulation did not affect the pH gradient across the membrane of

DNP-treated red cells. However, if protons had been passively distributed after adrenergic stimulation, the decrease in the pH gradient across the membrane of DNP-treated cells should have been similar to that of untreated cells. Second, when valinomycin-treated red cells were adrenergically stimulated, the membrane potential, measured by TPP^+ distribution, did not change, but the chloride distribution ratio and red cell water content increased. The chloride distribution ratio can change at a constant membrane potential if two conditions are fulfilled. (1) The conductive pathway for chloride should not be the major charge-carrying pathway across the red cell membrane. Generally, the potassium conductance is the major determinant of membrane potential in valinomycin-treated red cells (e.g. Lassen, 1977). Since the extracellular TPP^+ concentration in the present study responded to variations in the external potassium concentration, the same is probably also true for the cells used in this study. (2) Chloride distribution should not be passive. The electroneutral sodium/proton exchange generates a disequilibrium distribution for protons, with the consequence that intracellular pH increases. This increase causes a disequilibrium for the chloride/bicarbonate exchanger, with a net efflux of bicarbonate and net influx of chloride. Both these exchanges are electrically silent, so chloride ions will be displaced from electrochemical equilibrium. Thus, both the above conditions appear to be fulfilled. As a consequence, the chloride distribution across the red cell membrane need not be passive in adrenergically stimulated salmonid red cells. Any changes in membrane potential are secondary to the net movements of sodium *via* the sodium/proton exchanger and net movements of chloride *via* the anion exchange pathway, either as a result of an increased activity of the sodium pump (e.g. Bourne and Cossins, 1982) or because of the changes in the distribution ratios of the ions that are at disequilibrium. If, as in human red cells (e.g. Hoffman and Laris, 1974), the conductive permeability of chloride is far greater than the conductive permeability of cations, the membrane potential is mostly a function of the disequilibrium distribution (i.e. the electrochemical gradient) of chloride.

The species differences in the adrenergic pH changes of the red cells (Salama and Nikinmaa, 1989) can be explained on the basis of the present experiments. Whereas the rate of uncatalysed extracellular dehydration of carbonic acid to carbon dioxide is the same in the different species, the activity of the sodium/proton exchanger may be markedly different in different species. As a consequence, the adrenergically induced pH changes will also vary, because the greater the activity of the sodium/proton exchanger, the larger the increase in extracellular proton (or bicarbonate) concentration which is required before the rate of carbonic acid dehydration becomes equal to the rate of proton extrusion *via* the sodium/proton exchanger, and net proton extrusion stops.

The present results also partly explain why the adrenergic response can be reduced in red cells incubated in physiological salines. In many instances, the suspensions of teleost red cells in saline are slightly haemolyzed. This will liberate intracellular carbonic anhydrase into the extracellular compartment and reduce the effect of adrenergic stimulation on the pH gradient.

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